UNIVERZITA PALACKÉHO V OLOMOUCI

Přírodovědecká fakulta

Katedra biochemie

Purinové a pyrazolopyrimidinové inhibitory cyklin-dependentních kinas

DISERTAČNÍ PRÁCE

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Studijní obor:	Biochemie
Forma studia:	Prezenční
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„Na tomto místě bych rád poděkoval svému školiteli doc. RNDr. Vladimíru Kryštofovi, Ph.D. za odborné vedení mé disertační práce, vstřícnost, trpělivost a všechny cenné rady a připomínky, které mi poskytl při její tvorbě. Dále děkuji všem svým kolegům a kolegyním z Laboratoře růstových regulátorů v čele s vedoucím pracoviště prof. Ing. Miroslavem Strnadem, DrSc., především však Mgr. Martině Paprskářové, Ph.D. za její všestrannou odbornou pomoc. Stejnou mírou bych rád poděkoval i všem pracovníkům Katedry biochemie. V neposlední řadě bych rád vyslovil svůj dík Olze Hustákové, Janě Hudcové a Bc. Ditě Parobkové za jejich technickou podporu v řadě prováděných experimentů.“

„Prohlašuji, že jsem předloženou disertační práci vypracoval samostatně za použití citované literatury.“

V Olomouci dne 15.4.2011 …….

Bibliografické informace

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Název práce	Purinové a pyrazolopyrimidinové inhibitory cyklin-dependentních kinas
Typ práce	Disertační
Pracoviště	Laboratoř růstových regulátorů, Přírodovědecká fakulta Univerzity Palackého & Ústav experimentální botaniky AV ČR, v.v.i.
Vedoucí práce	doc. RNDr. Vladimír Kryštof, Ph.D.
Rok obhajoby práce	2011

Abstrakt

Cyklin-dependentní kinasy (CDK) jsou serin/threoninové proteinkinasy, které hrají významnou roli v regulaci buněčného cyklu, transkripce, posttrankripčních modifikacích, buněčné diferenciaci a buněčné smrti. Inhibice CDK nízkomolekulárními inhibitory je považována za vhodnou strategii léčby mnoha typů onemocnění včetně rakoviny. Molekula purinu se stala jednou z prvních, systematicky studovaných skeletů nových inhibitorů CDK, která vedla až k objevení roskovitinu, nejznámějšího purinového inhibitoru CDK. Díky roskovitinu následně začalo intenzivní hledání nových inhibitorů CDK odvozených od purinu. Tato doktorská práce je zaměřena na charakterizaci nového pyrazolo[4,3-*d*]pyrimidinového bioisosteru roskovitinu a hodnocení jeho biologických účinků (kinasová selektivita, defosforylace retinoblastomového proteinu, buněčná proliferace, akumulace nádorového supresoru p53, indukce apoptosy, inhibice homologní rekombinace) v porovnání s roskovitinem jako referenční sloučeninou. Některé inhibitory CDK jsou schopny blokovat buněčný cyklus a potlačovat viabilitu parazita leishmanie, proto byla analyzována knihovna 6,9-disubstituovaných purinů a isomerních 3,7-disubstituovaných pyrazolo[4,3-*d*]pyrimidinů pro svoji potenciální anti-leishmaniální aktivitu a inhibici CRK3 (cdc-2 related kinase). Poslední část práce se zabývá potenciální farmakologickou inhibicí CDK9 ve spojitostí se srdeční hypertrofií a nádorovou angiogenezí.

Klíčová slova	Purin, pyrazolo[4,3-*d*]pyrimidin, cyklin-dependentní kinasa, cyklin, inhibitor, bioisoster, leishmanie
Počet stran	52
Počet příloh	8
Jazyk	Český

Bibliographic information

Autor's name	Mgr. Radek Jorda
Title	Purine and pyrazolopyrimidine inhibitors of cyclin-dependent kinases
Type of thesis	Ph.D.
Department	Laboratory of Growth Regulators, Faculty of Science, Palacky University & Institute of Experimental Botany AS CR
Supervisor	doc. RNDr. Vladimír Kryštof, Ph.D.
The year of presentation	2011

Abstract

Cyclin-dependent kinases (CDK) are serine/threonine kinases that participate in processes of cell cycle control, transcription, posttranscriptional modifications, cell differentiation and cell death. Inhibition of CDK with small molecules has been suggested as a strategy for a treatment of several human diseases including cancer. The purine heterocycle became one of the first systematically investigated scaffolds of CDK inhibitors leading to the discovery of roscovitine. Inspired by the success of roscovitine, further exploration of purine-derived CDK inhibitors has been established. This doctoral thesis describes a novel bioisostere of roscovitine with the pyrazolo[4,3-*d*]pyrimidine core and its evaluation in diverse biological assays (kinase selectivity, dephosphorylation of the retinoblastoma protein, cell proliferation, accumulation of the tumor suppressor protein p53, induction of apoptosis, inhibition of homologous recombination) in comparison with roscovitine as a reference molecule. Furthermore, several CDK inhibitors have been shown to block cell cycle and reduce viability of leishmania, therefore a library of 6,9-disubstituted purines and 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines was screened for leishmanicidal activity and for inhibition of CRK3 (cdc-2 related kinase). Finally, the thesis suggests CDK9 as a target for pharmacological modulation in cardiac hypertrophy and tumour angiogenesis.

Keywords	Purine, pyrazolo[4,3-*d*]pyrimidine, cyclin-dependent kinase, cyclin, inhibitor, bioisostere, leishmania
Number of pages	52
Number of appendices	8
Language	Czech

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2. Cíle disertační práce

Cílem disertační práce bylo vypracování literární rešerše týkající se vývoje, charakterizace a terapeutické využitelnosti inhibitorů cyklin-dependentních kinas (CDK) se zaměřením na purinové inhibitory a jejich deriváty. Dalším cílem pak byla biologická a biochemická charakterizace pyrazolo[4,3-*d*]pyrimidinového bioisosteru roskovitinu a popsání potenciální aplikace purinových a pyrazolo[4,3-*d*]pyrimidinových inhibitorů CDK jako antileishmaniálních sloučenin.

3. Cyklin-dependentní kinasy

3.1. Biologické funkce CDK

Cyklin-dependentní kinasy (CDK) jsou serin/threoninové proteinkinasy, které hrají významnou roli především v regulaci buněčného cyklu, při transkripci a post-transkripčních modifikacích, ale také v procesech buněčné diferenciace či buněčné smrti (Knockaert et al., 2002; Malumbres & Barbacid, 2005). Pro jejich aktivaci je vyžadována přítomnost vazebného partnera, kterým je ve většině případů cyklin. Analýzou lidského genomu byla zjištěna existence 11 genů kódujících CDK, dalších 9 kódujících kinasy příbuzné CDK a dále 29 genů, které mohou být označovány jako cykliny (díky přítomnosti cyklinové domény), přestože funkce některých z nich zatím zůstává nevyjasněna (Malumbres & Barbacid, 2005).

Regulace buněčného cyklu je zajišťována čtyřmi hlavními kinasami CDK4/6/2/1 společně s cykliny D/E/A/B, určitou roli (přechod G0/G1) hraje i CDK3/cyklin C (Ren & Rollins, 2004). CDK7/cyklin H/MAT1 se zapojuje nejen do regulace buněčného cyklu (fosforylace CDK1/2), ale společně s kinasami CDK8/9 s cykliny C/K/T také do kontroly transkripce (fosforylace RNA polymerasy II) (Fisher, 2005; Marshall & Grana, 2006; Galbraith et al., 2010). CDK5 v komplexu s proteiny p35/p39 se převážně podílí na vývoji a diferenciaci neuronů (Maccioni et al., 2001; Cicero & Herrup, 2005). Méně prostudované kinasy CDK10 a CDK11/cyklin L se účastní některých procesů během mitosy a transkripce RNA (Kasten & Giordano, 2001; Hu et al., 2007).

3.2. Genetická analýza funkcí CDK

Role jednotlivých CDK v savčí buňce, interakce se substráty, inhibitory a jinými proteiny, byla primárně studována biochemickými přístupy. Tyto studie vedly k objasnění zapojení CDK a dalších proteinů do regulace buněčného cyklu a pochopení celého mechanismu. V poslední době však k objasnění ontogenetických funkcí CDK napomáhají i genetické analýzy *in vivo* v myších modelech. V současné době je popsáno množství modelů s cíleným umlčením jedné i více CDK současně (Ortega et al., 2002; Malumbres et al., 2004; Malumbres et al., 2008; Malumbres et al., 2009). Spousta těchto experimentů vede k embryonální letalitě v různých fázích vývoje a poukazuje tak na důležitost některých cyklin-dependentních kinas.

Cílené vyřazení CDK4 nebo CDK6 není sice embryonálně letální, i když deficience těchto kinas ovlivňuje proliferaci některých typů buněk. Ztráta exprese CDK4 například brání proliferaci pankreatických β-buněk a buněk laktotrofů hypofýzy v postnatálním vývoji (Rane et al., 1999; Tsutsui et al., 1999), potlačení exprese CDK6 pak vede k poklesu produkce červených krvinek (Malumbres et al., 2004). Kombinace $CDK6^{-/-}$ a $CDK4^{-/-}$ způsobuje pozdní embryonální letalitu z důvodu poškození krvetvorby (Malumbres et al., 2004).

Experimenty zabývající se cíleným vyřazením kinasy CDK2 ukázaly, že nedochází k výrazným poruchám proliferace některých typů buněk, což vedlo k závěru, že CDK2 není přímo vyžadována pro proces buněčného dělení. Tato postradatelnost je vysvětlována schopností kompenzace hladiny CDK2 mitotickou kinasou CDK1 (Aleem et al., 2005; Cai et al., 2006a). Negativní vliv deficience CDK2 byl pozorován při vývoji obou typů pohlavních buněk, které nebyly schopny dokončit první meiotické dělení, což následně vedlo ke sterilitě narozených myší (Berthet et al., 2006; Ortega et al., 2002). Kombinací mutantů $CDK2^{-/-}$ a $CDK4^{-/-}$ došlo k potlačení proliferace a diferenciace kardiomyocytů (Barriere et al., 2007; Berthet et al., 2006), přičemž ani u jednoho ze samostatných mutantů toto nebylo pozorováno. Kombinace mutantů $CDK2^{-/-}$ a $CDK6^{-/-}$ nevedla k žádným dalším efektům v porovnání s mutanty jednotlivých kinas (Malumbres et al., 2004).

Potlačení exprese mitotické CDK1 vede k okamžité embryonální letalitě během prvního buněčného dělení (Santamaria et al., 2007), stejně jako v případě vyřazení cyklinů A2 (Murphy et al., 1997) nebo B1 (Brandeis et al., 1998). Tento jev je pravděpodobně dán nezbytností CDK1 v mitotickém dělení a také nemožností její kompenzace v některých procesech (Malumbres & Barbacid, 2009). Embryonální letalita ve stádiu blastocysty v důsledku potlačení průběhu mitosy byla pozorována i u mutanta $CDK11^{-/-}$ (Lu et al., 2004).

Cílené vyřazení CDK5 vede k výrazným abnormalitám ve vývoji a struktuře nervového systému spojených s perinatální smrtí jedince (Ohshima et al., 1996). Podobný fenotyp byl pozorován i v případě umlčení vazebných partnerů p35/p39 (Ko et al., 2001).

Všechny zmíněné *in vivo* experimenty svědčí především o nepostradatelnosti CDK1/5/11, naopak CDK2/4/6 zřejmě nejsou esenciální pro regulaci buněčného cyklu, jelikož jejich ztráta se projevila jen ve vývoji některých specializovaných buněk v určité fázi myší embryogeneze (Malumbres & Barbacid, 2009).

Stejnou mírou jako zmiňované genetické manipulace *in vivo* přispívají k lepšímu pochopení funkce dané cyklin-dependentní kinasy i experimenty na nádorových buněčných liniích *in vitro* (siRNA, shRNA) odrážející přímý dopad vyřazení určité/ých CDK na nádorovou buňku. Genetické studie ukázaly na nevýznamnost CDK2 během buněčné proliferace a podobné závěry byly publikovány i při vyřazení CDK2 ve vybraných typech nádorových buněčných linií (kolorektálního karcinomu, osteosarkomu a karcinomu děložního čípku) metodou RNA-interference (Tetsu & McCormick, 2003; Payton et al., 2006). Nicméně potlačení CDK2 pomocí RNAi vedlo k zastavení buněčné proliferace melanocytů a předpokládá se, že CDK2 by mohla hrát důležitou roli ve vývoji kožního melanomu (Du et al., 2004), pravděpodobně díky nedávno prokázané výrazně zvýšené expresi u tohoto typu nádorových buněk (Abdulah et al., 2011).

Podobné experimenty byly prováděny i s ostatními cyklin-dependentními kinasami. Vyřazení CDK1 v nádorové linii U2OS či NCI-H1299 vedlo k očekávanému výraznějšímu nárůstu G2/M populace buněk bez indukce apoptózy (Cai et al., 2006a; Payton et al., 2006), nicméně u linie MDA-MB-453 vedlo vyřazení CDK1 společně s G2/M blokem k nárůstu apoptotické populace buněk o více než 150% (Payton et al., 2006). Protože mnoho inhibitorů CDK je skupinově selektivních vůči CDK2 i CDK1, zkoumal se i vliv současného vyřazení obou těchto enzymů. Tímto řešením byla navíc i potlačena vzájemná kompenzovatelnost obou kinas; ta byla potvrzena přítomností anomálních komplexů CDK2/cyklin B nebo CDK1/cyklin E (Cai et al., 2006a). Současným vyřazením CDK1 a CDK2 dle předpokladu výrazně vzrostlo zastoupení buněk v G2/M fázích buněčného cyklu v porovnání s vyřazením samotné CDK1, navíc došlo k výraznému narůstu apoptotické populace buněk (Cai et al., 2006a).

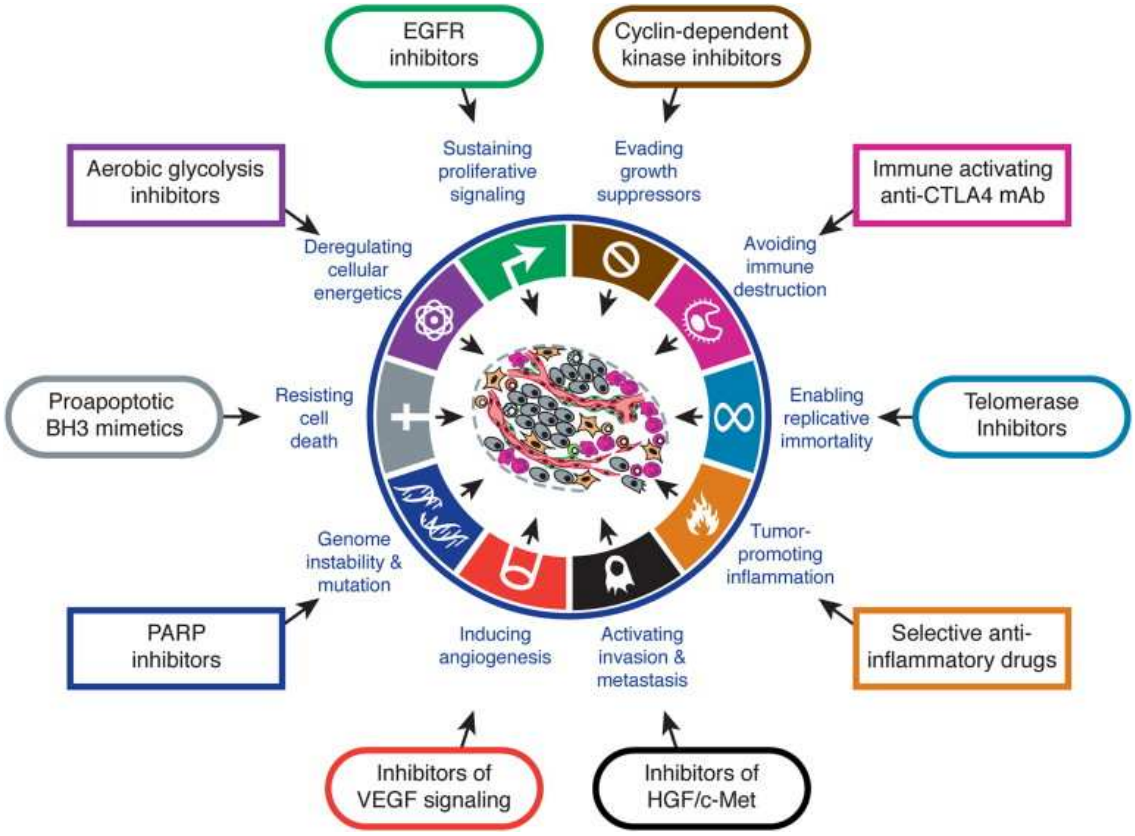
Vyřazení CDK4/6/2 bylo například studováno na lymfomových buňkách LY18 (Gumina et al., 2010). Umlčení uvedených CDK jednotlivě nevedlo k výrazným změnám, což opět potvrzuje jejich vzájemnou zastupitelnost. K akumulaci buněk v G1 fázi buněčného cyklu a inhibici proliferace došlo až při potlačení exprese všech CDK, nebo kombinace CDK4/6 (Gumina et al., 2010).

Zvýšení apoptotické populace buněk na 30% bylo pozorováno při vyřazení CDK5 v krysích nervových buňkách E-18 (Zheng et al., 2007), k potlačení proliferace došlo u linie odvozené od karcinomu štítné žlázy (Lin et al., 2007). Jiný efekt, potlačení migrace buněk jako výsledek umlčení CDK5, byl prokázán na prostatické linii DU145 (Strock et al., 2006).

Vyřazení CDK9, pozitivního regulátoru aktivity RNA polymerasy II, v některých buněčných liniích vedlo především k nárůstu apoptotické populace a okamžitému poklesu buněčné transkripce (Cai et al., 2006b; Manohar et al., 2010), což by mohlo svědčit o nenahraditelnosti CDK9. Snížení exprese CDK7 bylo provedeno nepřímo přes cílené umlčení cyklinu H, který se jeví jako klíčový pro stabilitu celého komplexu CDK7/cyklin H/MAT1 (Patel & Simon 2010). Přes výrazný pokles hladiny všech tří uvedených proteinů však nedošlo k žádným změnám v buněčném cyklu, ani k poklesu úrovně fosforylací CDK1/2 a RNA polymerasy II (Patel & Simon, 2010). Možným vysvětlením může být nedostatečné utlumení aktivity CDK7, jejíž reziduální aktivita může být postačující pro plnění všech funkcí, nebo kompenzace jinou kinasou. Tyto domněnky však zatím nebyly experimentálně potvrzeny, nicméně některé studie naznačují možnost fosforylace C-terminální domény (CTD) RNA polymerasy II kinasami CDK2/cyklin E a CDK1/cyklin B (Cisek et al., 1989; Deng et al., 2002).

3.3. CDK a nádorová transformace

Mezi základní vlastnosti nádorové buňky (Obrázek 1) patří nezávislost na mitogenních faktorech a také snížená citlivost odpovědi na růstové inhibiční signály (Hanahan & Weinberg, 2011). Obě tyto vlastnosti jsou spjaty se zvýšenou buněčnou proliferací, která souvisí s genetickými nebo epigenetickými změnami proteinů zasahujících do regulace buněčného cyklu. Tyto poruchy mohou souviset (i) s jednotlivými regulátory buněčného cyklu nebo (ii) s nadřazenými signálními drahami, které tyto regulátory ovlivňují. Nádorovou transformaci výrazně podporují změny proteinových hladin regulátorů CDK, především přirozených inhibitorů Cip/Kip a INK4, cyklinů D/E/A, fosfatasy Cdc25A/B, ale také substrátů CDK (např. retinoblastomový protein Rb nebo nukleofosmin) (Malumbres & Carnero, 2003; Shapiro, 2006). Přímé genetické změny CDK jsou spíše výjimkou. První případ onkogenní aktivace CDK4 bodovou mutací (R24C) byl popsán u maligního melanomu (Wölfel et al., 1995), v řadě jiných nádorových onemocnění byla prokázána zvýšená exprese CDK4 díky amplifikaci genu (Ortega et al., 2002), přičemž některé z nich vykazovaly i společnou amplifikaci s genem pro Mdm-2 (Reis et al., 2000; Lopes et al., 2001; Simon et al., 2002). CDK6 je nadměrně exprimována jen u některých typů spinocelulárních, gliomových a lymfomových karcinomů, opět jako důsledek genových amplifikací (Malumbres & Carnero, 2003). Rovněž byly popsány případy nádorových onemocnění, u kterých



Obrázek 1: Charakteristické znaky nádorové buňky (převzato z Hanahan & Weinberg, 2011; s povolením vydavatelství Elsevier).

dochází ke zvýšené expresi jak CDK4, tak CDK6 vlivem bodových mutací ve vazebných místech jejich přirozených inhibitorů (Zuo et al., 1996; Easton et al, 1998). Naopak nádorová onemocnění vycházející z alterací CDK2 nebo CDK1 zatím popsány nebyly. Případná zvýšení aktivity těchto dvou kinas jsou většinou spojena se zvýšenou expresí cyklinu E či inaktivací p27 (u CDK2) nebo s inaktivací p27 a p21 (u CDK1) (Malumbres & Carnero, 2003; Malumbres & Barbacid, 2009).

Cykliny D, zapojené do signální dráhy Cyklin D-CDK4/6-INK4-Rb-E2F, jsou prokázanými onkogeny v celé řadě nádorů (Ortega et al., 2002), jejich overexprese však také souvisí se zvýšenou aktivací nadřazených onkogenních signálních drah vlivem mutací například v onkogenech *Ras* a *Myc* (mitogenně aktivovaná dráha - MAPK), *β-kateninu* (dráha Wnt/β-katenin) nebo genu *Erbb2* (receptorová rodina RTK) (Malumbres & Carnero, 2003). U cyklinu E a A nedochází často ke genetickým alteracím, jejich zvýšená exprese je spojována se sníženou expresí p21 a p27, přirozených inhibitorů CDK rodiny Cip/Kip (Porter et al., 1997).

U těchto inhibitorů se setkáváme s genetickou alterací také jen zřídka. Prokázaná delece v genu pro p21 byla pozorována jen v některých případech karcinomů štítné žlázy (Shi et al., 1996), mutace v genu pro p27 pak v některých případech prsního adenokarcinomu (Spirin et al., 1996). Potlačení exprese p21 a p27 je dáno především mutací nadřazeného nádorového supresoru p53 (v případě p21), ubikvitinligasy APC (v případě p27) nebo epigenetickým umlčením (Malumbres & Carnero, 2003). U dalšího přirozeného inhibitoru p57 byla pozorována translokace genu vedoucí ke ztrátě CDK-inhibiční domény (Hatada et al., 1996; Lee at al., 1997). Inhibitory rodiny INK4 bývají v nádorech velmi často umlčované z důvodu delece, mutace a epigenetické hypermethylace promoteru (Ruas & Peters, 1998). Nejčastěji se setkáváme s těmito změnami u inhibitoru p16, naopak genetické alterace u inhibitorů p18 a p19 jsou pozorovány jen zřídka (Gemma et al., 1996; Lapointe et al., 1996).

Zvýšená exprese regulačních fosfatas Cdc25A/B popsaná u řady nádorových onemocnění vede k hyperaktivaci CDK (Boutros et al., 2007) a je většinou spojena s aktivací protoonkogenu *Myc* a následnou zvýšenou expresí cyklinů D (Galaktionov et al., 1996; Sato et al., 2001).

3.4. Strategie použití inhibitorů CDK

Všechny výše uvedené genetické i epigenetické alterace podporující nádorovou transformaci a buněčnou proliferaci naznačují, že právě CDK by se eventuálně mohly stát cílem protinádorové terapie. Proto se stala cílená inhibice CDK selektivními inhibitory jedním z intenzivně zkoumaných přístupů, jak zabránit nekontrolovatelné proliferaci nádorové buňky. V této souvislosti byly primárně studovány všechny CDK zahrnuté do regulace buněčného cyklu (CDK1/2/4/6) jako potenciální terapeutické cíle. Nicméně v poslední době se předmětem zájmu stala i CDK5 a transkripční CDK7/9, jejichž zvýšená exprese byla prokázaná u některých typů nádorových onemocnění (Romano & Giordano, 2008; Liu et al., 2010) a jejichž potenciální inhibice by tudíž mohla mít přínosný terapeutický potenciál.

Některé inhibitory CDK, kromě přímého vlivu na progresi buněčného cyklu prostřednictvím inhibice CDK1/2/4/6, omezují proliferaci a viabilitu nádorových buněk i jinými mechanismy. Zejména se jedná o schopnost některých inhibitorů CDK snižovat aktivitu CDK9 (Chao & Price, 2001; Shima et al., 2003; Heredia et al., 2005; MacCallum et al., 2005; Cai et al., 2006b) a ovlivňovat tak transkripci mRNA

(Ljungman & Paulsen, 2001; Blagosklonny, 2004). Potlačení transkripce vede ke snížení exprese řady antiapoptotických proteinů (Bcl-2, Mcl-1, survivin, XIAP) a podporuje indukci apoptosy. U některých maligních onemocnění se toto potlačení hladiny antiapoptotických proteinů roskovitinem (Alvi et al., 2005; Hahntow et al., 2004; McCallum et al., 2005; Raje et al., 2005), flavopiridolem (Kőnig et al., 1997; Kitada et al., 2000; Gojo et al., 2002; Chen et al., 2005), SNS-032 (Chen et al., 2009), P276-00 (Manohar et al., 2010) nebo AT7519 (Santo et al., 2010) ukázalo jako terapeuticky velmi nadějné.

Inaktivace CDK9 nízkomolekulárními inhibitory CDK však nemá vliv pouze na hladinu antiapoptotických proteinů, projevuje se i nepřímo akumulací proteinu p53 jako důsledek poklesu transkripce jeho negativního regulátoru Mdm-2 (Lu et al., 2001; Demidenko & Blagosklonny, 2004; Kryštof et al., 2005; **Příloha I**). Protein p53 je význačným transkripčním faktorem, který zasahuje do mnoha buněčných procesů, včetně regulace buněčného cyklu, opravy DNA nebo apoptosy (Oren, 2003; Woods & Lane, 2003). S aktivací p53 je spojena následná exprese přirozeného inhibitoru p21 či aktivace proapoptotických proteinů Puma a Noxa. Tímto způsobem se může zvyšovat protinádorový účinek sloučenin, které jsou schopny inhibovat i transkripční CDK7/9, jak bylo například ukázáno v jedné z přiložených prací (**Příloha I**).

Další důležitou vlastností, kterou se mohou inhibitory CDK podílet na potlačení růstu nádoru a jeho expanzi, je jejich schopnost potlačovat angiogenezi. U některých inhibitorů CDK byl prokázán negativní vliv na angiogenezi potlačením exprese VEGF (vascular endothelial growth factor) (Mellilo et al., 1999; Ali et al., 2007; Stockwin et al., 2009). Nicméně u některých inhibitorů za potlačením angiogeneze pravděpodobně stojí inhibice transkripce obecně, a také omezení migrace endoteliálních buněk, tedy procesů, kde se aktivně účastní CDK9 a CDK5 (Radhakrishnan & Gartel, 2006; Ali et al., 2007; Liebl et al., 2010; Zahler et al., 2010; **Příloha II**).

V souvislosti s hledáním vhodných inhibitorů CDK však prozatím nebyla vyřešena otázka selektivity příslušných inhibitorů; zda-li je výhodnější inhibovat jen jednu určitou CDK, všechny či přesně definované kombinace některých CDK. Podobný problém vyvstává u volby vhodného nádorového modelu při využití různě selektivních inhibitorů CDK a úspěšném převedení experimentů s buněčnými kulturami a myšími modely do klinické podoby. Použití inhibitorů CDK je totiž omezeno v závislosti na typu nádorové tkáně, genetickém pozadí a citlivosti k danému inhibitoru. Tyto

nevyjasněné otázky jsou příčinou relativně pomalého průběhu klinických experimentů s inhibitory CDK a zejména nejednoznačné terapeutické účinnosti.

U každého inhibitoru CDK je nutné zvažovat (ne)selektivitu především i z hlediska možných jiných proteinkinasových cílů, které by nemusely prospívat žádoucímu terapeutickému účinku. Některé očekávané toxické účinky spojené s inhibicí CDK4 (inhibice pankreatických β-buněk vedoucí k cukrovce), CDK2 (nedokončení meiotického dělení pohlavních buněk vedoucí ke sterilitě) nebo CDK6 (ztráta proliferace erytrocytů vedoucí k anémii), které byly prokázány v genetických modelech (Malumbres & Barbacid, 2009), mohou být akceptovatelné již pro dospělého jedince, naopak terapie cílená proti CDK1 (případně i CDK7 a CDK11) by mohla znamenat komplexní toxicitu výrazně se neodlišující od dnešních cytostatik (Malumbres & Barbacid, 2009). Další možnou komplikací by mohly být vedlejší účinky spojené s inhibicí méně studovaných kinas CDK10 a CDK11, které zatím nebývají součástí testů selektivity nových inhibitorů CDK. Bylo prokázáno, že inhibice CDK10 vede k nežádoucí aktivaci ERK/MAPK kinasové dráhy (Iorns et al., 2008) a potlačení exprese CDK11 zvyšuje genetickou nestabilitu a podporuje tumorogenezi (Chandramouli et al., 2007). Z aminokyselinové sekvence residuí formující aktivní místo kinas CDK10/11 je však patrná dostatečná odlišnost od ostatních CDK **(Tabulka 1)**, čímž se pravděpodobnost společné inhibice některých CDK společně s CDK10 nebo CDK11 poněkud snižuje.

I přes komplexnost posuzovaní vhodné selektivity inhibitorů CDK v závislosti na příslušném genetickém modelu se daří nacházet některé vhodné diagnózy pro experimentální léčbu. Jedním z příkladů může být cílená inhibice CDK4 u prsního karcinomu za předpokladu zvýšené exprese *Erbb2* a *Ras* onkogenu, která naopak není účinná v případě výskytu *Myc* a *Wnt-1* onkogenů (Yu et al., 2001). Naopak v případě *Myc*-indukované rakoviny kůže se jeví inhibice CDK4 jako efektivní (Miliani de Marval et al., 2004). Další možnou aplikací se ukázala i inhibice CDK1, prokázaná ale jen v případě nádorových onemocnění charakterizovaných overexpresí onkogenu *Myc* (Goga et al., 2007). Inhibice CDK2 by zase mohla najít uplatnění při léčbě kožního melanomu podporovaného expresí transkripčního faktoru MITF (melanocyte lineage transcription factor) (Du et al., 2004).

Tabulka 1. Srovnání aminokyselin formujících aktivní místa vybraných lidských a leishmaniových CDK. Barvy značí fyzikálně-chemické vlastnosti aminokyselin: červená - hydrofobní, modrá - kyselé, oranžová - bazické, zelené - ostatní polární. Nejvíce zastoupené aminokyseliny ve všech uvedených kinasách jsou označeny šedě.

	65	82	82	83	84	85	87	90	131	134	144	145
*Lmj*CRK1	V	F	E	Y	L	D	D	K	Q	L	G	D
	82	99	100	101	102	103	105	108	149	152	162	163
*Lmj*CRK3	V	F	E	Y	V	E	D	K	A	L	A	D
	64	80	81	82	83	84	86	89	132	135	145	146
CDK1	V	F	E	F	L	S	D	K	Q	L	A	D
	64	80	81	82	83	84	86	89	131	134	144	145
CDK2	V	F	E	F	L	H	D	K	Q	L	A	D
	64	80	81	82	83	84	86	89	131	134	144	145
CDK3	V	F	E	F	L	S	D	K	Q	L	A	D
	77	93	94	95	96	97	99	102	144	147	157	158
CDK4	V	F	E	H	V	D	D	T	E	L	A	D
	64	80	81	82	83	84	86	89	130	133	143	144
CDK5	V	F	E	F	C	D	D	K	Q	L	A	D
	77	98	99	100	101	102	104	107	149	152	162	163
CDK6	V	F	E	H	V	D	D	T	Q	L	A	D
	75	91	92	93	94	95	97	100	141	144	154	155
CDK7	I	F	D	F	M	E	D	V	N	L	A	D
	79	97	98	99	100	101	103	106	155	158	172	173
CDK8	I	F	D	Y	A	E	D	H	A	I	A	D
	79	103	104	105	106	107	109	112	153	156	166	167
CDK9	V	F	D	F	C	E	D	G	A	L	A	D
	101	117	118	119	120	121	123	126	167	170	180	181
CDK10	L	M	G	Y	C	E	D	S	S	L	A	D
	488	504	505	506	507	508	510	513	553	557	567	568
CDK11	V	M	N	Y	V	E	D	S	S	L	G	D

4. Inhibitory CDK

Se zjištěním, že by se CDK mohly stát vhodným cílem protinádorové terapie, začal v posledních dvaceti letech intenzivní výzkum v oblasti hledání inhibitorů CDK. Mezi tzv. CDK-inhibitory první generace se řadí roskovitin (Meijer et al., 2006) a flavopiridol (Sedláček, 2001; Blagosklonny, 2004), jejichž struktury vychází z přírodních látek, a které jako první významné inhibitory CDK vstoupily do klinického testování. Následně

bylo různými syntetickými přístupy vyvinuto obrovské množství dalších strukturních motivů (Malumbres et al., 2008; Dickson & Schwartz, 2009; Malumbres et al., 2009; Wesierska-Gadek et al., 2009; Kryštof & Uldrian, 2010; Lapenna & Giordano, 2010; Németh et al., 2011), ze kterých byly odvozeny inhibitory CDK označované jako inhibitory druhé generace, z nichž některé jsou v současnosti také testovány v klinických experimentech (**Tabulka 2; Obrázek 2**).

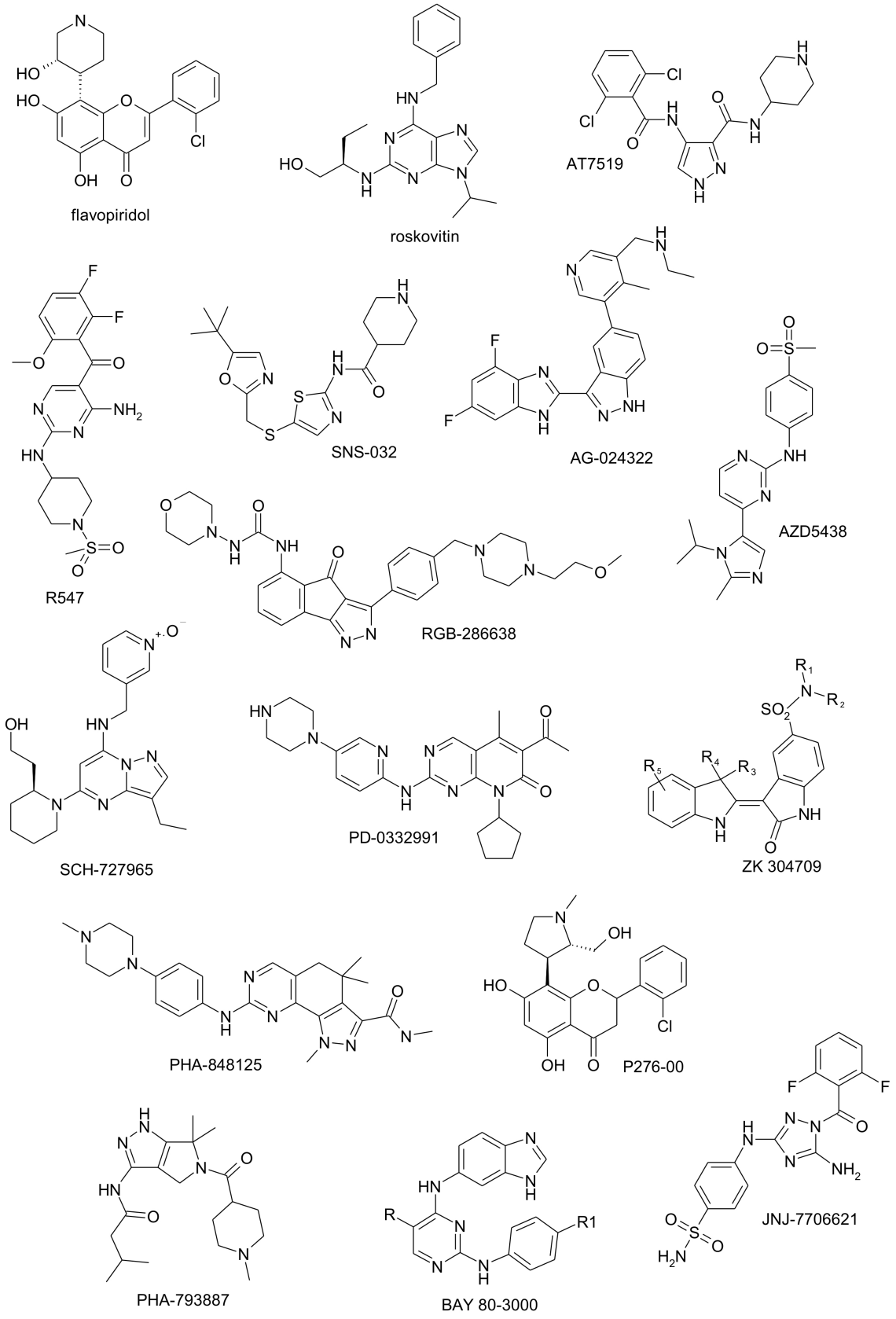
Díky postupující genotypizaci jednotlivých typů rakovin a pokročilému mapování funkcí všech známých CDK se počítá v budoucnu s vývojem tzv. CDK-inhibitorů třetí generace, které by už měly být syntetizovány cíleně jako inhibitory určitých CDK (jejich kombinací a případně i jiných kinas) dle závislosti na celkovém genetickém pozadí daného nádorového onemocnění (Malumbres et al., 2008).

Mezi první systematicky popsané nízkomolekulární inhibitory CDK patří 2,6,9-trisubstituované puriny (Meijer & Raymond, 2003), které tvoří stěžejní základ této doktorské práce. Různými syntetickými přístupy byla získána celá řada purinových derivátů (Veselý et al., 1994; De Azevedo at al., 1997; Havlíček et al., 1997; Schow et al., 1997; Chang et al., 1999; Vermeulen et al., 2002).

Tabulka 2. Přehled inhibitorů CDK nacházejících se v různých fázích klinického testování (Malumbres et al., 2008; Dickson & Schwartz, 2009; Malumbres et al., 2009; Brasca et al., 2010; Wesierska-Gadek et al., 2009; Kryštof & Uldrian, 2010; Lapenna & Giordano, 2010; Németh et al., 2011).

Inhibitor	Cílená CDK	Inhibitor	Cílená CDK
Flavopiridol	2/4/6/9	ZK 304709[b]	1/2/4/7/9
Roscovitin	1/2/5/7/9	AZD5438	1/2/5/6/9
AT7915[a]	1/2/4/5	TG02[e]	1/2/7/9
R547	1/2/4	BAY 1000394	1/2/4/9
PD-0332991	4/6	LEE011	4/6
SNS-032[a]	2/7/9	P276-00	1/4/9
AG-024322	1/2/4	PHA-793887	1/2/4/5/7/9
RGB 286638	1/2/4/5/7/9	BAY 80-3000	1/2
P 1446A-05	4	JNJ-7706621[c]	1/2
PHA-848125[d]	1/2/4	SCH-727965	1/2/5/9

prokázaná inhibice dalších proteinkinas: [a]GSK3; [b]VEGFR1/2/3, PDGFR-β, Flt-3; [c]AUR1/2; [d]TRKA; [e]Flt-3, JAK2, ERK5



Obrázek 2: Struktury inhibitorů CDK nacházejících se v různých fázích klinického testování. Struktury TG02, BAY 1000394, LEE011 a P 1446A-05 nebyly doposud zveřejněny.

Jedním z nich byl roskovitin (CYC202; Seliciclib), který jako první a zatím nejvýznamnější inhibitor CDK z řady trisubstituovaných purinů vstoupil do klinických testů a v současnosti se nachází v různých fázích klinického testování jako samostatné léčivo proti nemalobuněčnému karcinomu plic a karcinomu nosohltanu nebo v kombinaci s gemcitabinem proti různým typům progredujícího solidního tumoru (cyclacel.com; clinicaltrials.gov).

4.1. Roskovitin

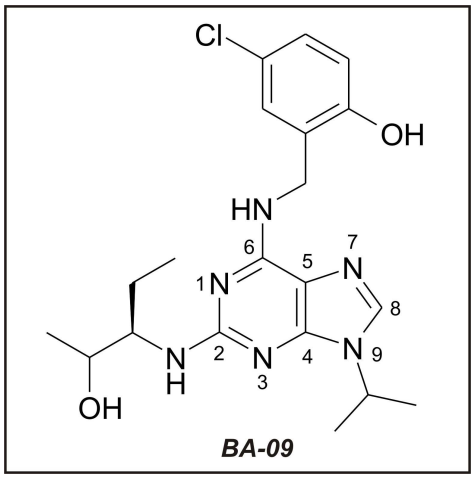
Roskovitin vykazuje výrazné inhibiční účinky proti CDK1/2/5/7/9 (McClue et al., 2002; Kryštof et al., 2005), které se následně odrážejí v jeho antiproliferačních a cytotoxických schopnostech (Meijer, 2006). Vliv roskovitinu na buněčný cyklus je spojován s defosforylací retinoblastomového proteinu i s poklesem hladiny některých cyklinů a CDK (Barrie et al., 2003; Whittaker et al., 2004; Raynaud et al., 2005; McCallum et al., 2005; Paprskářová et al., 2009). Díky inhibici CDK7 a CDK9 dochází v buňkách k poklesu aktivity RNA polymerázy II a tím k potlačení transkripce (Ljungman & Paulsen, 2001; McCallum et al., 2005; **Příloha I**), což se projevuje snižováním hladin proteinů s krátkou stabilitou, především s antiapoptotickou funkcí (Mcl-1, survivin, Mdm-2) a dochází tak k indukci apoptózy (McCallum et al., 2005; Raje et al., 2005). Potlačením exprese proteinu Mdm-2 dochází v buňkách ovlivněných roskovitinem k akumulaci nádorového supresoru p53 v jádře (David-Pfeuty, 1999; Paprskářová et al., 2009). Roskovitin negativně ovlivňuje také další buněčné cíle, např. mitogenně aktivované kinasy ERK1/2 (Whittaker et al., 2004), pyridoxalkinasu (Bach et al., 2005; Tang et al., 2005) či mitotické kinasy AUR1/2 a PLK1 (Whittaker et al., 2007), jejichž inhibice se taky podílí na antiproliferativních účincích.

4.2. Další purinové inhibitory CDK

Díky poměrně silným inhibičním vlastnostem, selektivitě a řadě významných biologických účinků se roskovitin stal motivací mnoha studií věnujících se dalšímu vývoji inhibitorů CDK. Vývoj v oblasti purinových inhibitorů CDK se ubírá převážně třemi směry. Prvním z nich je snaha o obměnu substituovatelných pozic purinového skeletu (především pozic 2, 6 a 9), druhou pak je změna samotného purinového skeletu náhradou či doplněním dusíkových atomů za vzniku nových heterocyklických systémů,

tzv. purinových bioisosterů. Třetím přístupem je pak kombinace dvou předešlých inovativních směrů.

Ve snaze zesílit inhibiční a antiproliferační vlastnosti purinových inhibitorů CDK bylo připraveno velké množství derivátů s nejrůznějšími typy substituentů (Schow et al., 1997). Podařilo se tak získat některá účinnější analoga CVT313 (Brooks et al., 1997), purvalanol (Gray et al., 1998; Chang et al., 1999), olomoucin II (Kryštof et al., 2005), H717 (Dreyer et al., 2001), NU2058 a NU6102 (Davies et al., 2002; Hardcastle et al., 2004), CR8 (Bettayeb et al., 2008a) nebo skupinu biarylových derivátů (Oumata et al., 2008; Trova et al., 2009a; Trova et al., 2009b). I přes vysokou účinnost řady inhibitorů (až stonásobné zlepšení inhibiční a antiproliferační aktivity oproti roskovitinu) nebyla žádná sloučenina vybrána pro klinické experimenty.



BA-09

Pracoviště Laboratoře růstových regulátorů taktéž dlouhodobě studuje inhibitory CDK odvozené od roskovitinu. Snahou však není jen zvýšení inhibičních a proliferačních vlastností nově vznikajících derivátů, ale i zvýšení jejich metabolické stability. Farmakokinetické studie totiž ukázaly, že roskovitin je v systému *in vivo* poměrně rychle metabolizován (Nutley et al., 2005; Raynaud et al., 2005; McClue & Stuart, 2007). Jeden z hlavních metabolitů vzniká oxidací primární hydroxyskupiny na C2 postranním řetězci až na karboxylát a tím úplně ztrácí inhibiční vlastnosti vůči CDK. Tato metabolická deaktivace pak vede ke snižování terapeutického účinku roskovitinu *in vivo* (Benson et al., 2007). Na základě těchto skutečností byla v naší laboratoři připravena série nových purinových derivátů obsahujících sekundární a terciární hydroxyskupinu na C2 postranním řetězci, která by měla být chráněna proti metabolické oxidaci. Důležité přitom je také zachování afinity k CDK a proapoptotického působení v nádorových buněčných liniích, které bylo prokázáno se sloučeninou BA09, zástupcem této série **(Příloha III)**.

4.3. Roskovitinem inspirované purinové bioisostery

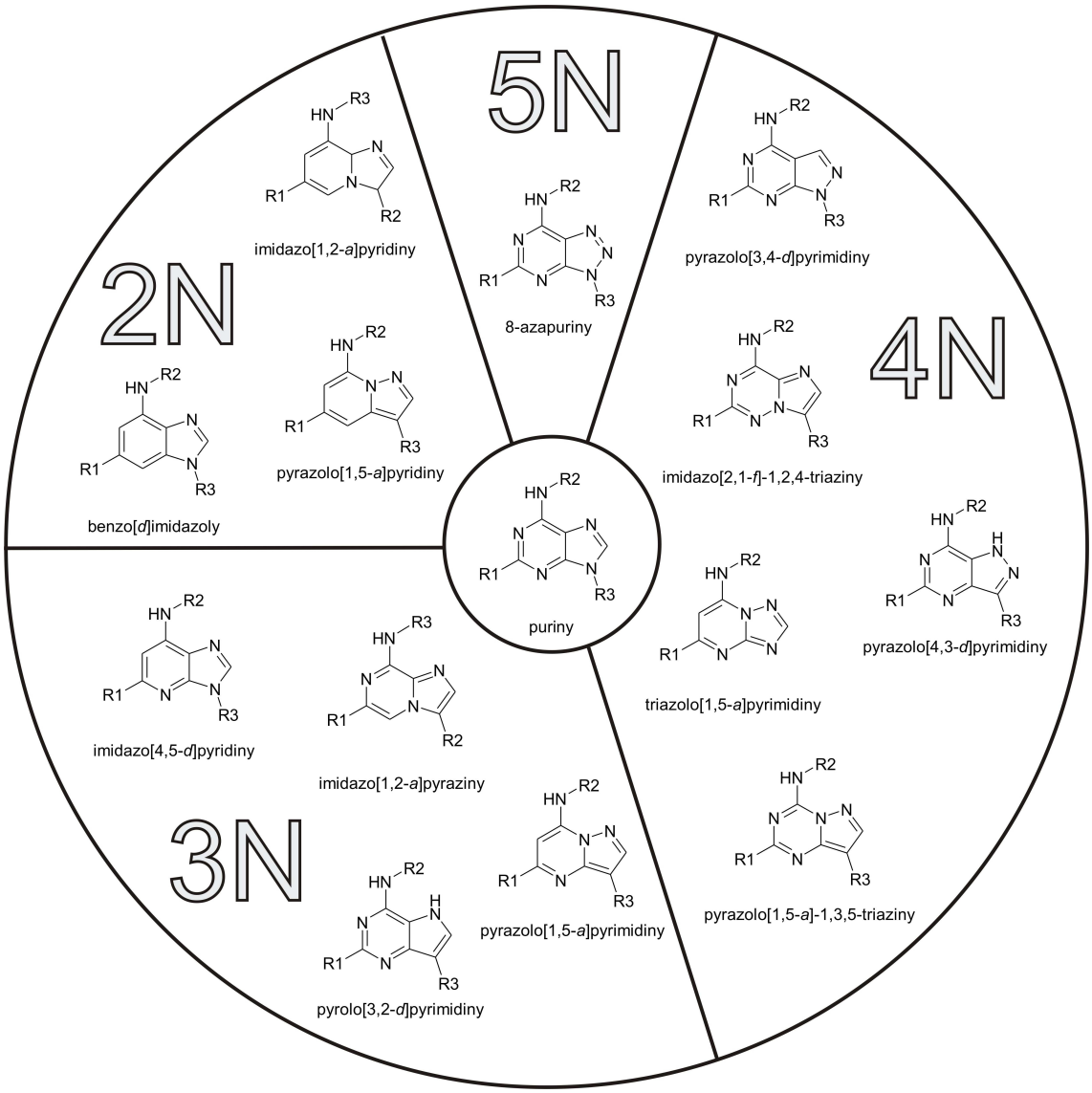
Jednou ze strategií racionálního přístupu k vývoji nových léčiv (Patani & LaVoie, 1996; Lima & Barreiro, 2005) je syntéza a studium bioisosterů, neboli strukturně podobných

sloučenin lišících se některými funkčními skupinami či atomy nebo jen vzájemným uspořádáním jednotlivých atomů v molekule. Tyto modifikace obvykle vedou ke změnám fyzikálně-chemických vlastností nově vzniklých sloučenin, které se mohou projevit (i) zlepšením metabolické stability, (ii) potlačením určitých nežádoucích efektů, (iii) změnami farmakokinetických vlastností, (iv) zlepšením biodostupnosti (Popowycz et al., 2009). Tímto přístupem bylo v posledních letech syntetizováno několik tříd bioisosterů purinových inhibitorů CDK **(Obrázek 3)**. Největší skupinu tvoří bioisostery se zachovaným počtem čtyř dusíkových atomů v základním skeletu. Vznikly však i skupiny isomerů obsahující pouze tři nebo dokonce dva atomy dusíku v molekule, stejně tak i jedna skupina sloučenin s pěti atomy dusíku.

Jen u mála z těchto bioisosterů došlo k výraznějšímu zvýšení aktivity vůči CDK. Většina bioisosterů vykazuje stejné vlastnosti jako purinové látky. Mezi takové patří imidazo[2,1-*f*]-1,2,4-triaziny (Bettayeb et al, 2008b; Popowycz et al, 2009), pyrolo[3,2-*d*]pyrimidiny (Čapek et al., 2003) triazolo[1,5-*a*]pyrimidiny (Richardson et al., 2006; WO/2004108136), imidazo[4,5-*d*]pyridiny (WO 2009/034411), imidazo[1,2-*a*]pyraziny (WO/2004/026877) a imidazo[1,2-*a*]pyridiny (WO/2004/026867; Fischmann et al., 2008). Souběžně však vznikaly i purinové bioisostery, u kterých došlo k úplnému potlačení inhibičního potenciálu vůči CDK. Jsou jimi pyrazolo[3,4-*d*]pyrimidiny (Kim et al., 2003), 8-azapuriny (Havlíček et al., 2005) a benzo[*d*]imidazoly (Jain et al., 2011).

Byly však také připraveny čtyři typy bioisosterů, které výrazně převyšují inhibiční schopnosti analogických purinů. Jsou jimi pyrazolo[1,5-*a*]-1,3,5-triaziny (Bettayeb et al, 2008b; Popowycz et al, 2009; WO/2005/082908), pyrazolo[1,5-*a*]pyrimidiny (Williamson et al., 2005; Paruch et al., 2007; Ali et al., 2009; Heathcote et al., 2010; WO/2004/087707; WO2008/027220; WO/2008/151304), pyrazolo[1,5-*a*]pyridiny (Fischman et al., 2008; WO/2004/026872) a pyrazolo[4,3-*d*]pyrimidiny (Kryštof et al., 2006; Sroka et al., 2010, **Přílohy IV, V**). Aby mohlo dojít k co nejpřesnějšímu porovnání vlastností jednotlivých bioisosterů, byl u některých skupin vytvořen přímý isomer roskovitinu **(Příloha IV - Figure 1)**.

Za vyšší účinnost těchto čtyř tříd purinových bioisosterů pravděpodobně stojí zejména uspořádání dusíkových atomů na pětičlenném kruhu. Z vazby roskovitinu a dalších purinových inhibitorů v aktivním místě CDK2 (Davies et al., 2002) je totiž patrná důležitost atomu dusíku (pozice 7 u purinu), neboť se podílí na vodíkové vazbě s aminoskupinou Leu83 podobně jako dusík N6 s karbonylem Leu83 a vytváří tak

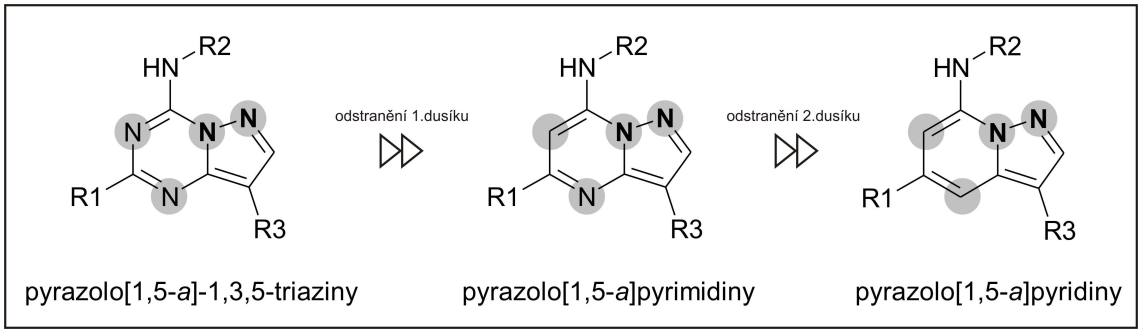


Obrázek 3: Strukturní motivy známých purinových bioisosterů cíleně syntetizovaných a zkoumaných jako inhibitory CDK.

nezbytný donor-akceptorový motiv v páteřní oblasti CDK2. Přítomnost dusíku v této pozici tedy zajišťuje i u ostatních purinovým bioisosterů podobné inhibiční vlastnosti jako u purinových molekul. Pokud tento dusík chybí jako v případě pyrazolo[3,4-*d*]pyrimidinů (Kim et al., 2003), pozorujeme výrazné snížení aktivity vůči CDK. Avšak přítomnost dalšího dusíku na vedlejší pozici mění elektrostatický potenciál vazebné části inhibitoru (Popowycz et al, 2009), což se projevuje výrazným zvýšením afinity k CDK u zmíněných čtyř skupin bioisosterů obsahujících tento motiv.

Nicméně není to jen pozice dvou dusíků, která by rozhodovala o síle inhibičních vlastností jednotlivých tříd purinových bioisosterů. Dalším významným faktorem se zdá

být i celkový počet dusíkových atomů v pětičlenném kruhu, který je blíže páteři CDK, a jehož atomy se účastní přímé vazebné interakce. U skupiny triazolo[1,5-*a*]pyrimidinů a 8-azapurinů totiž došlo zavedením třetího dusíku do pětičlenného kruhu ke značnému zeslabení inhibice CDK (Havlíček et al., 2005; Richardson et al., 2006). Naopak v případě šestičlenného kruhu můžeme uvažovat o dostatečné variabilitě v obsazenosti jednotlivých poloh atomy dusíku, neboť v některých případech nemá odebrání jednoho či více atomů dusíku z některých pozic šestičlenného kruhu výrazný vliv na potlačení inhibice CDK. Příkladem může být série pyrazolo[1,5-*a*]-1,3,5-triazinů (4N), pyrazolo[1,5-*a*]pyrimidinů (3N) a pyrazolo[1,5-*a*]pyridinů (2N) (**Obrázek 4**), u jejichž zástupců lze pozorovat zachování srovnatelných inhibičních vlastností.



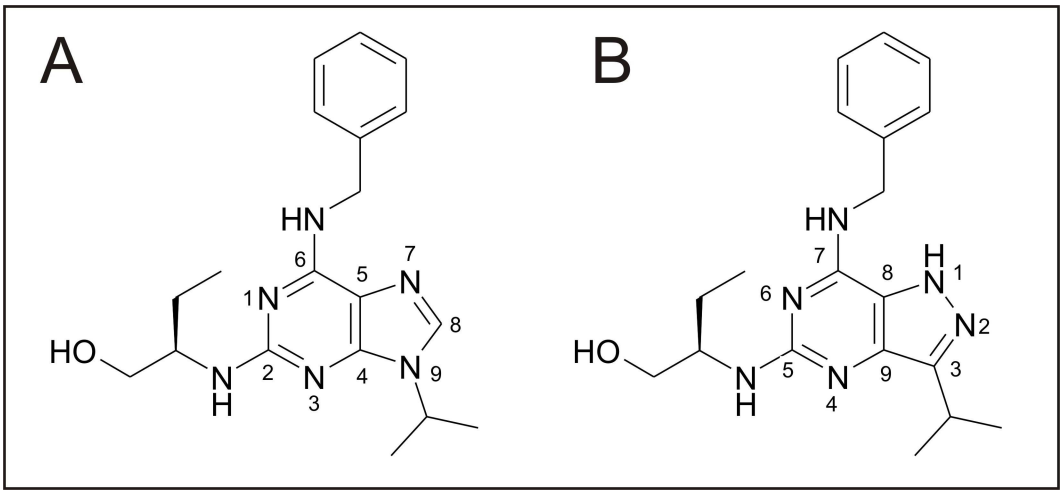
Obrázek 4: Schématické znázornění tří skupin purinových bioisosterů, u kterých nedochází k výrazným změnám v inhibici CDK po odebrání atomu/ů dusíku z vyznačených poloh.

4.4. Pyrazolo[4,3-*d*]pyrimidiny

Pracoviště Laboratoře růstových regulátorů se zabývá syntézou a zkoumáním biologických vlastností dvou tříd purinových bioisosterů, 8-azapurinů a pyrazolo[4,3-*d*]pyrimidinů. Zatímco u azapurinů došlo k zeslabení inhibičních účinků vůči CDK (Havlíček et al., 2005), u pyrazolo[4,3-*d*]pyrimidinů byl pozorován opačný efekt.

U nejdříve popsaných 3,7-disubstituovaných pyrazolo[4,3-*d*]pyrimidinů byla prokázána zvýšená inhibice CDK1 v porovnání se sérií purinů s identickými substituenty (Moravcová et al., 2003). U nejúčinnějších látek však byla dosažená inhibice CDK1 srovnatelná s trisubstituovanými puriny roskovitinem či olomoucinem II. Dále se snaha organických chemiků soustředila na zavedení třetího substituentu do pozice 5 pyrazolo[4,3-*d*]pyrimidinového kruhu a byla vytvořena knihovna 3,5,7-trisubstituovaných pyrazolo[4,3-*d*]pyrimidinů, u kterých byla zjištěna podstatně vyšší biologická účinnost v porovnání s puriny. Vztahy mezi strukturou a aktivitou těchto

bioisosterů purinu zatím nebyly uceleně publikovány, nicméně protinádorové účinky některých trisubstituovaných pyrazolo[4,3-*d*]pyrimidinů již byly popsány (Kryštof et al., 2006, Sroka et al., 2010; **Přílohy IV, V**). Jedna sloučenina z této série (LGR1406) byla dokonce studována v souvislosti s potlačením abnormálního růstu buněk hladkého svalstva (Sroka et al., 2010), což by mohlo být významné při hypotetickém využití v léčbě restenosy. Přestože přímý vliv LGR1406 na CDK nepřevyšuje účinky roskovitinu, inhibice růstu svalových buněk je přibližně pětkrát vyšší. Navíc dochází k inhibici proliferace buněk hladkého svalstva i po mitogenní stimulaci.



Obrázek 5: Roskovitin (A) a jeho pyrazolo[4,3-d]pyrimidinový bioisoster LGR1404 (B).

Exaktní srovnání biochemických účinků roskovitinu a jeho pyrazolo[4,3-*d*]pyrimidinového bioisosteru LGR1404 (**Obrázek 5**) bylo jedním ze stěžejních cílů této práce **(Příloha IV)**. Pro potvrzení přímé interakce LGR1404 s CDK2 byla analyzována krystalová struktura (záznam v Protein Data Bank: 3PJ8), dále byla studována selektivita vůči řadě proteinkinas, vliv na proliferaci, indukci apoptózy a některé další účinky spojované s inhibicí CDK.

Další inhibitor na bázi pyrazolo[4,3-*d*]pyrimidinu připravený a charakterizovaný během tohoto doktorského studia je LGR1492, zatím nejúčinnější inhibitor CDK2 ze současné knihovny těchto bioisosterů (**Příloha V**). Vyznačuje se podobným profilem selektivity jako roskovitin, dosahuje však silnějších inhibičních a antiproliferačních účinků (přibližně 15-krát, IC_{50} pro CDK činí přibližně 10 nM). Podobně jako roskovitin inhibuje replikaci DNA, potlačuje transkripci mRNA, zastavuje buněčný cyklus,

způsobuje jadernou akumulaci proteinu p53. Odlišnost je patrná jen v průběhu buněčné smrti. Pyrazolo[4,3-*d*]pyrimidin LGR1492 se totiž vyznačuje částečně neobjasněným mechanismem v závislosti na použité koncentraci, pravděpodobně důsledkem ztráty selektivity při vyšších koncentracích a interakci s jiným buněčným cílem. Podobný jev v závislosti na koncentraci látky byl popsán s roskovitinem, ale bez vysvětlení mechanismu účinku (Ishimaru et al., 2010).

4.5. Pyrazolo[1,5-*a*]-1,3,5-triaziny

Další zajímavá skupina purinových bioisosterů je postavena na pyrazolo[1,5-*a*]-1,3,5-triazinovém heterocyklu. Jednou z připravených látek byl i roskovitinový bioisoster označovaný jako N&N1 (Bettayeb et al, 2008b; WO/2005/082908). V porovnání s roskovitinem má zvýšenou kinasovou selektivitu, silnější antiproliferační vlastnosti *in vitro* (Bettayeb et al, 2008b) a vyšší účinnost v experimentech na modelu *in vivo* (Popowycz et al, 2009). Poznatky těchto studií rovněž poukazují na potenciál bioisosterní strategie vývoje nových látek vedoucí k překonání limitujících vlastností původních sloučenin, v tomto případě roskovitinu.

4.6. Pyrazolo[1,5-*a*]pyrimidiny

U druhého účinnějšího roskovitinového bioisosteru (látka BS-193; ICEC00167) ze skupiny účinných pyrazolo[1,5-*a*]pyrimidinů nebyly do dnešního dne popsány žádné další biologické účinky mimo inhibice CDK (Heathcote et al., 2010; WO/2008/151304). Důvodem patrně je, že se tato pracovní skupina začala zabývat mnohem účinnějším inhibitorem této série (látka 4k; Heathcote et al., 2010). Látka 4k představuje orálně dostupný nanomolární inhibitor CDK, selektivní pro CDK1/2/5/9, vysoce účinný v modelech *in vivo* (Heathcote et al., 2010).

Jedním z dalších významných pyrazolo[1,5-*a*]pyrimidinů je inhibitor dinaciclib (SCH727965; Paruch et al., 2010). Podobně jako látka 4k vykazuje i dinaciclib selektivitu vůči CDK1/2/5/9 a vylepšené biologické vlastnosti *in vitro* a *in vivo* (Parry et al., 2010). Navíc je u dinaciclibu pozorován výrazný dlouhotrvající terapeutický efekt *in vivo* vznikající po krátkodobém podání látky (Parry et al., 2010). Na základě celkového biologického profilu je v současnosti dinaciclib testován v několika režimech v různých fázích klinických experimentů (Dickson & Schwartz, 2009; clinicaltrials.gov).

Na význačnost této skupiny purinových bioisosterů poukazují i studie, které se zaměřily na hledání selektivního inhibitoru CDK7 (WO/2008/151304; Ali et al., 2009).

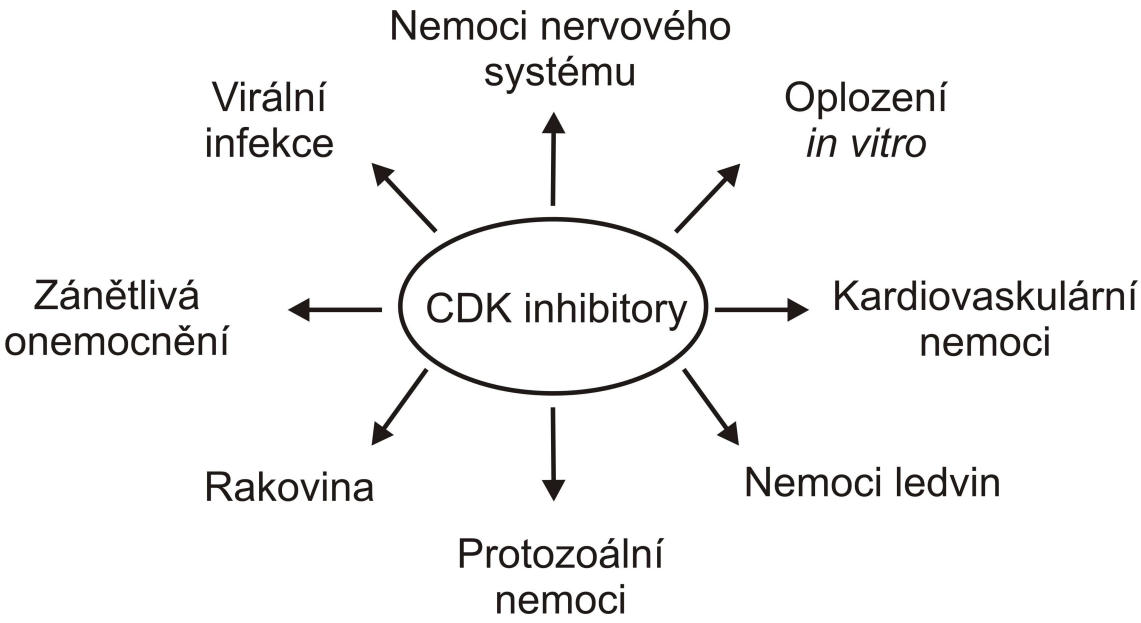
5. Možná další uplatnění inhibitorů CDK

Přestože inhibitory CDK jsou primárně vyvíjeny jako možná protinádorová léčiva, objevují se v současnosti v literatuře i případy možného využití v léčbě jiných onemocnění a poruch (**Obrázek 6**). Zdánlivě nesourodé oblasti však spojují právě CDK.

Nemoci nervového systému (např. Alzheimerova choroba) jsou pravidelně spojovány s narušenou funkcí CDK5, která hraje význačnou roli ve vývoji a diferenciaci neuronů. V nich je CDK5 často zvýšeně aktivována svými vazebnými partnery p29 a p25, kteří vznikají proteolytickou aktivací proteinů p39 a p35, a podílí se tak na hyperfosforylaci cytoskeletálních proteinů, což vede k buněčné smrti. Cílená inhibice CDK5 by proto mohla být základem léčby některých neurodegenerativních onemocnění. V některých případech se poukazuje i na možnou výhodnost společné inhibice CDK5 s dalšími kinasami, CDK1 nebo GSK3β (Knockaert et al., 2002). Samotná inhibice CDK4 se zase jeví jako schůdné řešení při léčbě následků mozkové mrtvice (Osuga et al., 2000).

Virová onemocnění se odvíjí od závislosti viru na metabolickém aparátu hostitelské buňky. Některé viry pro svou replikaci aktivují hostitelské CDK (např. lidský cytomegalovirus), jiné jsou schopny vytvářet svůj vlastní cyklin (např. herpesviry), další pak využívat transkripční aparát hostitelské buňky (HIV) (Knockaert et al., 2002). To jsou hlavní důvody zkoumání inhibitorů CDK jako potenciálních virostatik (Wang et al., 2001; Schang, 2002). Protivirový efekt má i roskovitin, který potlačuje prostřednictvím inhibice CDK2 transkripci viru HIV (Agbottah et al., 2005), replikaci cytomegaloviru a herpesviru (Bresnahan et al., 1997; Schang et al., 2000; Davido et al., 2002). Nicméně potlačení transkripce HIV je spíše spojováno s inhibicí CDK9, která je důležitá pro funkci virálního transaktivátoru Tat. Tento jev prokázaný studií inhibitoru CDK9 flavopiridolu na replikaci HIV (Chao et al., 2000) tak ukázal na potenciální využití inhibitorů CDK9 při léčbě HIV (Canduri et al., 2008; Wang & Fischer, 2008; Németh et al., 2011).

I při léčbě kardiovaskulárních nemocí jakými jsou ateroskleróza, restenosa, nebo hypertrofie srdce se rovněž mohou uplatňovat různě selektivní inhibitory CDK. Srdeční



Obrázek 6: Schéma potenciálního terapeutického a biotechnologického využití CDK (převzato z Knockaert et al., 2002; Galons et al., 2010).

hypertrofie je charakterizována zvětšenou velikostí diferenciovaných srdečních myocytů v důsledku intenzivní transkripce a translace. Urychlení transkripce je spojováno s chronickou aktivací CDK9, která se podílí na fosforylaci CTD RNA polymerasy II a stimuluje tak transkripční elongaci (Kulkarni et al., 2004). Selektivní inhibitory CDK9 by se tak mohly stát novým nástrojem pro potenciální léčbu srdeční hypertrofie. Tato myšlenka je zpracována v jedné z publikací autora (**Příloha VI**). Zabývá se strukturou a biologickou funkcí CDK9 především ve spojitosti s hypertrofií srdce; součástí je i přehled dostupných nízkomolekulárních inhibitorů CDK9, jejich selektivity a dále možnosti racionálního designu dalších specifických inhibitorů CDK9. Nemoci jako ateroskleróza či restenosa jsou charakterizovány zvýšenou proliferací buněk hladkého svalstva. Potlačení jejich abnormálního růstu, které by mohlo najít uplatnění při léčbě restenosy, bylo úspěšně prokázáno např. u inhibitorů CDK flavopiridolu (Ruef et al., 1999; Jaschke et al., 2004), purinu CVT-313 (Brooks et al., 1997) nebo již zmíněného pyrazolo[4,3-*d*]pyrimidinu LGR1406 (Sroka et al., 2010).

Protozoální infekce způsobené jednobuněčnými parazity (*Leishmania, Plasmodium, Trypanosoma*) patří mezi nejzávažnější celosvětová onemocnění. U těchto parazitů bylo pozorováno mnoho společných znaků s nádorovými buňkami, jakými jsou nekontrolovaná proliferace, nezávislost na exogenních faktorech nebo rezistence k apoptose (Fuertes et al., 2008). Tento fakt vedl k předpokladu, že by se látky původně

vyvíjené pro onkologické aplikace mohly uplatnit i jako potenciální antiparazitika (Klinkert & Heussler, 2006; Fuertes et al., 2008). Díky vysoké sekvenční i funkční homologii některých lidských a parazitických CDK se tak podařila prokázat antiparazitická aktivita některých inhibitorů CDK (Harmse et al., 2001; Grant et al., 2004; Reichwald et al., 2008; Xingi et al., 2009). Například flavopiridol je schopen blokovat buněčný cyklus v G2/M fázi prostřednictvím inhibice CRK3 (cdc-2 related kinase) u *Leishmania mexicana* (Hassan et al., 2001). Nicméně existují určité strukturní odlišnosti mezi CDK a CRK (**Tabulka 1**), které by měly umožnit vývoj inhibitorů specifických pro parazita s minimálním vlivem na hostitelskou buňku a podstatně tak zvýšit terapeutický potenciál současně vyvíjených sloučenin. I naší skupinou byla publikována studie zaměřená na hledání potenciálních antileishmaniálních sloučenin mezi 2,6-disubstituovanými puriny a stejně substituovanými pyrazolo[4,3-*d*]pyrimidiny (**Příloha VII**). Tyto látky byly dříve popsány jako inhibitory CDK1 (Moravcová et al., 2003), a proto byly testovány v různých funkčních i interakčních systémech s homologní leishmaniální kinasou CRK3/CYC6 (**Příloha VIII**), důležitým regulátorem buněčného cyklu parazita (Naula et al., 2005). U některých inhibitorů byla prokázána interakce s tímto proteinovým komplexem, která se následně projevila i v inhibici růstu amastigotů *Leishmania donovani*, což vedlo k potvrzení výsledků dřívějších prací (Hassan et al., 2001). Tato publikace tak naznačuje na možnosti využití některých inhibitorů, především účinných pyrazolo[4,3-*d*]pyrimidinů, jako potenciálních antileishmanik a může tak pomoci k vývoji nových antiparasitických sloučenin.

Zánětlivé, imunitní procesy vznikají v buňce jako odezva na její poškození nebo napadení nějakým patogenem. V některých případech obrany však nedochází k vyvolání adekvátní protizánětlivé odpovědi, naopak může docházet k nesprávnému rozpoznání antigenů, jak je tomu u chronických a autoimunitních zánětlivých onemocnění, projevujících se zpravidla výskytem nepřiměřeně proliferujících lymfocytů, eosinofilů a neutrofilů (Leitch et al., 2009). Díky známým antiproliferačním a proapoptotickým vlastnostem inhibitorů CDK se nabízí jejich potenciální využití při léčbě různých typů zánětlivých onemocnění (Rossi et al., 2006; Leitch et al., 2009). V případě roskovitinu se předpokládá, že jeho protizánětlivé vlastnosti jsou navíc podporovány přímou inhibicí NF-κB regulovaných genů, mitogenní dráhy ERK či potlačením exprese Mcl-1 (Leitch et al., 2010; Berberich et al., 2011).

Také u nemoci ledvin označované jako glomerulonefritida, kde dochází k nadměrné proliferaci buněk ledvinových glomerulů, se podařilo ukázat jistou aktivitu

některých inhibitorů CDK, včetně roskovitinu (Clough, 2002; Milovanceva-Popovska et al., 2005; Zoja et al., 2007).

Jednou z méně probádaných aplikací je i využití inhibitorů CDK při *in vitro* oplození a klonování (Knockaert et al., 2002). Použitím roskovitinu bylo například dosaženo vyšší úspěšnosti klonování u telecích embryí (Gibbons te al., 2002), na stejném modelu bylo využito i butyrolactonu I pro potlačení jaderné maturace samičího oocytu při *in vitro* oplození (Ponderato et al., 2001).

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7. Seznam použitých zkratek

APC	Anaphase-promoting complex
AUR	Aurora kinase
Bcl-2	B-cell CLL/lymphoma 2
CDK	Cyclin-dependent kinase
CRK	Cdc2-related kinase
CTD	C-terminal domain
ERK	Extracellular-signal-regulated kinase
GSK3-β	Glycogen synthase kinase 3β
HIV	Human immunodeficiency virus type 1
IC_{50}	50% inhibitory concentration
MAPK	Mitogen-activated protein kinase
MAT1	Ménage à trois 1
Mcl-1	Myeloid-cell leukemia 1
Mdm-2	Murine double minute 2
MITF	Melanocyte lineage transcription factor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PDGFR	Platelet-derived growth factor receptor
PLK	Polo-like kinase
P-TEF	Positive transcription elongation factor
Rb	Retinoblastoma protein
RTK	Receptor tyrosine kinase
mRNA	Messenger ribonucleic acid
shRNA	Short hairspin ribonucleic acid
siRNA	Small interfering ribonucleic acid
TFII	Transcription factor IIF
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis

8. Curriculum Vitae

Osobní údaje

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Dokončené vzdělání

2002-2005, bakalářský program oboru biochemie PřF UP v Olomouci

2005-2007, magisterský program oboru biochemie PřF UP v Olomouci

Pedagogická činnost

2008, 2010 – Laboratorní cvičení z biochemie (KBC/BCHC)

Vedení diplomové práce: Mgr. Eva Řezníčková (obor molekulární a buněčná biologie, obhájeno 2010)

Přehled absolvovaných vědeckých stáží

Structural Biochemistry Group (prof. M. D. Walkinshaw), Centre for Translational and Chemical Biology, School of Biological Sciences, The University of Edinburgh, UK (říjen - prosinec, 2008; leden - duben, 2009).

Zapojení do grantových projektů (řešitel)

1. Analýza markerů buněčného cyklu průtokovou cytometrií, FRVŠ 1743/2011 (G3)

Zapojení do grantovych projektů (odborný pracovník)

1. Modulace signálních a regulačních drah normálních a nádorových buněk, MSM6198959216 (2005-2011)
2. Biologická aktivita syntetických inhibitorů cyklin-dependentních kinas, GACR 2004/08/0511 (2008-2010)
3. AescuLab - systematické laboratorní vzdělávání studentů a pracovníků VaV v oblasti Life Sciences s podporou e-learningu OP VK 2.3 CZ.1.07/2.3.00/09.0018 (2009 - 2012)

Přednášky na zahraničních univerzitách

1. Biochemical and cellular effect of a novel cyclin-dependent kinase inhibitor (15.10.2008 - The University of Edinburgh)
2. CRK1, expression, purification and crystallization (5.12.2008 - The University of Edinburgh)
3. Leishmanial cyclin-dependent related kinases (CRKs) (27.3.2009 - The University of Glasgow)
4. Leishmanial cdc-2 related kinases (CRKs) as a potencial target of a novel kinase inhibitors (3.7.2009 - Protein Kinase Research, Helsinky)

Seznam publikovaných prací

1. Paprskářová M, Kryštof V, **Jorda R**, Džubák P, Hajdúch M, Węsierska-Gądek J, Strnad M. Functional p53 in cells contributes to the anticancer effect of the cyclin-dependent kinase inhibitor roscovitine. *J Cell Biochem*. 2009 Jun 1;107(3):428-37.
2. Kryštof V, Chamrád I, **Jorda R**, Kohoutek J. Pharmacological targeting of CDK9 in cardiac hypertrophy, *Med Res Rev*. 2010 Jul;30(4):646-66.
3. Frébortová J, Novák O, Frébort I, **Jorda R.** Degradation of cytokinins by maize cytokinin dehydrogenase is mediated by free radicals generated by enzymatic oxidation of natural benzoxazinones *Plant J*. 2010 Feb 1;61(3): 467–81.
4. **Jorda R**, Havlíček L, McNae IW, Walkinshaw MD, Voller J, Šturc A, Navrátilová J, Kuzma M, Mistrík M, Bártek J, Strnad M, Kryštof V. Synthesis and biological evaluation of 3,5,7-trisubstituted pyrazolo[4,3-*d*]pyrimidines, novel selective inhibitors of cyclin-dependent kinases with antiproliferative activity. *J Med Chem*. 2011, v tisku, http://dx.doi.org/10.1021/jm200064p.

Práce v recenzním řízení

1. Kryštof V, Rárová L, Liebl J, Zahler S, **Jorda R**, Voller J, Cankař P. The selective P-TEFb inhibitor CAN508 targets angiogenesis. *ACS Chem Biol*. 2011, v recenzním řízení.
2. **Jorda R**, Sacerdoti-Sierra N, Voller J, Havlíček L, Kráčalíková K, Nowicki MW, Nasereddin A, Kryštof V, Strnad M, Walkinshaw MD, Jaffe ChL. Antileishmanial activity of disubstituted purines and related pyrazolo[4,3-*d*]pyrimidines. *Bioorg Med Chem Lett*. 2011, v recenzním řízení.

Konferenční příspěvky

1. **Jorda R**, Kryštof V, Havlíček L, Strnad M: Biochemical and cellular effects of a novel cyclin-dependent kinase inhibitor. *20th Meeting of the European-Association-for-Cancer-Research*, July 5–8, 2008, Lyon, France, *European Assoc Canc Res*; EJC SUPPLEMENTS Vol 6: pp 84-84.
2. **Jorda R**, Nowicki M W, Charles Ch L, Havlíček L, Kryštof V, Strnad M, Walkinshaw M D. Antileishmanial activity of disubstituted purines and related pyrazolo[4,3-*d*]pyrimidines. *6th International Conference: INHIBITORS OF PROTEIN KINASES*, June 27-July 1, 2009, Warsaw, Poland (in *Biochimica Polonica*, 56 Supplement 1/2009).
3. Řezníčková E, **Jorda R**, Kryštof V, Havlíček L, Strnad M: A novel pyrazolo[4,3-*d*]pyrimidine inhibitor of cyclin-dependent kinases: antiproliferative and proapoptotic effects. *22nd EORTC-NCI-AACR symposium on Molecular targets and Cancer Therapeutics*, Nov 16-18, 2010, Berlin, Germany, *European Assoc Canc Res*; EJC SUPPLEMENTS Vol 8: pp 160-160.
4. **Jorda R**, Zatloukal M, Řezníčková E, Vymětalová L, Kryštof V, Strnad M: Novel derivatives of cyclin-dependent kinase inhibitor roscovitine. *Cell Cycle Regulators/Inhibitors and Cancer*, Feb 5-8, 2011, Vienna, Austria.
5. Řezníčková E, **Jorda R**, Kryštof V, Havlíček L, Strnad M: Antiproliferative and proapoptotic effects of a new pyrazolo[4,3-*d*]pyrimidine inhibitor of cyclin-dependent kinases. *The Student Scientific Conference on Cancer Research*, Apr 7-8, 2011, Brno, Czech Republic.

9. Seznam příloh

I. Paprskářová M, Kryštof V, **Jorda R**, Džubák P, Hajdúch M, Węsierska-Gądek J, Strnad M. Functional p53 in cells contributes to the anticancer effect of the cyclin-dependent kinase inhibitor roscovitine. *J Cell Biochem*. 2009 Jun 1;107(3):428-37.

II. Kryštof V, Rárová L, Liebl J, Zahler S, **Jorda R**, Voller J, Cankař P. The selective P-TEFb inhibitor CAN508 targets angiogenesis. *ACS Chem Biol*. 2011, v recenzním řízení.

III. **Jorda R**, Zatloukal M, Řezníčková E, Vymětalová L, Kryštof V, Strnad M: Novel derivatives of cyclin-dependent kinase inhibitor roscovitine. *Cell Cycle Regulators/Inhibitors and Cancer*, Feb 5-8, 2011, Vienna, Austria.

IV. **Jorda R**, Havlíček L, McNae IW, Walkinshaw MD, Voller J, Šturc A, Navrátilová J, Kuzma M, Mistrík M, Bártek J, Strnad M, Kryštof V. Synthesis and biological evaluation of 3,5,7-trisubstituted pyrazolo[4,3-*d*]pyrimidines, novel selective inhibitors of cyclin-dependent kinases with antiproliferative activity. *J Med Chem*. 2011, v tisku, http://dx.doi.org/10.1021/jm200064p.

V. Řezníčková E, **Jorda R**, Kryštof V, Havlíček L, Strnad M: A novel pyrazolo[4,3-*d*]pyrimidine inhibitor of cyclin-dependent kinases: antiproliferative and proapoptotic effects. *22nd EORTC-NCI-AACR symposium on Molecular targets and Cancer Therapeutics*, Nov 16-18, 2010, Berlin, Germany, *European Assoc Canc Res*; EJC SUPPLEMENTS Vol 8: pp 160-160.

VI. Kryštof V, Chamrád I, **Jorda R**, Kohoutek J. Pharmacological targeting of CDK9 in cardiac hypertrophy, *Med Res Rev*. 2010 Jul;30(4):646-66.

VII. **Jorda R**, Sacerdoti-Sierra N, Voller J, Havlíček L, Kráčalíková K, Nowicki MW, Nasereddin A, Kryštof V, Strnad M, Walkinshaw MD, Jaffe ChL. Antileishmanial activity of disubstituted purines and related pyrazolo[4,3-*d*]pyrimidines. *Bioorg Med Chem Lett*. 2011, v recenzním řízení.

VIII. **Jorda R**, Nowicki M W, Charles Ch L, Havlíček L, Kryštof V, Strnad M, Walkinshaw M D. Antileishmanial activity of disubstituted purines and related pyrazolo[4,3-*d*]pyrimidines. *6th International Conference: INHIBITORS OF PROTEIN KINASES*, June 27-July 1, 2009, Warsaw, Poland (in *Biochimica Polonica*, 56 Supplement 1/2009).

10. Potvrzení spoluautorů o podílu uchazeče na společných pracích

I. Paprskářová M, Kryštof V, **Jorda R**, Džubák P, Hajdúch M, Węsierska-Gądek J, Strnad M. Functional p53 in cells contributes to the anticancer effect of the cyclin-dependent kinase inhibitor roscovitine. *J Cell Biochem*. 2009 Jun 1;107(3):428-37.

Radek Jorda se podílel na analýze exprese proteinů pomocí western blottingu a na přípravě vzorků pro měření pomocí průtokové cytometrie.

II. Kryštof V, Rárová L, Liebl J, Zahler S, **Jorda R**, Voller J, Cankař P. The selective P-TEFb inhibitor CAN508 targets angiogenesis. *ACS Chem Biol.* 2011, v recenzním řízení.

Radek Jorda měřil a vyhodnocoval vliv látky CAN508 na celkovou hladinu mRNA a RNA v ovlivněných buňkách MCF-7 a HMEC-1.

III. **Jorda R**, Zatloukal M, Řezníčková E, Vymětalová L, Kryštof V, Strnad M: Novel derivatives of cyclin-dependent kinase inhibitor roscovitine. *Cell Cycle Regulators/Inhibitors and Cancer*, Feb 5-8, 2011, Vienna, Austria.

Radek Jorda navrhoval většinu experimentů, prováděl cytometrické analýzy a detekci exprese proteinů pomocí western blottingu. Výrazně se podílel na psaní textu a grafickém zpracování. Osobně prezentoval data na uvedené konferenci.

IV. **Jorda R**, Havlíček L, McNae IW, Walkinshaw MD, Voller J, Šturc A, Navrátilová J, Kuzma M, Mistrík M, Bártek J, Strnad M, Kryštof V. Synthesis and biological evaluation of 3,5,7-trisubstituted pyrazolo[4,3-*d*]pyrimidines, novel selective inhibitors of cyclin-dependent kinases with antiproliferative activity. *J Med Chem*. 2011, v tisku, http://dx.doi.org/10.1021/jm200064p.

Radek Jorda se podílel na většině prováděných experimentů, prováděl cytometrické analýzy, biochemické detekce aktivity kaspas, prováděl imunofluorescenční analýzu a stanovení exprese proteinů pomocí western blottingu. Výrazně se podílel na psaní rukopisu, odesílal jej do redakce a vyřizoval příslušnou korespondenci.

V. Řezníčková E, **Jorda R**, Kryštof V, Havlíček L, Strnad M: A novel pyrazolo[4,3-*d*]pyrimidine inhibitor of cyclin-dependent kinases: antiproliferative and proapoptotic effects. *22nd EORTC-NCI-AACR symposium on Molecular targets and Cancer Therapeutics*, Nov 16-18, 2010, Berlin, Germany, *European Assoc Canc Res*; EJC SUPPLEMENTS Vol 8: pp 160-160.

Radek Jorda navrhoval většinu experimentů, zavedl a optimalizoval dvouparametrovou analýzu exprese vybraných regulátorů buněčného cyklu pomocí průtokové cytometrie a metodu stanovení mRNA a RNA pomocí radioaktivně značených nukleosidů. Výrazně se podílel na psaní textu a grafickém zpracování. Osobně prezentoval data na uvedené konferenci.

VI. Kryštof V, Chamrád I, **Jorda R**, Kohoutek J. Pharmacological targeting of CDK9 in cardiac hypertrophy, *Med Res Rev*. 2010 Jul;30(4):646-66.

Radek Jorda provedl kompletní literární rešerši dostupných inhibitorů CDK9, provedl její zpracování a podílel se na psaní rukopisu.

VII. **Jorda R**, Sacerdoti-Sierra N, Voller J, Havlíček L, Kráčalíková K, Nowicki MW, Nasereddin A, Kryštof V, Strnad M, Walkinshaw MD, Jaffe ChL. Antileishmanial activity of disubstituted purines and related pyrazolo[4,3-*d*]pyrimidines. *Bioorg Med Chem Lett*. 2011, v recenzním řízení.

Radek Jorda navrhoval většinu experimentů a syntézu některých sloučenin, experimentálně prováděl purifikaci rekombinantní proteinkinasy CRK3/CYC6, podílel se na optimalizaci kinasové inhibični reakce. Výrazně se podílel na psaní rukopisu, odesílal jej do redakce a vyřizoval příslušnou korespondenci.

VIII. **Jorda R**, Nowicki M W, Charles Ch L, Havlíček L, Kryštof V, Strnad M, Walkinshaw M D. Antileishmanial activity of disubstituted purines and related pyrazolo[4,3-*d*]pyrimidines. *6th International Conference: INHIBITORS OF PROTEIN KINASES,* June 27-July 1, 2009, Warsaw, Poland (in *Biochimica Polonica*, 56 Supplement 1/2009).

Radek Jorda navrhoval a prováděl většinu experimentů, prováděl purifikaci rekombinantní proteinkinasy CRK3/CYC6 a testoval její inhibici s nově vyvíjenými inhibitory v různých funkčních i interakčních systémech (thermal-shift assay, surface plasmon resonance). Osobně prezentoval data na uvedené konferenci.

Souhlasím s uvedenými informacemi:

Prof. Ing. Miroslav Strnad, DrSc. .....

Doc. RNDr. Vladimír Kryštof, Ph.D. .....

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13 April 2011

To whom it may concern

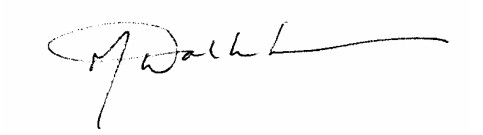
We confirm that we have collaborated with Radek Jorda on the following papers and he made a significant contribution to this work.

1. Jorda R, Havlícek L, McNae IW, Walkinshaw MD, Voller J, Sturc A, Navrátilová J, Kuzma M, Mistrik M, Bartek J, Strnad M, Krystof V. Synthesis and biological evaluation of 3,5,7-trisubstituted pyrazolo[4,3-d]pyrimidines, novel selective inhibitors of cyclin-dependent kinases with antiproliferative activity. J Med Chem. 2011, in press, http://dx.doi.org/10.1021/jm200064p.

2. Jorda R, Sacerdoti-Sierra N, Voller J, Havlícek L, Krácalíková K, Nowicki MW, Nasereddin A, Kryštof V, Strnad M, Walkinshaw MD, Jaffe ChL. Antileishmanial activity of disubstituted purines and related pyrazolo [4,3-d]pyrimidines. Bioorg Med Chem Lett. 2011, reviewed.

3. Jorda R, Nowicki M W, Charles Ch L, Havlícek L, Kryštof V, Strnad, M, Walkinshaw M D. Antileishmanial activity of disubstituted purines and related pyrazolo[4,3-d]pyrimidines. 6th International Conference: INHIBITORS OF PROTEIN KINASES, June 27-July 1, 2009, Warsaw, Poland(in Biochimica Polonica, 56 Supplement 1/2009).

Yours sincerely



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Ihr Zeichen, Ihre Nachricht vom
xxxx

Unser Zeichen
xxxx

München, 14.04.2011

Hereby we confirm that Radek Jorda contributed to preparation of the following manuscript:

Kryštof V, Rárová L, Liebl J, Zahler S, Jorda R, Voller J, Cankař P. The selective P-TEFb inhibitor CAN508 targets angiogenesis. Submitted to ACS Chem Biol.

Dr. Stefan Zahler

Dr. Johanna Liebl

The Faculty of Medicine הפקולטה לרפואה
ביה"ס לרפואה של האוניברסיטה העברית והדסה

האוניברסיטה העברית בירושלים
The Hebrew University of Jerusalem

המכון למחקר רפואי - ישראל-קנדה
המחלקה למיקרוביולוגיה וגנטיקה מולקולרית
Department of Microbiology and Molecular Genetics

April 14, 2011

Institute for Medical Research Israel-Canada
INNOVATIONS TOUCHING LIVES

To whom it may Concern:

This letter certifies that Radek Jorda made major contributions to the design, work and writing of the following research publications:

1). Jorda R, Sacerdoti-Sierra N, Voller J, Havlíček L, Kráčalíková K, Nowicki MW, Nasereddin A, Kryštof V, Strnad M, Walkinshaw MD, Jaffe ChL. Antileishmanial activity of disubstituted purines and related pyrazolo[4,3-d]pyrimidines. Bioorg Med Chem Lett. 2011, reviewed.

2). Jorda R, Nowicki M W, Charles Ch L, Havlíček L, Kryštof V, Strnad M, Walkinshaw M D. Antileishmanial activity of disubstituted purines and related pyrazolo[4,3-d]pyrimidines. 6th International Conference: INHIBITORS OF PROTEIN KINASES, June 27-July 1, 2009, Warsaw, Poland (in Biochimica Polonica, 56 Supplement 1/2009).

Sincerely yours,

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PŘÍLOHA I.

Paprskářová M, Kryštof V, **Jorda R**, Džubák P, Hajdúch M, Węsierska-Gądek J, Strnad M. Functional p53 in cells contributes to the anticancer effect of the cyclin-dependent kinase inhibitor roscovitine. *J Cell Biochem*. 2009 Jun 1;107(3):428-37.

ARTICLE
Journal of Cellular Biochemistry 107:428–437 (2009)

Journal of Cellular Biochemistry

Functional p53 in Cells Contributes to the Anticancer Effect of the Cyclin-Dependent Kinase Inhibitor Roscovitine

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ABSTRACT

Inhibitors of cyclin-dependent kinases (CDKs) undergoing clinical trials as anticancer agents usually target several CDKs in cells. Some of them are also able to increase cellular levels of p53 protein and to activate p53-regulated transcription. To define the role of p53 in the anticancer effect of selective CDK inhibitors, two related compounds roscovitine and olomoucine II were studied. Roscovitine differs functionally from its congener olomoucine II only in the selectivity towards transcriptional CDK9. Action of both compounds on proliferation, cell-cycle progression, and apoptosis was examined in RPMI-8226 cells expressing the temperature-sensitive mutant of p53 and in MCF-7 cells with wild-type p53. Both compounds blocked proliferation, decreased phosphorylation of RNA polymerase II, downregulated antiapoptotic protein Mcl-1 in both cell lines in a dose-dependent manner, and also activated p53 in MCF-7 cells. Moreover, we showed that the anticancer efficiency of CDK inhibitors was enhanced by active p53 in RPMI-8226 cells kept at permissive temperature, where downregulation of Mcl-1, fragmentation of PARP-1, and increased caspase-3 activity was detected with lower doses of the compounds. The results confirm that functional p53 protein may enhance the anticancer activity of roscovitine that could be beneficial for anticancer therapy. J. Cell. Biochem. 107: 428–437, 2009.

KEY WORDS: APOPTOSIS; CYCLIN-DEPENDENT KINASE; OLOMOUCINE II; p53; ROSCOVITINE

Several small molecular inhibitors of cyclin-dependent kinases (CDK) are currently undergoing clinical evaluation as a new generation of anticancer agents [Malumbres and Barbacid, 2001; Fischer and Gianella-Borradori, 2005; Malumbres et al., 2008]. Activities of CDKs are usually elevated in cancers, due to both genetic and epigenetic alterations of CDKs themselves or of the proteins they interact with, including activating cyclins and phosphatases (cdc25), inhibitors (INK4, CIP/KIP), and substrates (pRBs and E2Fs) [Malumbres and Barbacid, 2001]. These alterations provide a basis for pharmacological interventions by synthetic compounds specifically targeting hyperactive cell-cycle regulators in cancer cells.

The 2,6,9-trisubstituted purine derivative roscovitine belongs to the most advanced anti-CDK drugs [Meijer and Raymond, 2003; Fischer and Gianella-Borradori, 2005]. It has been long assumed that roscovitine, like many other CDK inhibitors, arrests and kills transformed cells as a direct consequence of the inactivation of cell-cycle CDKs. However, several recent articles also point to an involvement of the transcriptional CDK7 and CDK9 [Demidenko and Blagosklonny, 2004; MacCallum et al., 2005; Cai et al., 2006a; Gao et al., 2006; Hajduch et al., 2007]. Besides direct inhibition of CDK2 and CDK1 by roscovitine associated with cell-cycle arrest in G_1/S or G_2/M transitions, there are indirect mechanisms by which roscovitine can block the proliferation. For example, interaction

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Grant Agency of the Czech Republic; Grant number: 204/08/0511; Grant sponsor: Ministry of Education, Youth and Sports of the Czech Republic; Grant number: MSM6198959216.

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Received 15 September 2008; Accepted 17 February 2009 • DOI 10.1002/jcb.22139 • 2009 Wiley-Liss, Inc.
Published online 23 March 2009 in Wiley InterScience (www.interscience.wiley.com).

with constitutively active CDK7, which phosphorylates the threonine residue in the activation loop of CDKs (e.g., Thr161 in CDK1), reduces levels of active CDKs [Hajduch et al., 2007]. An unphosphorylated activation loop closes the catalytic site and the activities of CDK1 and CDK2 then decrease. But most of the indirect mechanisms stem from changed gene expression profiles following treatment with roscovitine. Like several other CDK inhibitors, roscovitine also reduces levels of cyclins D1, B, and A, while simultaneously upregulating expression of the natural CDK inhibitor $p21^{waf1}$ [Kotala et al., 2001; Lu et al., 2001; Whittaker et al., 2004; Lacrima et al., 2005]. More recently, cDNA microarray analysis demonstrated that other important cell-cycle regulatory genes also change expression. For example, markedly reduced mRNA expression was found for mitotic regulators, including Aurora A and B, Polo-like kinase, Wee1 and Cdc25C [Whittaker et al., 2007]. These alterations also contribute to cell-cycle arrest and accumulating evidence suggests that roscovitine-sensitive CDK7 and CDK9 are responsible for the reduced expression [Whittaker et al., 2004; Lacrima et al., 2005; MacCallum et al., 2005].

CDKs 7 and 9 stimulate initiation and elongation of mRNA transcription, respectively, by phosphorylating the C-terminal domain of RNA polymerase II. Suppressed transcription and decreased phosphorylation of RNA polymerase II were documented in cells treated not only with roscovitine, but also with other CDK inhibitors [Ljungman and Paulsen, 2001; Demidenko and Blagosklonny, 2004; Whittaker et al., 2004]. The transcripts that are most sensitive to CDK9 inhibition are those with short half-lives such as cell-cycle regulators, mitotic kinases, NF-κB-regulated genes, and apoptosis regulators, because their levels decrease rapidly when initiation and elongation of transcription are inhibited. One of them is MDM2, an E3-ubiquitin ligase negatively regulating tumor suppressor p53 protein level [Momand et al., 1992]. p53 is a stress-inducible transcription factor that controls hundreds of genes involved in a variety of cellular functions, including cell-cycle arrest, DNA repair, and apoptosis [Wei et al., 2006]. Due to amplification or overexpression, MDM2 contributes to cancer development. Disruption of the interaction between p53 and MDM2 provides a rationale for therapeutic p53 activation in cancers bearing wild-type p53 gene. Interestingly, an alternative mechanism of anticancer activity of roscovitine and some other CDK inhibitors is based on accumulation of tumor suppressor protein p53 in nuclei in a transcriptionally active form [David-Pfeuty, 1999; Kotala et al., 2001; Lu et al., 2001; Wojciechowski et al., 2003; Demidenko and Blagosklonny, 2004; Wesierska-Gadek et al., 2005]. The effect may be due to downregulation of MDM2, as reduced expression of MDM2 helps to stabilize p53 level. An increase of p53-dependent transcription is also frequently observed, with the concomitant accumulation of $p21^{waf1}$, one of the endogenous CDK inhibitors [Kotala et al., 2001; Lu et al., 2001].

In this study, we describe effects of roscovitine in human cancer cell lines differing in p53 status. As shown recently, it has a greater potency against p53 wild-type cell lines than against p53 mutant cell lines [McClue et al., 2002; Krystof et al., 2005]. To define the role of p53 in the anticancer effect of CDK inhibitors, we selected the RPMI-8226 cell line, expressing a temperature-sensitive mutant of p53 that allowed us to study the function of p53 in the same genetic background under different temperatures, and the MCF-7 cell line with wild-type p53. Structurally distinct compounds with surmised functional similarities (i.e., with a common molecular target) are often used for understanding and validating their molecular impact. However, although these distinct compounds usually help to reveal off-target effects, they may not only differ substantially in affinity to the primary target, but also in other features such as polarity, stability, and cellular uptake that could unknowingly limit the comparative work. Therefore, our approach has been based on use of two structurally related compounds, the functional analogues roscovitine and olomoucine II, that strongly and equally inhibit kinases CDK2 and CDK7. Importantly for this study, both congeners differ in selectivity towards transcriptional CDK9, which is more sensitive to olomoucine II [Krystof et al., 2005]. Based on our data, we demonstrate a crucial role of CDK9 in the upregulation of p53 and p53-connected anticancer properties of roscovitine. Our results provide another insight into the cellular effects of roscovitine and support its therapeutic application in cancers with wild-type p53.

MATERIALS AND METHODS

DRUGS AND ANTIBODIES

R-roscovitine (6-benzylamino-2-[(*R*)-(1-ethyl-2-hydroxyethylamino)]-9-isopropylpurine) and *R*-olomoucine II (6-(2-hydroxybenzylamino)-2-{[(1*R*)-(hydroxymethyl)propyl]amino}-9-isopropylpurine) were synthesized according to published procedures [Havlicek et al., 1997; Krystof et al., 2002]. For cell treatment, compounds were made up as 100 mM stocks in dimethylsulfoxide (DMSO) and diluted prior to application in culture media. The maximum concentration of DMSO in the medium never exceeded 0.1%.

The following specific antibodies were used to detect the relevant proteins: anti-p53 (clone DO-1), anti-cyclin D1, anti-CDK4, anti-MDM2 (clone 2A9), anti-PCNA (clone PC-10), and anti-$p21^{waf1}$ (clone 118, all gifts from B. Vojtesek); anti-α-tubulin (clone DM1A; Sigma–Aldrich, Prague, Czech Republic); anti-Mcl-1 (clone S-19), anti-PARP-1 (clone F-2), anti-cyclin E (clone HE12), and anti-bcl-2 (clone 100; all from Santa Cruz Biotechnology, Santa Cruz, CA); anti-RNA polymerase II (clone ARNA-3; Millipore, Prague, Czech Republic); anti-RNA polymerase II phosphorylated on Ser-5 (clone H14) and anti-RNA polymerase II phosphorylated on Ser-2 (clone H5, both from Abcam, Cambridge, UK); fluorescein-labeled anti-BrdU (Becton–Dickinson, Prague, Czech Republic).

CELL CULTURES AND VIABILITY ASSAY

Human MCF-7 breast carcinoma and RPMI-8226 multiple myeloma cell lines purchased from American Type Culture Collection were maintained in a humidified CO_2 incubator at 37 °C in DMEM or RPMI medium, respectively, supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Viability assays were performed in triplicates in 96-well microtiter plates with cells seeded at densities appropriate for their respective cell sizes and doubling times. Twelve hours after seeding, tested compounds in threefold dilutions were added in triplicate and the treatment lasted continuously for 72 h. At the end of this period, the cells were fed for 1 h with Calcein AM solution

(Invitrogen, Carlsbad, CA) and the fluorescence of the live cells was measured at 485/538 nm (ex/em) with a Fluoroskan Ascent microplate reader (Labsystems, Helsinki, Finland). The drug concentrations lethal to 50% of the cells (IC_{50} values) were determined from the dose–response curves using GraphPad Prism software (GraphPad Software, La Jolla, CA).

BrdU INCORPORATION AND CELL-CYCLE ANALYSIS

Before harvesting, cells were pulse labeled with 10 μM 5-bromo-2′ deoxyuridine (BrdU) for 30 min. The cells were trypsinized, fixed with ice-cold 70% ethanol, incubated on ice for 30 min, washed with PBS, and resuspended in 2 M HCl for 30 min at room temperature to denature their DNA. Following neutralization with 0.1 M $Na_2B_4O_7$, the cells were washed with PBS containing 0.5% Tween-20 and 1% BSA. They were then stained with fluorescein-labeled anti-BrdU antibody for 30 min at room temperature in the dark. The cells were then washed with PBS, incubated with propidium iodide (0.1 mg/ml) and RNAse A (0.5 mg/ml) for 1 h at room temperature in the dark, and finally analyzed by flow cytometry using a 488 nm single beam laser (FACSCalibur; Becton–Dickinson).

CASPASES-3/7 ASSAY

Treated cells were harvested by centrifugations and homogenized in an extraction buffer (10 mM KCl, 5 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.2% CHAPS, inhibitors of proteases, pH 7.4) on ice for 20 min. The homogenates were clarified by centrifugation at 10,000*g* for 20 min at 4°C, the proteins were quantified by the Bradford method and diluted to the same concentration. Lysates were then incubated for 1 h with 100 μM Ac-DEVD-AMC as substrate (Sigma–Aldrich) in an assay buffer (25 mM PIPES, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, pH 7.3). For negative controls, the lysates were supplemented with 100 μM Ac-DEVD-CHO as a caspase-3/7 inhibitor (Sigma–Aldrich). The fluorescence of the product was measured using a Fluoroskan Ascent microplate reader (Labsystems) at 346/442 nm (ex/em).

SDS–POLYACRYLAMIDE GEL ELECTROPHORESIS AND IMMUNOBLOTTING

For immunoblotting, harvested cells were lysed in RIPA buffer (20 mM Tris–HCl, pH 7.4, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 30 μM PMSF, 1 mM DTT, 10 μg/ml of aprotinin and leupeptin). Proteins in lysates were quantified by the Bradford method and then diluted with Laemmli electrophoresis buffer. Proteins were then separated on SDS-polyacrylamide gels, transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Prague, Czech Republic) and stained with Ponceau S to check equal protein loading. The membranes were blocked with 5% low-fat milk and 0.1% Tween-20 in PBS or in 3% BSA and 0.1% Tween-20 in TBS (for the detection of phosphoproteins), respectively, for 2 h and probed with the specific primary antibodies overnight. After washing in PBS/TBS and PBS/TBS with 0.1% Tween-20, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies and vizualized with chemiluminescent detection reagent ECL+ (Amersham Biosciences, Prague, Czech Republic). To confirm equal protein loading, immunodetection was performed with the anti-α-tubulin monoclonal antibody.

RESULTS

ANTIPROLIFERATE EFFECTS OF ROSCOVITINE AND OLOMOUCINE II

The MCF-7 breast cancer and multiple myeloma RPMI-8226 cell lines were treated with the CDK inhibitors roscovitine and olomoucine II, and the percentage of surviving cells was measured by the Calcein AM viability assay. Cells were incubated with increasing concentrations of both drugs for 72 h and then the IC_{50} values were determined. As shown in Figure 1, neither roscovitine nor olomoucine II affected viability of cells at low concentrations

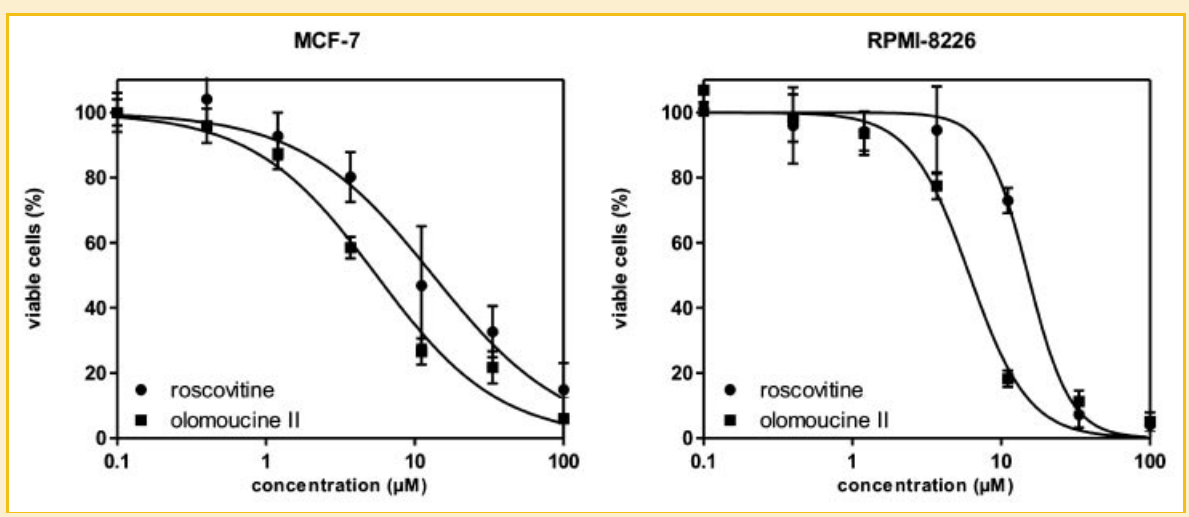

Fig. 1. Roscovitine and olomoucine II reduce the number of living cells. Human MCF-7 breast cancer and RPMI-8226 multiple myeloma cell lines were treated for 72 h with increasing concentrations of roscovitine and olomoucine II. Then, the number of viable cells was determined by a Calcein AM assay. Results represent the average ± SD for three independent experiments.

over the 72 h incubation period. The IC_{50} values are 15.2 μM for roscovitine and 6.1 μM for olomoucine II in RPMI-8226 cells. The sensitivity of the MCF-7 cell line is comparable to that of multiple myeloma; the IC_{50} of roscovitine is 13.3 and 5.5 μM for olomoucine II in MCF-7 cells. The results show that olomoucine II is at least twofold more effective than roscovitine in both cell lines.

CDK INHIBITORS ARREST CELL CYCLE IN MULTIPLE PHASES

To further test the antiproliferative effects of CDK inhibitors, we treated both cell lines with either roscovitine or olomoucine II for 24 h and analyzed their dose-dependent effect on the cell division cycle. The cells, pulse labeled with BrdU, were doubly stained with PI and anti-BrdU antibody and analyzed by flow cytometry. As shown in Figure 2, CDK inhibitors arrested both cell lines in G_2/M phases and decreased the size of their S-phase populations. In addition, RPMI-8226 cells also partially accumulated in the G_1 phase upon treatment with higher concentrations of both inhibitors. Importantly, both CDK inhibitors also markedly decreased the population of cells actively replicating DNA (i.e., BrdU-positive cells).

ROSCOVITINE AND OLOMOUCINE II INDUCE CASPASE-DEPENDENT CELL DEATH

The analysis of the DNA profiles of RPMI-8226 myeloma cells revealed an accumulation of sub-G_1 cells treated with both CDK inhibitors indicating that cells undergo apoptosis. The appearance of hypoploid cells is usually attributable to caspase-dependent fragmentation of chromatin. Therefore, we determined the activity of caspase-3/7 in RPMI-8226 cells exposed to roscovitine or olomoucine II using a fluorogenic substrate Ac-DEVD-AMC. In the first series of experiments, cells were treated in a time-dependent manner with fixed drug concentrations exceeding their IC_{50} values (40 μM roscovitine and 20 μM olomoucine II). Roscovitine strongly induced the activity of caspase-3/7; after treatment for only 3 h a threefold increase of the effector caspases was observed compared with the untreated control; this increased to a sevenfold increase after 6 h (Fig. 3A). Unlike roscovitine, olomoucine II only weakly affected the activity of caspases-3/7; a threefold enhancement of the activity was detected after 6 h and after longer treatment the caspase-3/7 activity decreased.

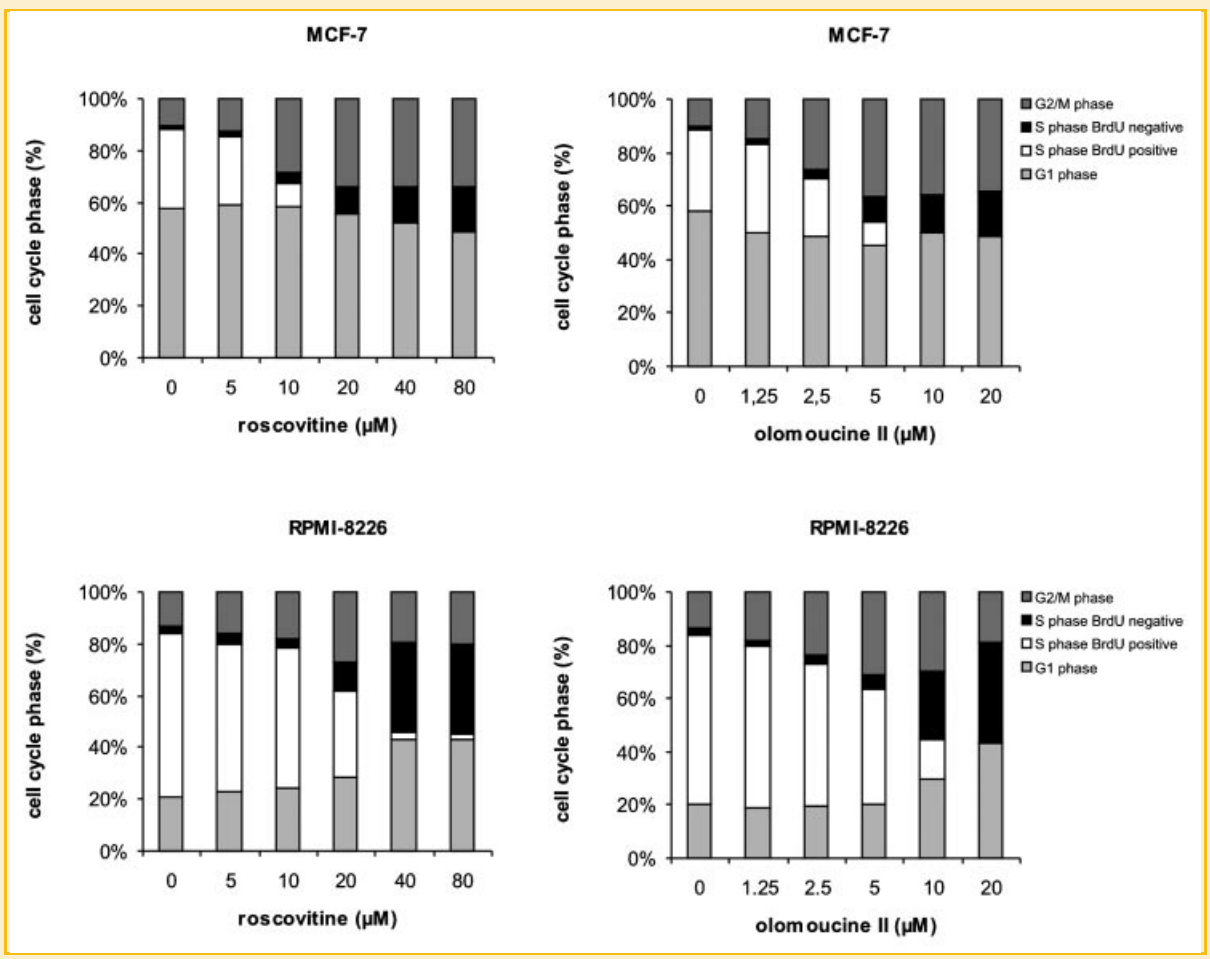

Fig. 2. Roscovitine and olomoucine II induce cell-cycle arrest. MCF-7 and RPMI-8226 cells were treated for 24 h with increasing concentrations of roscovitine and olomoucine II. Half an hour before the end point, BrdU was added to the culture media. The cells were collected, fixed, and stained with propidium iodide and anti-BrdU antibodies. DNA content and the percentage of BrdU-labeled cells were quantified by flow cytometry.

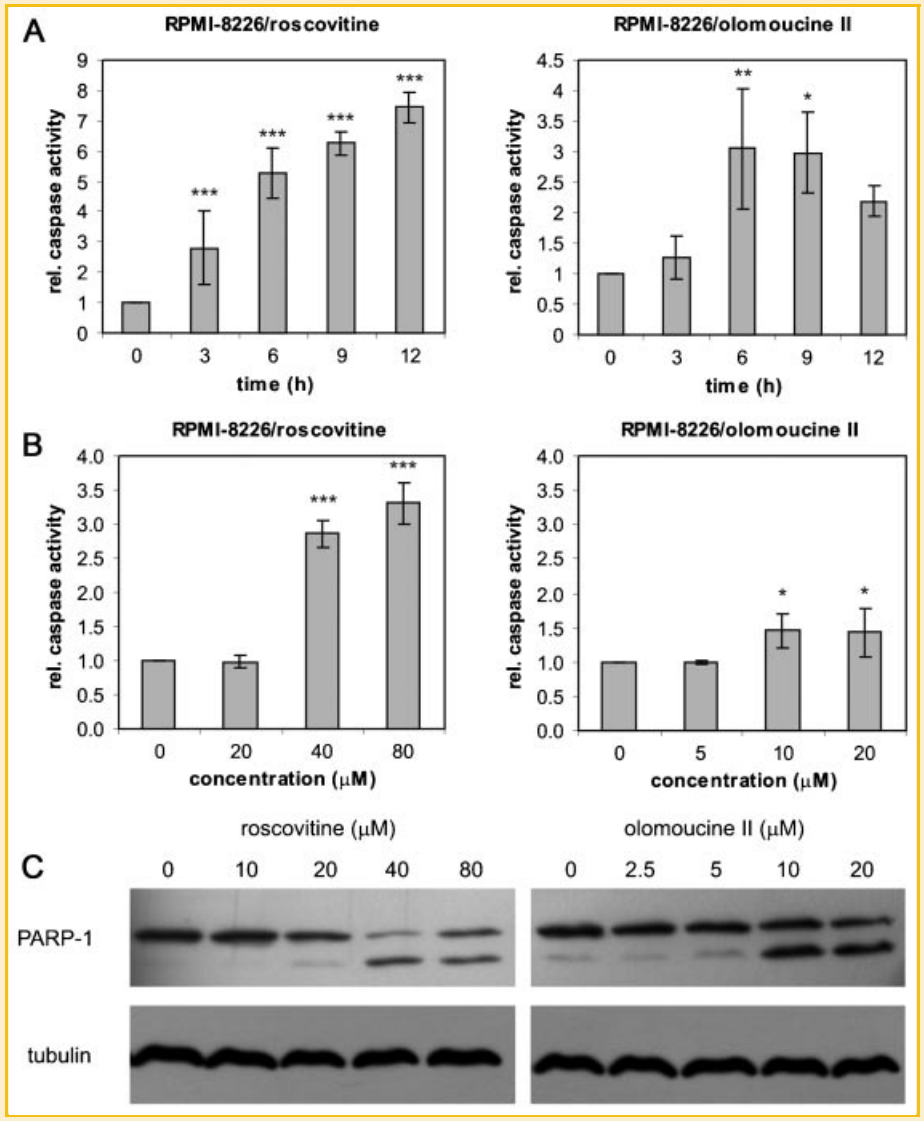

Fig. 3. Roscovitine and olomoucine II cause cell death. RPMI-8226 cell line was treated in a time (A) or concentration-dependent (B,C) manner with roscovitine and olomoucine II, harvested and whole cell lysates were prepared. The activities of caspases-3/7 were measured using specific fluorogenic peptide substrate Ac-DEVD-AMC (A,B), or the fragmentation of PARP-1 was detected by immunoblotting (C). The significance (treatment vs. control) was determined using Dunnett's multiple comparison test: $^{*}P < 0.05$ (significant); $^{**}P < 0.01$ (very significant); $^{***}P < 0.001$ (extremely significant).

In a concentration-dependent experiment, the cells were treated for 24 h. As shown in Figure 3B, 40 and 80 μM roscovitine induced a strong activation of procaspases-3/7, whereas olomoucine II activated procaspases-3/7 only weakly in RPMI-8226 cells.

Monitoring of the cleavage of PARP-1, a nuclear target of caspase-3, confirmed the above results. An appearance of the caspase-3-cleaved PARP-1 fragment at 89 kDa after cell exposure to 40 μM roscovitine was associated with a diminution of its full-length form (Fig. 3C; left panel). Fragmentation of PARP-1 was also detected after treatment of RPMI-8226 cells with olomoucine II at higher doses (10 and 20 μM). However, the intensity of full-length PARP-1 remained almost unchanged (Fig. 3C; right panel).

Taken together, our results clearly evidence that roscovitine strongly activates pro-caspases-3/7 in RPMI-8226 cells in a time- and concentration-dependent manner. However, the results for olomoucine II are inconsistent and its stimulatory effect on pro-caspase-3/7 is only weak. Our data suggest that olomoucine II may initiate cell death by a mechanism different from that of roscovitine.

EXPRESSION OF CELL-CYCLE AND APOPTOTIC PROTEINS

To better understand the mechanisms leading to the cell-cycle arrest and induction of apoptosis by roscovitine and olomoucine II, we next analyzed changes in selected proteins by immunoblotting (Fig. 4). In MCF-7 cells, treatment with equiactive doses of roscovitine and olomoucine II (20 and 5 μM, respectively) resulted in a significant reduction in CDK4 expression and when higher doses were used, the level of its positive regulator cyclin D1 also declined. In RPMI-8226 cells, the level of CDK4 was rapidly decreased, but cyclin D1 expression remained unchanged. Reduction of cyclin E, a regulatory subunit of CDK2 controlling G_1/S phase transition, also

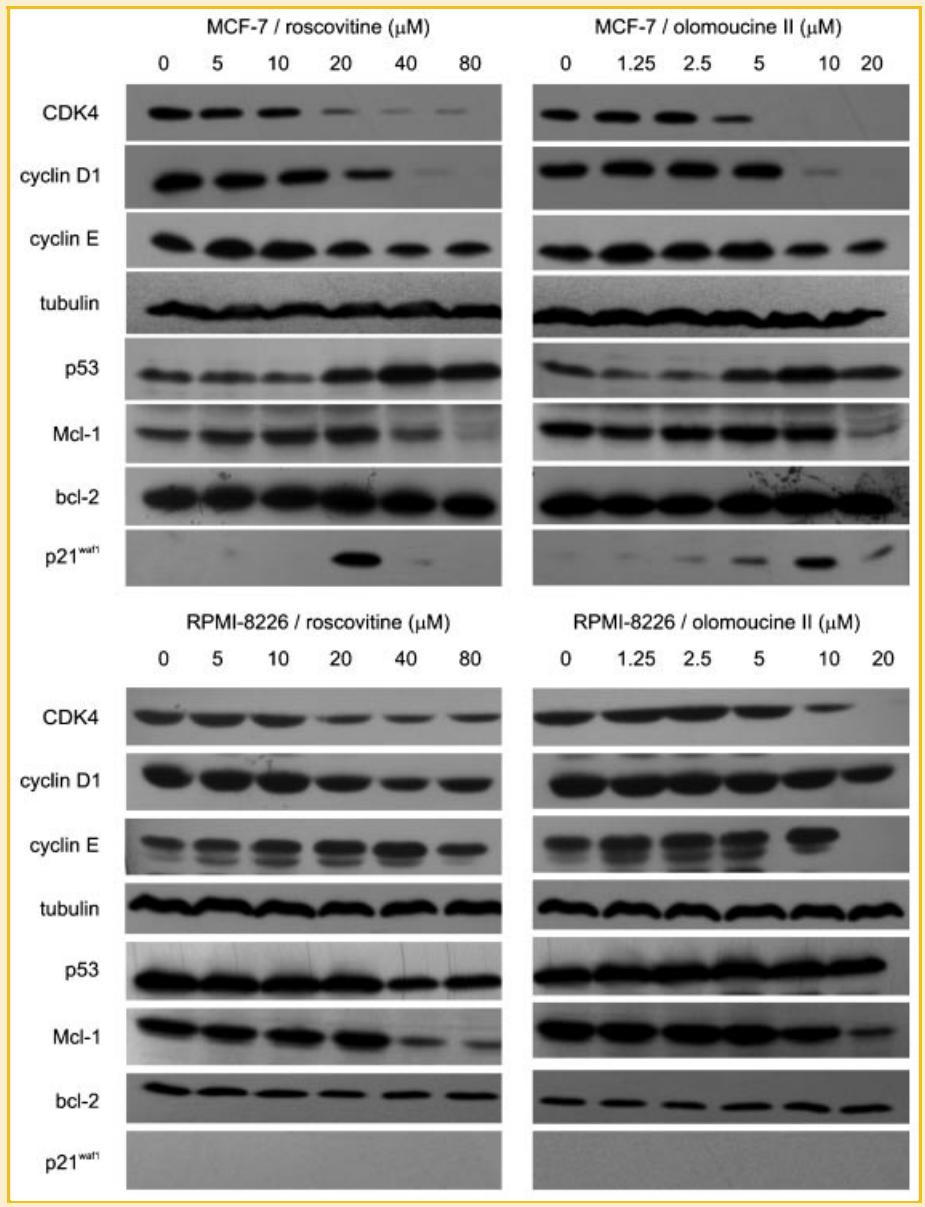

Fig. 4. Roscovitine and olomoucine II change the expression of the cell-cycle proteins and regulators of apoptosis. Cell lines were treated in a concentration-dependent manner with roscovitine and olomoucine II for 24 h, harvested, and whole cell lysates were prepared. The changes in the expression of various cell-cycle regulators in MCF-7 cell line expressing wild-type p53 and RPMI-8226 cell line with mutant p53 were analyzed by immunoblotting.

occurred in both cell lines. These observations correlate with flow cytometry results, where the rapid G_1 block and declined S phase population (in RPMI-8226 cells) and increasing of G_2/M arrest and decreased S phase (in MCF-7) were seen following treatment with CDK inhibitors.

In line with previously published data, both roscovitine and olomoucine II were also able to cause the accumulation of p53 in MCF-7 cells and activate $p21^{waf1}$ expression (Fig. 4). Interestingly, $p21^{waf1}$ expression diminished with the highest concentrations of CDK inhibitors, probably as a result of inhibition of general mRNA transcription. Interestingly, in RPMI-8226 cells with inactive p53, levels of neither p53 nor $p21^{waf1}$ changed. In contrast, the antiapoptotic protein Mcl-1 decreased in both cell lines following treatment, whereas no changes were detected in the levels of another antiapoptotic factor bcl-2 (Fig. 4) or proapoptotic protein bax (not shown).

DEPHOSPHORYLATION OF THE RNA POLYMERASE II

Previous studies have shown that treatment of cells with roscovitine leads to dephosphorylation of the carboxyl-terminal domain (CTD) of RNA polymerase II, particularly at serine 2 and 5, which consequently decreases transcription rate. The phosphorylation of the CTD of RNA polymerase II is caused by two CDKs, namely CDK9, which is specific for Ser-2 phosphorylation, and CDK7, which phosphorylates both sites. Also, the phosphorylation at Ser-5, but

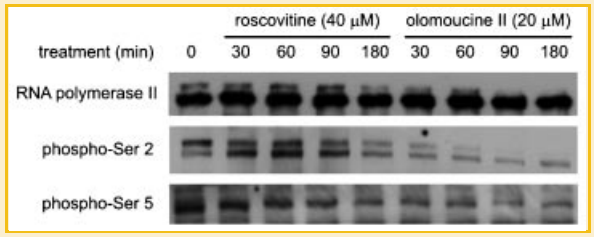

Fig. 5. Roscovitine and olomoucine II modulate expression of RNA polymerase II and its phosphorylation status in RPMI-8226 cells. Cells were incubated with drugs at the indicated time points, harvested, and whole cell lysates were analyzed by immunoblotting.

not at Ser-2, is responsible for prevention of RNA polymerase II ubiquitylation.

To confirm our assumption that not only roscovitine but also olomoucine II inhibits CTD phosphorylation, RPMI-8226 cells were treated with both CDK inhibitors in a time-dependent manner (Fig. 5). The expression of total RNA polymerase II did not change during treatment with both CDK inhibitors for 3 h. However, phosphorylated Ser-2 was markedly reduced after 60 min in olomoucine II-treated cells, while only moderate roscovitine-induced diminution of the Ser-2 phosphorylation was detected after 1.5 h and at its twofold higher concentration. The longer exposure (12 or 24 h) of cells to the drugs resulted in degradation of total RNA polymerase II level.

REACTIVATION OF WILD-TYPE p53 PROTEIN IN RPMI-8226 CELLS SENSITIZES THEM TO INDUCTION OF APOPTOSIS

The cell lines examined in this study differ in their p53 status; MCF-7 cells express wild-type p53, while in RPMI-8226 cells bearing temperature-sensitive mutant of p53 (E285K), p53 protein switches between mutant and wild-type conformation in a temperature-dependent manner. At restrictive (37 °C) temperature mutated p53 protein occurs. After shift to a permissive (30 °C) temperature, the protein is folded to functional p53. To analyze the functional involvement of p53 in the efficiency of the treatment with CDK inhibitors, we cultivated RPMI-8226 cells independently at two different temperatures (restrictive or permissive), and exposed them to increasing doses of roscovitine or olomoucine II. The cell lysates were prepared and the levels of cell-cycle regulators were analyzed by immunoblotting.

Both CDK inhibitors caused accumulation of wild-type p53 protein in cells maintained at permissive temperature, but did not change the levels of mutant p53 in cells cultivated at restrictive temperature. This indicates that both CDK inhibitors promote stabilization of wild-type p53 protein. Moreover, the monitoring of the expression of p53 targets such as $p21^{waf1}$ and MDM2 revealed their appearance following treatment with CDK inhibitors solely in cells maintained at permissive temperature, but not in cells kept at restrictive temperature (Fig. 6).

Remarkably, cells under permissive conditions underwent apoptosis even at lower concentrations of roscovitine and olomoucine II (Fig. 6). We have observed fragmentation of PARP-1 to occur in cells treated with about twofold lower drug concentrations that in cells kept in restrictive temperature (Fig. 6A). Similarly, expression of antiapoptotic protein Mcl-1 was also suppressed more efficiently at 30 °C by both inhibitors (Fig. 6A). More importantly, activities of caspases-3/7 extracted from cells kept under different conditions significantly differed, that is, they were higher in lysates prepared from cells kept at 30 °C (Fig. 6B). These data evidence that wild-type p53 protein expressed in RPMI-8226 cells cultured at permissive temperature facilitates induction of apoptosis upon treatment with roscovitine and olomoucine II.

DISCUSSION

Observations reported in many articles have provided the rationale for therapeutical applications of roscovitine, a 2,6,9-trisubstituted purine inhibitor of cyclin-dependent kinases CDK1, CDK2, CDK3, CDK7, and CDK9. This drug has entered clinical trials as a potential agent for treatment of several different cancers, including B-cell malignancies, lung and breast cancers [Meijer and Raymond, 2003; Meijer et al., 2006]. Roscovitine has been shown to arrest the cell cycle in proliferating cells, a response associated with pRb dephosphorylation [Whittaker et al., 2004]. The antiproliferative effects of roscovitine are caused by its ability to target multiple CDKs simultaneously rather than only one of them. The ability to induce cell arrest and death through depletion of the activity of a single CDK is weak and often cell line dependent, but cooperative pharmacological inactivation of several CDKs results in stronger antiproliferative effects in cancer cell lines [Cai et al., 2006b]. Similarly, recent genetic experiments demonstrated that multiple CDKs are not essential for mouse embryonic cells, where only mitotic CDK1 is required [Santamaría et al., 2007]. Interestingly, in cells lacking multiple CDKs, unusual compensatory complexes of CDK1 with cyclins E or D were detected [Aleem et al., 2005; Cai et al., 2006b; Santamaría et al., 2007]. Therefore, CDK1 can apparently compensate for the lack of, but itself cannot be replaced by, any interphase CDK in normal cells [Santamaría et al., 2007]. The situation in human adult or transformed cells is still not completely clear, but available data suggest that only combined inhibition of CDK1, CDK2, and CDK9 in some cancer cell lines significantly decreases cell proliferation and enhances apoptosis, too. Notably, CDK1, CDK2, and CDK9 represent a rational subset of CDK family members for drug targeting [Cai et al., 2006a,b] and this subset overlaps well with the selectivity of roscovitine [McClue et al., 2002; Krystof et al., 2005].

In line with published results, we found that not only roscovitine but also olomoucine II arrested the cell cycle in MCF-7 and RPMI-8226. Both CDK inhibitors blocked the G_2/M transition and brought about a concentration-dependent reduction in BrdU incorporation. Furthermore, the blocking of G_1 was evident at higher concentrations of both drugs in the RPMI-8226 cell line. The cell-cycle arrest was accompanied by decreased levels of cyclin D1, cyclin E, and CDK4 in both cell lines, as well as enhanced expression of transcription factor p53 and its downstream target $p21^{waf1}$ in MCF-7. There were no substantial differences in the effects of both compounds, other than the higher efficiency of olomoucine II over roscovitine attributable to stronger CDK9 inhibition.

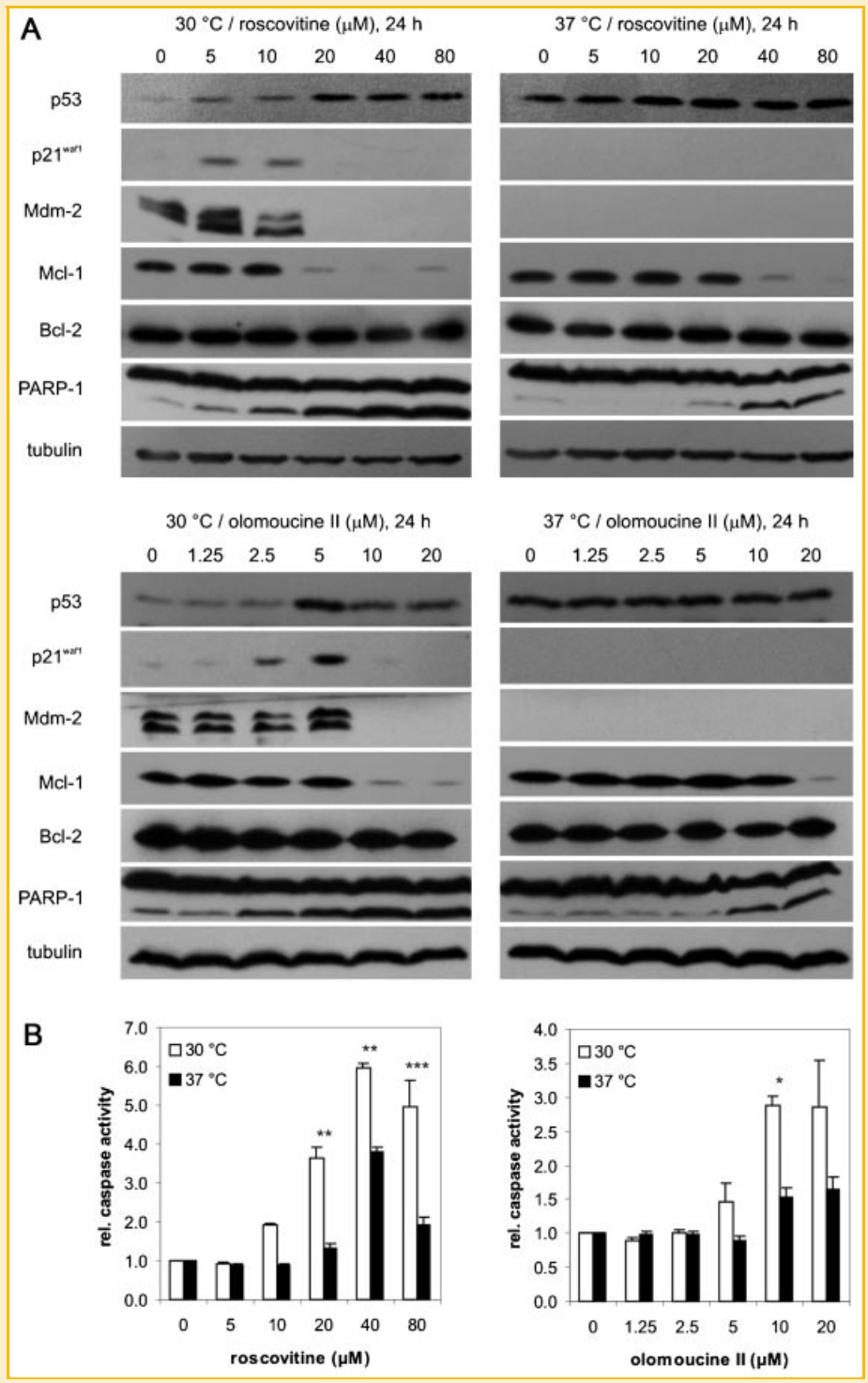

Fig. 6. Roscovitine and olomoucine II induce reactivation of temperature-sensitive mutant p53 in RPMI 8826. RPMI-8226 cells were treated for 24 h in a concentration-dependent manner with roscovitine and olomoucine II at 30 or 37°C, harvested, and whole cell lysates were prepared. The expression of cell-cycle regulators and proteins involved in apoptosis were detected by immunoblotting (A). The activities of caspases-3/7 in treated cells were measured using specific fluorogenic peptide substrate Ac-DEVD-AMC (B). The significance (treatment at 30°C vs. 37°C) was determined using Bonferroni's multiple comparison test: $^{*}P < 0.05$ (significant); $^{**}P < 0.01$ (very significant); $^{***}P < 0.001$ (extremely significant).

However, the exact mechanism by which roscovitine induces apoptosis has not been clearly defined and it is still uncertain which CDKs (or other targets) are critical for its anticancer effects and to what extent. Several studies have shown that CDK9 is an important target of some pharmacological CDK inhibitors, including roscovitine, flavopiridol, SU9516, and AZ703 [Gojo et al., 2002; MacCallum et al., 2005; Raje et al., 2005; Gao et al., 2006; Cai et al., 2006a]. As well as others, all these drugs are capable of

inhibiting phosphorylation of the C-terminus of RNA polymerase II and therefore act as global transcriptional repressors. RNA polymerase II is regulated by phosphorylation of Ser-5 and Ser-2 within the CTD by CDK7 and CDK9 and probably also by cell-cycle-related CDK1 and CDK2 [Cai et al., 2006b]. Hence, the major impact of these pharmacological inhibitors is manifested primarily on short-lived proteins such as Mcl-1, XIAP, or $p21^{waf1}$. Of these, Mcl-1 plays a critical role in the survival of cancer cells being an important antiapoptotic protein [MacCallum et al., 2005; Yang-Yen, 2006]. Both Mcl-1 protein and its mRNA have very short half-lives and therefore repression of transcription leads to its relatively rapid elimination from cells and promotes apoptosis. Downregulation of Mcl-1 by roscovitine and olomoucine II in the two cell lines shown here is therefore consistent with observations by others [Gojo et al., 2002; MacCallum et al., 2005; Raje et al., 2005; Gao et al., 2006]. Indeed, both CDK inhibitors were able to induce apoptosis in RPMI-8226 cells, as evidenced by increased caspase-3/7 activity and fragmentation of PARP-1. Surprisingly, despite weaker caspase-3/7 activation by olomoucine II, the cells were still more sensitive to olomoucine II (cf. to Fig. 1) as shown by enhanced PARP-1 fragmentation. The results suggest that different cell death pathways (proteases) may be involved in the apoptosis induced by both structurally related CDK inhibitors. Due to different inhibitory potency of the compounds towards CDK9, we may speculate about the role of CDK9 (or an unknown target of the inhibitors) in caspase-3/7 activation and alternative mechanisms of cell death. The existence of an alternative mechanism of cell death would explain why MCF-7 cells also respond to olomoucine II more rapidly than to roscovitine, even though they lack functional caspase-3 [Simstein et al., 2003].

Inhibition of RNA polymerase II-dependent transcription also leads to accumulation of p53 by blocking its degradation by MDM2 [Kotala et al., 2001; Lu et al., 2001]. Recently, it was described that p53 restoration can be strongly potentiated by combinations of p53-activating agents with different actions, such as nutlin-3a, a specific inhibitor of MDM2, with nongenotoxic CDKs inhibitors, like DRB and roscovitine [Cheok et al., 2007]. We showed here the influence of roscovitine and its derivative olomoucine II on reactivation of the temperature-sensitive E285K mutant of p53 in RPMI-8226 cells. The lower temperature allows stabilization of active p53 and subsequent expression of $p21^{waf1}$, enhanced by roscovitine and olomoucine II, as also shown here. Simultaneously, reduced expression of antiapoptotic Mcl-1 and fragmentation of PARP-1 were observed with at least twofold lower doses of the compounds at permissive conditions. Nearly the same effect of both drugs was also observed following measurement of caspase-3/7 activity, together suggesting that cells die more easily if they possess functional p53, as suggested by our previous study [Krystof et al., 2005].

However, blockage of transcription and accumulation of p53 resulting from diminished levels of MDM2 is definitely not the only reason for induction of programmed cell death by CDK inhibitors [O'Hagan and Ljungman, 2004; Wesierska-Gadek et al., 2005, 2007]; specific posttranslational modifications of p53 are necessary for its particular functions. It has been evidenced that roscovitine activates HIP2 kinase, modifying p53 at Ser-46, and that overexpression of HIP2 kinase increases rate of apoptosis in MCF-7 cells, involving p53AIP1 protein, the downstream target of p53 [Wesierska-Gadek et al., 2007]. This pathway probably helps to overcome lack of functional caspase-3 in MCF-7 cells and increase the efficacy of drug-induced apoptosis. Similarly, our results also indicate that CDK inhibitors are able to induce different apoptotic pathways, depending on the status of p53 and/or caspase-3 in the two cell lines used here.

Moreover, it seems to be possible that interplays between p53 and other antiapoptotic/prosurvival molecules also contribute to roscovitine-induced apoptosis in cancer cells. For example, recent article suggests that p53-dependent apoptosis is potentiated by NF-κB suppression and shows that both these pathways are targeted simultaneously by roscovitine [Dey et al., 2008]. Or alternatively, it was shown recently that mitochondrial p53 displaces Mcl-1 from the complex with Bak upon cell stress, resulting in release of cytochrome *c* from mitochondria and induction of apoptosis [Leu et al., 2004]. p53 negatively regulates translationally controlled tumor protein TCTP, which normally binds to Mcl-1 and thus prevents its ubiquitinylation by ARF-BP/Mule [Zhong et al., 2005]. Stabilized and activated p53 may therefore influence Mcl-1 activity directly by disrupting its pro-survival function in mitochondria and indirectly by allowing its ubiquitinylation [Zhong et al., 2005]. Our finding confirms that simultaneous inactivation of the CDKs involved in the regulation of the cell cycle, as well as transcription, seems to be beneficial for anticancer therapy and further suggests that active p53 may enhance the anticancer activity of roscovitine through multiple mechanisms.

ACKNOWLEDGMENTS

The authors wish to thank D.A. Morris for his suggestions and critical reading of the manuscript as well as J. Hudcová, O. Hustáková, and D. Parobková for technical assistance.

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PŘÍLOHA II.

Kryštof V, Rárová L, Liebl J, Zahler S, **Jorda R**, Voller J, Cankař P. The selective P-TEFb inhibitor CAN508 targets angiogenesis. *ACS Chem Biol*. 2011, v recenzním řízení.

The selective P-TEFb inhibitor CAN508 targets angiogenesis

Journal:	*ACS Chemical Biology*
Manuscript ID:	cb-2011-00067f
Manuscript Type:	Letter
Date Submitted by the Author:	24-Feb-2011

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Title: The selective P-TEFb inhibitor CAN508 targets angiogenesis

Running title: CDK9 as an angiogenesis target

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ABSTRACT

Small molecule inhibitors of cyclin-dependent kinases (CDK) have been developed as anticancer drugs with cytostatic and cytotoxic properties, but some of them have also been shown to limit angiogenesis. Here, we report that the 3,5-diaminopyrazole CAN508 inhibits endothelial cell migration and tube formation. In addition, it reduces phosphorylation of the C-terminus of RNA polymerase II and inhibits mRNA synthesis in endothelial cells, in accordance with previous observations that it has high selectivity towards the positive transcriptional regulator P-TEFb. Moreover, CAN508 reduces expression of vascular endothelial growth factor by several human cancer cell lines. The findings suggest that P-TEFb may be an attractive target for antiangiogenic therapy.

Key words

angiogenesis; cancer; transcription; inhibitor; cyclin-dependent kinase 9; P-TEFb

INTRODUCTION

Members of the cyclin-dependent kinase (CDK) family have well-documented roles in the regulation of cell growth, gene transcription and cell death. Consequently, they have attracted considerable attention as possible novel therapeutic agents for treating a wide range of diseases. In the last two decades many CDK-selective inhibitors have been identified either by screening natural compounds or through directed medicinal chemistry programs. Exposure to CDK inhibitors can induce cell cycle arrest, apoptosis, or both, depending on the selectivity of the inhibitor: anti-proliferative effects result from inhibition of cell cycle CDKs 1, 2, 4 and 6, whereas inhibition of the transcriptional CDKs 7 and 9 has been shown to promote apoptosis.[1]

To date, ca. 20 potent CDK inhibitors have been registered for clinical trials in patients with different cancers.[2,3] One of these, ZK 304709, is a relatively promiscuous kinase inhibitor that targets not only multiple CDKs, but also vascular endothelial growth factor (VEGF) receptor tyrosine kinases 1-3 and platelet-derived growth factor receptor β tyrosine kinase (PDGFRB).[4] Hence, ZK 304709 has been proposed to exert its anticancer effects *in vivo* through interfering with tumour angiogenesis, which is widely regarded as a key stage in carcinogenesis.

Several other CDK inhibitors have also been shown to influence angiogenesis. For example, the potent, selective CDK inhibitor SNS-032 prevents migration of human umbilical vein endothelial cells (HUVECs) and inhibits formation of a capillary network from these cells in culture.[5] The mechanism responsible for the anti-angiogenic properties of this drug has not been determined, but its action can be partially ascribed to down-regulation of both mRNA and protein levels of VEGF, the most potent tumour angiogenic factor.

The CDK inhibitor flavopiridol is currently considered to be a transcriptional inhibitor, but it has also been shown to prevent VEGF production in human monocytes.[6] Both SNS-032 and flavopiridol share similar kinase inhibition profiles, including targeting of CDK9; a subunit of Positive Transcription Elongation Factor b (P-TEFb), and hence an important regulator of the elongation phase of mRNA transcription. Connections between angiogenesis, mRNA transcription and CDK9 have been suggested by analyses of the effects of 4-amino-6-hydrazino-7-β-D-ribofuranosyl-7*H*-pyrrolo[2,3-*d*]-pyrimidine-5-carboxamide (ARC). This compound was originally identified as a general inhibitor of transcription, but it also potently induces apoptosis in human tumour cells and has strong anti-angiogenic activity *in vitro*.[7,8]

The aims of the study presented here were to evaluate the potential utility of disrupting the link between angiogenesis and P-TEFb, and more specifically the effects of CAN508, the most potent competitive 4-arylazo-3,5-diamino-1*H*-pyrazole inhibitor of P-TEFb subunit CDK9,[9] on human endothelial cells. Towards this end, we have examined the anti-angiogenic properties of CAN508 in several biochemical and cellular models, focusing on its transcriptional effects. Our results suggest that abrogation of P-TEFb activity could serve as a potential therapeutic anti-tumour angiogenesis strategy.

RESULTS

As mentioned above, the main aims of this study were to investigate the effects of disrupting the link between angiogenesis and p-TEFb, and more specifically to assess responses of human endothelial cells to the compound CAN508. CAN508 has already been shown to have anti-proliferative activity towards

four human cancer cell lines *in vitro*, albeit at high micromolar concentrations.[9] Further information about its anticancer activity was obtained from tests with NCI60, a panel of 59 cell lines used for high-throughput drug screening in the Developmental Therapeutics Program of the US National Cancer Institute (Bethesda, USA). The average GI_{50} value was 20 μM (range 4.1 to 49.2 μM) and the highest activities were observed in the leukemia and melanoma cell lines (Table S1).

We next determined its anti-proliferative activity against primary endothelial cells. As shown in Figure 1, reductions in both HMEC-1 and HUVEC cell numbers were observed (using Calcein AM staining) within 72 h of treatment with CAN508 concentrations > 50 μM. Thus, the effects of CAN508 on numbers of primary cells in the cultures were clearly weaker than its inhibitory effects on the growth of cancer cells. However, in further experiments the time frame and CAN508 concentration were limited to ≤24 h and < 50 μM, respectively.

Recent work has shown that some compounds that target CDK9 also possess significant anti-angiogenic activity *in vitro*.[5,7] We therefore evaluated the potential anti-angiogenic activity of CAN508 in cell migration and tube formation assays using HUVEC and HMEC-1 primary endothelial cells, respectively. As shown in Figure 2, treating the cells with CAN508 clearly inhibited cell migration and tube formation (both of which can be easily quantified by analysing images of cell cultures). The migration of VEGF-stimulated HUVECs across a scratched area was inhibited by CAN508 in a dose-dependent manner, and significant inhibition of migration was observed at concentrations > 10 μM. The experiments with HMEC-1 showed that CAN508 treatment also inhibited tube formation (reducing the total length of tubes by 20% and 30%, and their median length by 30% and 50%, at 20 μM and 40 μM, respectively; Figure 2D). However, CAN508 only marginally (and non-significantly) decreased the total number of tube nodes. In summary, the results show that CAN508 significantly reduced the angiogenic-like activity of endothelial cells in a dose-dependent fashion. Moreover, all of the observed effects were clearly apparent after 16 h treatment and in a concentration range that did not affect cell viability (cf. Figure 1).

Flavopiridol, SNS-032 and ARC have been shown to have anti-angiogenic activity, which was proposed to result from reduced expression of VEGF, an extracellular factor known to be required for activation of endothelial cells.[5,6,8] VEGF is an extracellular factor required for activation of endothelial cells. To determine whether CAN508 also reduces VEGF expression, we exposed the human carcinoma cell lines MCF7, MDA-MB-231 and DU145 to various doses of CAN508 for 24 h, then determined the quantity of VEGF released into their media by sandwich ELISA. The results, presented in Figure 3, show that at concentrations exceeding 10 μM CAN508 significantly reduced the amount of VEGF in the cell culture media. However, untreated HMEC-1 cells released very low levels of VEGF into their growth media (29 pg/ml ± 26 pg/ml), close to the assay's detection limit, hence the effects of CAN508 on these cells could not be reliably measured.

The CDK9 inhibitors flavopiridol and roscovitine have been shown to reduce expression of NF-κB target genes, probably through interference with general transcription, which may further potentiate the anticancer effects of these inhibitors.[10,11] One of the genes regulated by NF-κB and down-regulated by roscovitine is intercellular adhesion molecule-1 (ICAM-1),[10] expression of which contributes to cell adhesion, invasivity and angiogenesis. We therefore examined the effects of CAN508 treatment on

ICAM-1 expression in HMEC-1 cell, by stimulating the cells with TNFα for 30 min, applying various doses of CAN508, then examining their expression of ICAM-1 flow cytometrically 24 h later. The results show that CAN508 reduced ICAM-1 expression dose-dependently, with an approximate IC_{50} value of 20 μM (Figure 4).

CDK inhibitors that interfere with transcription have been found to be potent inhibitors of CDK7 and CDK9,[12,13] and we have previously shown that CAN508 treatment can reduce the activity of RNA polymerase II in cancer cells.[9] To explore consequences of this activity, in the present study we used pulse-labelling to determine the effects of CAN508 on the synthesis of both mRNA and total RNA in human MCF7 breast cancer and HMEC-1 cells (Figure 5). The level of newly synthesized RNA was found to be dose-dependently reduced after 2 h of treatment, with IC_{50} values of 15 μM and 20 μM for MCF7 and HMEC-1 cells, respectively. We next confirmed that this reduction of RNA transcription is accompanied by decreased phosphorylation of RNA polymerase II at its C-terminus. The human RNA polymerase II CTD is composed of 52 repeats of the heptad sequence $Y_1S_2P_3T_4S_5P_6S_7$, and phosphorylation of Ser5 and Ser2 (catalyzed by CDK7 and CDK9, respectively) is required for completion of the initiation and elongation phases of mRNA transcription. In both HMEC-1 and MCF7 cells treatment with CAN508 dose-dependently reduced Ser2 phosphorylation levels, and Ser5 phosphorylation to a lesser extent, indicating that the compound inhibits CDK9 more strongly than CDK7 (Figure 5).

Preliminary kinetic measurements with a small subset of human protein kinases, focused on CDKs, suggested that CAN508 selectively inhibits CDK9 (IC_{50}=0.35 μM), at least 40-fold more strongly than other CDKs.[9] To further characterise CAN508 selectivity we profiled its activity against a panel of 100 enzymes covering all protein kinase families, using a standard kinetic radioassay at a single dose of CAN508 (10 μM). The results, summarized in Table S2, show that in addition to CDK9, CDK2/cyclin A has substantial sensitivity to the compound (residual activity 27%). Of the other protein kinases tested, the activities of 28 were inhibited by more than 50% (residual activities: 17-48%) and 70 were inhibited by 50% or less. To explore the reasons for the observed selectivity, the structure of the CAN508-CDK9/cyclin T complex was determined, but the findings will be published in another paper (Baumli et al., manuscript in preparation).

DISCUSSION

Antitumour activities of small-molecule inhibitors of CDKs are highly complex and pleiotropic. The diversity of their effects is partly due to their ability to differentially block multiple CDKs involved in cell cycle regulation and transcription. Unexpectedly, some CDK inhibitors (including flavopiridol, SNS-032, roscovitine and ARC) currently under clinical evaluation as anticancer drugs, have also been shown to inhibit angiogenesis *in vitro*.[5-8,14] Furthermore, although their underlying mechanisms might differ somewhat due to differences in their kinase selectivity, they all share significant activity against CDK9. Recent bioinformatics analyses have suggested that the cancer selectivity and (especially) anti-angiogenic effects of ARC may be due to its ability to inhibit protein kinase C (PKC) in addition to CDK9.[8] However, flavopiridol, roscovitine and SNS-032 are poor PKC inhibitors,[15,16] as is CAN508, another small compound that targets CDK9. Furthermore, we recently found that CDK5 also plays an important

role in angiogenesis and that the antiangiogenic activity of several CDK inhibitors with different structures, including roscovitine, arises at least partially from interference with CDK5.[14,17]

Here, we show that CAN508 has the ability to block two essential angiogenic steps (cell migration and tube formation) in human endothelial cells *in vitro*. Given the kinase profile of CAN508, its anti-angiogenic properties are probably mediated by inhibition of CDK9-catalyzed phosphorylation of RNA polymerase II, leading to rapid down-regulation of RNA transcription. Subsequent reductions in expression of the strong angiogenic hormone VEGF and surface expression of the adhesion molecule ICAM-1 may contribute to the observed effects. In accordance with this hypothesis, reduced expression of VEGF by other CDK9-targeting compounds (including SNS-032 and ARC) is a postulated mechanism for their inhibition of the VEGF-dependent migration of, and tube formation by, endothelial cells.[5,8] On the other hand, the function of ICAM-1 has been studied mainly in the context of inflammatory diseases, as it is critical for the transmigration of leukocytes into tissues.[(18)] However, expression of ICAM-1 is also important for the activity of endothelial cells and their interaction with tumour cells, which is regulated mainly by the transcription factor NFκB.[19] Although we did not detect any evidence of a direct link between the action of CAN508 and NFκB, it is possible that exposure to CAN508 may lead to perturbations in the transcription of NFκB-regulated genes. In this respect, it is notable that the less selective CDK inhibitors flavopiridol and roscovitine (both of which target CDK9) can also suppress NFκB activation,[10,11] *inter alia* roscovitine can repress expression of the NFκB-regulated gene product ICAM-1.[10]

The suppression of transcription as a consequence of CDK9 inhibition by roscovitine, flavopiridol, CAN508 or ARC has also been linked to induction of the tumour suppressor p53.[7,9,13,20] The tumour-suppressive role of p53 in DNA repair and checkpoint activation has been well documented, but its roles in the modulation of angiogenesis and cell migration are not well understood.. However, there are several mechanisms whereby the accumulation of p53 could contribute to the observed anti-angiogenic properties of CDK inhibitors, including p53-dependent activation of SMAR1, which coordinates p53 and TGF-β pathways, leading to a reduction of cell migration,[21] or p53-dependent up-regulation of caldesmon, an actin-binding protein inhibitor of podosome/invadopodium formation.[22] Alternatively, CDK9 inhibitors may limit angiogenesis by decreasing levels of short-lived proteins, including cyclins.[20,23,24] In accordance with this hypothesis, a spliced cyclin D1a variant can reportedly inhibit RhoA-induced ROCK kinase activity (independently of CDKs 4 and 6) and thereby promote cellular migration.[25]

In conclusion, we show here that compound CAN508 has anti-angiogenic potential that is linked with inhibition of the transcription regulator P-TEFb. Since hexamethylene bisacetamide inducible protein 1 (HEXIM1), a negative regulator of P-TEFb activity, may also play an inhibitory role during angiogenesis,[26] abrogation of CDK9 activity could serve as a potential therapeutic strategy against tumour angiogenesis.

METHODS

Drugs and antibodies. CAN508 was synthesized and used as previously described.[9] The following specific antibodies were used to detect the corresponding proteins: anti-α-tubulin (clone DM1A, Sigma-

Aldrich), anti-RNA polymerase II (clone ARNA-3, Millipore), anti-RNA polymerase II phosphorylated at Ser-2 or Ser-5 (clones H5 and H14, Abcam) and fluorescein-labelled anti-ICAM1 (Invitrogen).

Cell cultures and viability assay. Human microvascular endothelial cells (HMEC-1 cells, a gift from Dr. F.J. Candal, CDC, Atlanta, GA) were cultured in endothelial cell growth medium (Provitro) supplemented with 10% fetal bovine serum. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins by collagenase digestion and used at passage two or three.[27] The human carcinoma cell lines MCF-7, MDA-MB-231 (both breast) and DU145 (prostate) were purchased from the American Type Culture Collection and cultured in DMEM medium supplemented with 10% fetal bovine serum. Viability assays were performed in 96-well microtiter plates using Calcein AM solution (Invitrogen) and a Fluoroskan Ascent microplate reader (Labsystems).

NCI60 cytotoxicity assay. The cytotoxic activity of CAN508 against the NCI60 cell panel was assayed by staff of the Developmental Therapeutics Program (DTP) of the US National Cancer Institute (Bethesda, MD) using the standard protocol (http://dtp.nci.nih.gov/docs/compare/compare_methodology.html). GI_{50} values (concentrations of the drug inducing a 50% reduction of growth) values were estimated from the dose response curves, and CAN508 was assigned NSC number 741614.

Migration assay. Confluent HUVECs were scratched and immediately treated with either starvation medium M199 (serum-free, negative control; PAN Biotech) or full endothelial cell growth medium (positive control). After incubation with various doses of CAN508 at 37 °C for 16 h, each well was photographed using a TILLvisiON-system (TILL Photonics) connected to an Axiovert 200 microscope (Carl Zeiss). Migration was expressed as the proportion of pixels that were not covered by cells in the image of the wound area using S.CORE Image Analysis software (S.CO LifeScience).

Tube formation. Ibidi µ-slides were coated with Matrigel® (Schubert & Weiss-OMNILAB) then suspensions of 1×10^4 HMEC-1 cells in growth medium supplemented with or without various doses of CAN508 were distributed into the wells of the slides. After incubation at 37 °C for 16 h, each well was photographed and analyzed as for the migration assay, then numbers of tubes (capillary structures) and nodes formed by treated cells and untreated controls were quantified and compared.

ELISA quantification of VEGF. Amounts of VEGF released from cultured cells into the medium were quantified using a Human VEGF ELISA Development Kit (Peprotech) according to the manufacturer's instructions. Briefly, cells seeded in 6-well plates were treated with various doses of CAN508 in 2 ml of complete medium, then after 24 h incubation the medium was collected, cleared by centrifugation, analyzed directly in triplicate, and the levels of VEGF present in the samples were quantified by comparison to a standard curve generated using recombinant VEGF supplied with the kit.

Flow cytometric analysis of the cell adhesion molecule ICAM-1. HMEC-1 cells were grown to confluence, CAN508 was added to various concentrations and 30 min later TNF-α (Sigma Aldrich) was added to a concentration of 10 ng/ml to all samples except controls. After 24 h, the cells were fixed by 4% formaldehyde and labelled with FITC-labelled anti-ICAM-1 antibody, then samples were prepared and analyzed flow cytometrically by a FACSCalibur system (Becton Dickinson) as previously described.[28] In each experiment, the fluorescence of cells exposed to all treatments was expressed relative to the mean fluorescence of cells treated with TNFα alone (set as 100%), and changes in the expression of

ICAM-1 on the cells' surfaces were expressed in terms of relative changes in the mean (logarithmic) index of fluorescence intensity. At least three different sets of experiments were performed in triplicate.

Measurement of RNA synthesis. To evaluate the effects of CAN508 on RNA synthesis, cells were prelabelled with [^{14}C]thymidine (60 Bq/ml in growth medium) 1 day before the measurements, and nascent RNA was labelled for the last 30 min of drug treatment by adding [^{3}H]uridine (7×10^5 Bq/ml). For measurements of total RNA synthesis, cell lysates were precipitated with ice-cold TCA, the TCA-insoluble material was collected on spin filters (Invitek), washed and then nucleic acids were eluted with 1 M NaOH. The ^{3}H and ^{14}C in the eluate were quantified simultaneously using an LS6500 liquid scintillation counter (Beckman Coulter). mRNA was isolated from cells using an Oligotex Direct mRNA kit (Qiagen), then relative total RNA synthesis and mRNA synthesis rates were determined by calculating the ^{3}H/^{14}C ratio for each sample and comparing it with the ratio from an untreated control sample. The data are presented here as percentages of the ^{3}H/^{14}C ratio for each treatment compared with the value determined for mock-treated control cells.

SDS-polyacrylamide gel electrophoresis and immunoblotting. For immunoblotting, harvested cells were lysed, proteins in lysates were quantified, diluted with Laemmli electrophoresis buffer to equal concentrations, separated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes and stained with Ponceau S to check for equal protein loading. The membranes were blocked and, after probing with individual antibodies and antigen–antibody complexes, visualized using ECL+ chemiluminescent detection reagent (Pierce).

Protein kinase assays. The kinase selectivity of CAN508 (at 10 μM) was profiled by screening under previously described conditions in assays initiated with 800 cpm/pmol [γ-^{33}P]ATP at 5, 20 or 50 μM, as indicated in the Supporting Information, to ensure that the ATP concentration was $\leq K_m$ value for each enzyme.[29] In each case the reaction was stopped by the addition of phosphoric acid and the mixture was spotted onto P81 filter plates. The experiment was performed in duplicate, and inhibition was expressed as residual kinase activities.

Statistical analysis. All experiments were performed in triplicate in at least two independent experiments. All quantitative data are presented as means ± standard deviation (SD), and for all comparisons of treatment means with controls, one-way ANOVA was used.

SUPPORTING INFORMATION

Supporting Information can be found with this article online at ....

ACKNOWLEDGMENTS

The authors have been supported by funds from several sources, including: the Ministry of Education, Youth and Sports of the Czech Republic (grant no. MSM6198959216), Czech Science Foundation (grant no. 204/08/0511), 6th European Framework Programme Protein Kinase Research (grant no. LSHB-CT-2004-503467). The authors confirm that there are no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Growth inhibitory effect of CAN508 on human microvascular endothelial cells. (HMEC-1) and umbilical vein endothelial cells (HUVECs). The cells were incubated for 72 h with the indicated doses of CAN508, then the number of cells remaining in each culture was quantified using Calcein AM staining and fluorescence measurement. The assay was performed in triplicate and repeated twice.

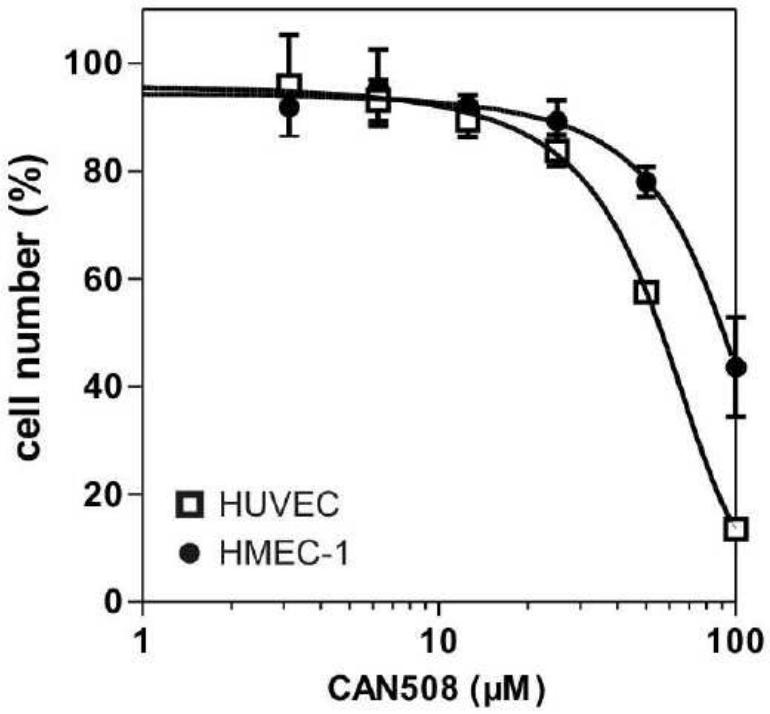
Figure 2. CAN508 has antiangiogenic effects *in vitro* on human endothelial cells. Confluent HUVEC cells were scratched and treated with/without CAN508. After 16 h, cells that migrated were stained and photographed (A), then quantified (B). For tube formation assays, HMEC-1 cells were seeded in Matrigel-coated dishes in the presence of indicated doses of CAN508 and incubated for 16 h to allow formation of a capillary network. The cells were photographed (C), then the length of tubes and number of nodes were measured and counted, respectively (D). For negative controls (NC), the cells were kept in a serum-free medium, for positive controls (PC) the cells were kept in a full medium. Both assays were repeated thrice. Representative microphotographs showing cell migration and capillary network formation are presented.

Figure 3. Expression of VEGF in MCF7, MDA-MB-231 and DU145 cell cultures is reduced in the presence of CAN508. Cells were exposed to indicated doses of CAN508 for 24 h, then the amount of VEGF released into the cell culture medium was determined by sandwich ELISA.

Figure 4. Expression of ICAM-1 on surfaces of HMEC-1 cells is reduced in the presence of CAN508. HMEC-1 cells were stimulated with TNFα for 30 min then treated with indicated doses of CAN508, and changes in ICAM-1 expression on cell surfaces after 24 h were measured and expressed as changes in the mean (logarithmic) index of fluorescent intensity quantified by flow cytometry. NC, control cells untreated with TNFα.

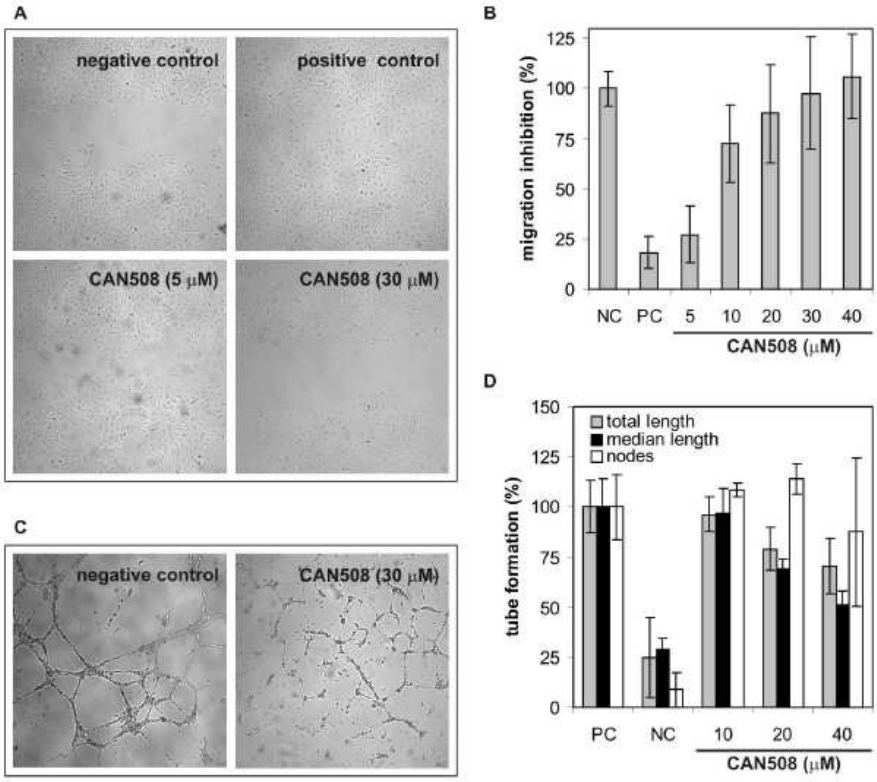
Figure 5. CAN508 inhibits transcription in HMEC-1 and MCF7 cells. HMEC-1 (A, B) and MCF7 (C, D) cells were labelled with [^{14}C]thymidine for 24 h, treated with CAN508 for 2 h, then pulse-fed with [^{3}H]uridine for 30 min, after which RNA was isolated and quantified (A, C). Phosphorylation of the CTD of RNA polymerase II at Ser2 and Ser5 in cells treated for 2 h with indicated doses of CAN508 was analyzed by immunoblotting (B, D).

Figure 1.



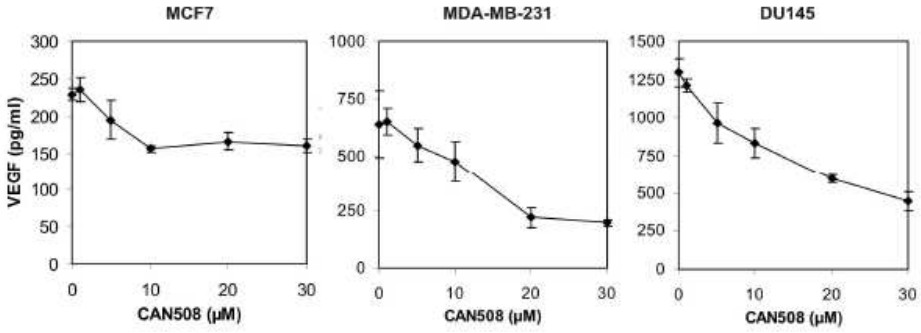
Growth inhibitory effect of CAN508 on human microvascular endothelial cells. (HMEC-1) and umbilical vein endothelial cells (HUVECs). The cells were incubated for 72 h with the indicated doses of CAN508, then the number of cells remaining in each culture was quantified using Calcein AM staining and fluorescence measurement. The assay was performed in triplicate and repeated twice.
66x60mm (300 x 300 DPI)

Figure 2.



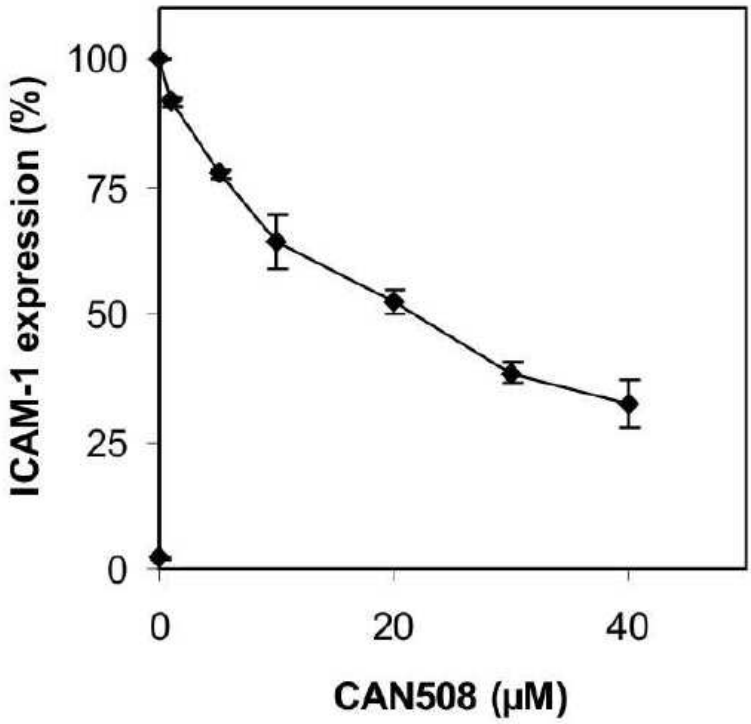
CAN508 has antiangiogenic effects in vitro on human endothelial cells. Confluent HUVEC cells were scratched and treated with/without CAN508. After 16 h, cells that migrated were stained and photographed (A), then quantified (B). For tube formation assays, HMEC-1 cells were seeded in Matrigel-coated dishes in the presence of indicated doses of CAN508 and incubated for 16 h to allow formation of a capillary network. The cells were photographed (C), then the length of tubes and number of nodes were measured and counted, respectively (D). For negative controls (NC), the cells were kept in a serum-free medium, for positive controls (PC) the cells were kept in a full medium. Both assays were repeated thrice. Representative microphotographs showing cell migration and capillary network formation are presented.
137x123mm (300 x 300 DPI)

Figure 3.



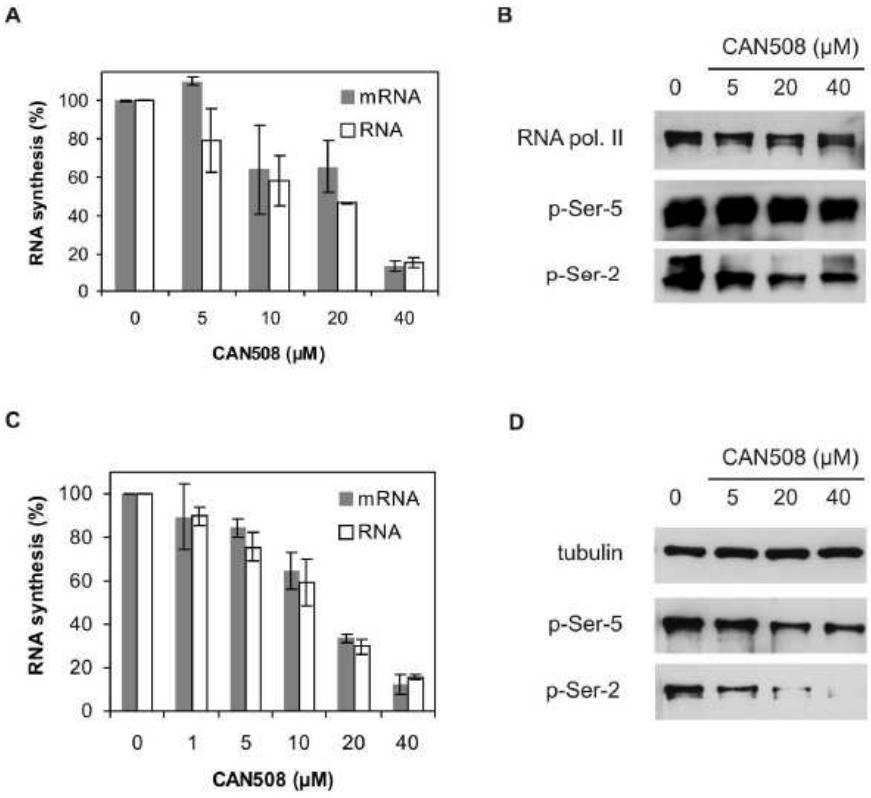
Expression of VEGF in MCF7, MDA-MB-231 and DU145 cell cultures is reduced in the presence of CAN508. Cells were exposed to indicated doses of CAN508 for 24 h, then the amount of VEGF released into the cell culture medium was determined by sandwich ELISA.
185x66mm (300 x 300 DPI)

Figure 4.



Expression of ICAM-1 on surfaces of HMEC-1 cells is reduced in the presence of CAN508. HMEC-1 cells were stimulated with TNFα for 30 min then treated with indicated doses of CAN508, and changes in ICAM-1 expression on cell surfaces after 24 h were measured and expressed as changes in the mean (logarithmic) index of fluorescent intensity quantified by flow cytometry. NC, control cells untreated with TNFα.
58x53mm (300 x 300 DPI)

Figure 5.



CAN508 inhibits transcription in HMEC-1 and MCF7 cells. HMEC-1 (A, B) and MCF7 (C, D) cells were labelled with [^{14}C]thymidine for 24 h, treated with CAN508 for 2 h, then pulse-fed with [^{3}H]uridine for 30 min, after which RNA was isolated and quantified (A, C). Phosphorylation of the CTD of RNA polymerase II at Ser2 and Ser5 in cells treated for 2 h with indicated doses of CAN508 was analyzed by immunoblotting (B, D).
139x128mm (300 x 300 DPI)

PŘÍLOHA III.

Jorda R, Zatloukal M, Řezníčková E, Vymětalová L, Kryštof V, Strnad M: Novel derivatives of cyclin-dependent kinase inhibitor roscovitine. *Cell Cycle Regulators/Inhibitors and Cancer*, Feb 5-8, 2011, Vienna, Austria.

NOVEL DERIVATIVES OF CYCLIN-DEPENDENT KINASE INHIBITOR ROSCOVITINE

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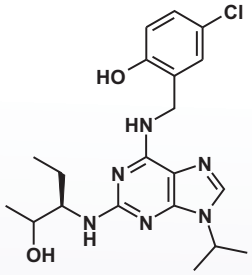


BA09

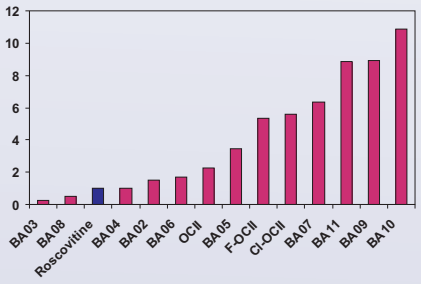
DEVELOPMENT OF BA09

Protein kinase selectivity

As roscovitine was previously described as a pan-selective CDK inhibitor of CDK1/2/5/7/9 kinases, we also evaluated the preference of its new derivatives for CDKs. As shown in Table I. the majority of compounds follow the kinase selectivity profile of roscovitine with marked preference to CDK2. Compounds BA03, 06 and 08 show only weak anti-kinase activity through all tested kinases, but most of other compounds significantly exceed the kinase inhibition of roscovitine. Especially halogenated compounds BA09 and BA10 were revealed as the most potent CDK inhibitors of this series with IC50's for CDK2/5/9 about 15-fold less than roscovitine.

Code	Kinase inhibition (nM)		
	CDK2	CDK5	CDK9
Roscovitine	180	1080	1600
OCII	50.8	270	815
Cl-OCII	9.4	150	285
F-OCII	19	240	106
BA02	20	740	1240
BA03	790	>2000	>2000
BA04	70	675	845
BA05	61	550	910
BA06	220	>2000	>2000
BA07	53	n.d.	n.d.
BA08	260	>2000	>2000
BA09	13.1	70.5	165
BA10	11.8	67	100
BA11	25	125	45

Table I. Kinase selectivity of new roscovitine derivatives.



Anticancer activity

Anticancer activity of all compounds was tested against four human cancer cell lines (K562, MCF-7, CEM, HCT-116). The results (Figure 1) clearly show stronger activity of majority of inhibitors over roscovitine. The most effective compounds BA09 and BA10 exert average anticancer activity in submicromolar range (2.67 μM and 2.19 μM, respectively) and are about 10-fold lower than roscovitine.

Figure 1. Average anticancer activity of new derivatives tested using Calcein AM assay against four cancer cell lines expressed as ratios of IC_{50} compared to IC_{50} of roscovitine

INDUCTION OF APOPTOSIS

For the evaluation of apoptotic effects of BA09 in HCT-116 and K562 cells we used fluorimetric-based caspase-3,7 activity assay and immunoblotting analyses of some apoptotic markers (Figure 2). Expression of proteins PUMA, caspase 3 and Bcl-2 remained unchanged after the treatment of cells. On the other hand the level of anti-apoptotic protein Mcl-1 showed the decrease upon the treatment of the highest tested concentration of BA09. Also the fragmentation of poly(ADP-ribose)polymerase (PARP) was seen.

The previous results were complemented by caspase-3,7 enzyme activity assay but only marginal activation of caspases 3,7 was observed after BA09 treatment of HCT-116 cells (Figure 3). This effect corresponds with immunoblotting analysis, where the cleavage of zymogene of caspase-3 was not observed at the same concentrations (Figure 2B). The ability of compound BA11 to induce apoptosis (in enzymatic assay) was also tested in both cell lines. In contrast to BA09, compound BA11 activated apoptosis more potently (Figure 3).

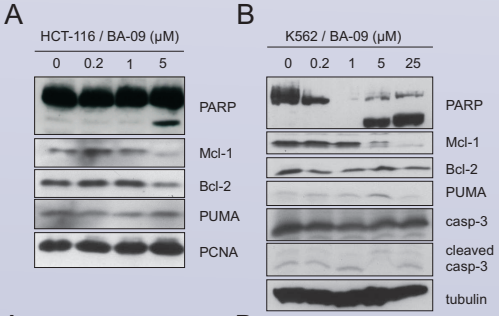


Figure 2. Immunoblotting analysis of apoptotic markers in HCT-116 and K562 cells treated with BA09 for 24 h. PCNA and tubulin levels were detected to verify equal protein loading.

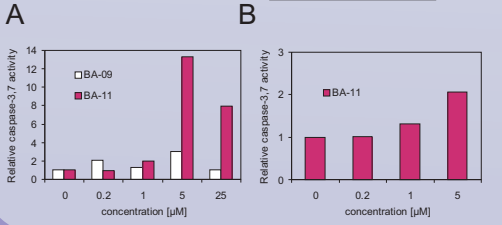


Figure 3. Fluorimetric caspase-3,7 enzyme activity assay (based on cleavage of specific Ac-DEVD-AMC peptide substrate) in HCT-116 (A) and K562 (B) cells upon treatment BA09 or BA11, respectively for 24 h.

Acknowledgments

The work was supported by grants GACR 204/08/0511 and MSM 6198959216.

ACTIVATION OF P53 AND P53-ACTIVATED TRANSCRIPTION

Treatment of cells harboring wild type tumor suppressor protein p53 with CDK inhibitors usually lead to the accumulation of p53 and an increase of p53-dependent transcription. Therefore we evaluated these effects upon treatment with one of the most effective compound, BA09, in colorectal carcinoma HCT-116 cells. BA09 rapidly increased the expression of p53 starting from 1 μM concentration (Figure 4A). Accumulation of p53 was accompanied with decreased expression of Mdm-2, which is a negative regulator of p53. The induction of $p21^{WAF1}$, a direct p53 transcriptional target, was also analyzed. The protein level increases slightly from 1 μM and reached maximum at 5 μM.

The effect of BA09 on p53-dependent transcriptional activity was confirmed by ß-galactosidase reporter assay using stable transfected cell line Arn-8. Roscovitine derivative BA09 exerted dose-dependent effect on p53 transcriptional activation, with maximum at 2 μM concentration (Figure 4B).

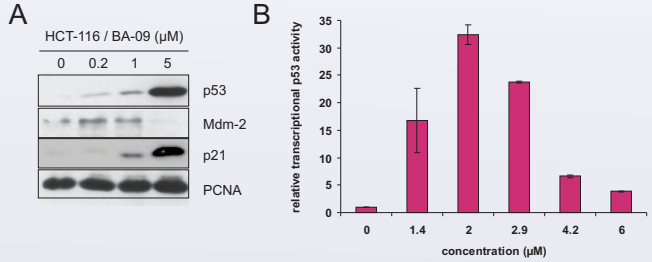


Figure 4: Analysis of p53 activation. (A) Immunoblot analysis of p53, $p21^{WAF1}$ and Mdm-2 in HCT-116 treated with BA09 for 24 h. PCNA level was detected to verify equal protein loading. (B) Analysis of relative p53-dependent transcriptional activity by ß-galactosidase reporter assay in Arn-8 cell line treated with BA09 for 24 h.

CELL CYCLE ANALYSIS

The antiproliferative activity of BA09 was analyzed in asynchronously growing colon carcinoma cell line HCT-116 and chronic myeloid leukemia cell line K562 stained with propidium iodide. As shown in Figure 5 inhibitor BA09 potently decreased population of cells in G1 phase of cell cycle and arrested cells in late S and G2/M phases. This effect was evident mainly in HCT-116 cells, where we observed about 15% more cells in G2/M phase of cell cycle compared to control cells after the treatment with 5 μM BA09.

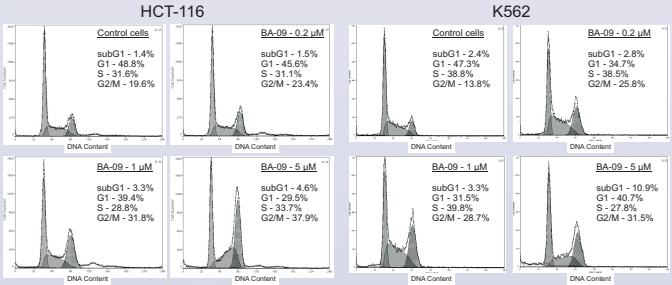


Figure 5. Flow cytometric analysis of cell cycle after propidium iodide staining in HCT-116 and K562 cell lines treated with BA09 for 24 hours.

REPLICATION AND TRANSCRIPTION

For the evaluation of transcription inhibition of BA09 in HCT-116 and K562 cells we use immunoblotting analysis of dephosphorylation of RNA polymerase II at Ser2 and 5, respectively. Already at the dose of 5 μM of BA09 we observed rapid decrease of transcription in both cell lines (Figure 6A,B).

The effect of BA09 on replication was analyzed by flow cytometric measurement of HCT-116 and K562 cells doubly stained with propidium iodide and 5-bromo-2'-deoxyuridine (BrdU). As shown in Figure 5C, BA09 rapidly decreased the population of cells actively replicating DNA (i.e. BrdU-positive cells) in a dose-dependent manner. Similarly, at the dose of 5 μM of BA09 we observed markedly decreased replication to 5% and 57% value of control in HCT-116 and K562 cells, respectively. Higher concentration (25 μM) then caused total block of replication.

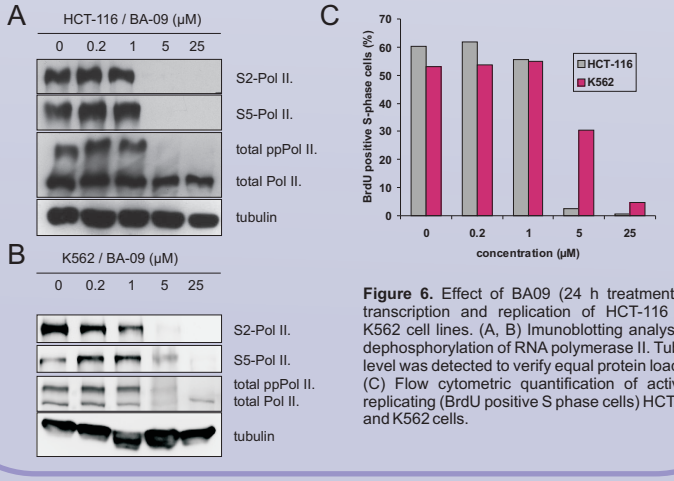


Figure 6. Effect of BA09 (24 h treatment) on transcription and replication of HCT-116 and K562 cell lines. (A, B) Imunoblotting analysis of dephosphorylation of RNA polymerase II. Tubulin level was detected to verify equal protein loading. (C) Flow cytometric quantification of actively-replicating (BrdU positive S phase cells) HCT-116 and K562 cells.

PŘÍLOHA IV.

Jorda R, Havlíček L, McNae IW, Walkinshaw MD, Voller J, Sturc A, Navrátilová J, Kuzma M, Mistrik M, Bartek J, Strnad M, Krystof V. Synthesis and biological evaluation of 3,5,7-trisubstituted pyrazolo[4,3-*d*]pyrimidines, novel selective inhibitors of cyclin-dependent kinases with antiproliferative activity. *J Med Chem*. 2011, v tisku, http://dx.doi.org/10.1021/jm200064p.

Journal of Medicinal Chemistry

ARTICLE

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Pyrazolo[4,3-*d*]pyrimidine Bioisostere of Roscovitine: Evaluation of a Novel Selective Inhibitor of Cyclin-Dependent Kinases with Antiproliferative Activity†

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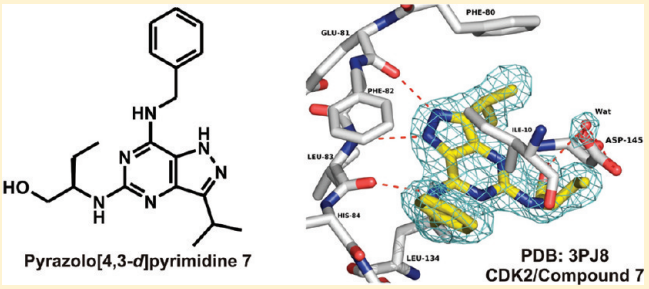
Supporting Information

ABSTRACT: Inhibition of cyclin-dependent kinases (CDKs) with small molecules has been suggested as a strategy for treatment of cancer, based on deregulation of CDKs commonly found in many types of human tumors. Here, a new potent CDK2 inhibitor with pyrazolo[4,3-*d*]pyrimidine scaffold has been synthesized, characterized, and evaluated in cellular and biochemical assays. 7-Benzylamino-5(*R*)-[2-(hydroxymethyl)-propyl]amino-3-isopropyl-1(2)*H*-pyrazolo[4,3-*d*]pyrimidine, compound **7**, was prepared as a bioisostere of the well-known CDK inhibitor roscovitine. An X-ray crystal structure of compound **7** bound to CDK2 has been determined, revealing a binding mode similar to that of roscovitine. Protein kinase selectivity profile of compound **7** and its biological effects (cell cycle arrest, dephosphorylation of the retinoblastoma protein, accumulation of the tumor suppressor protein p53, induction of apoptosis, inhibition of homologous recombination) are consistent with CDK inhibition as a primary mode of action. Importantly, as the anticancer activities of the pyrazolo[4,3-*d*]pyrimidine **7** exceed those of its bioisostere roscovitine, compound **7** reported here may be preferable for cancer therapy.



INTRODUCTION

A growing body of evidence has linked abnormal protein phosphorylation patterns with pathogenesis of various human diseases and encouraged the search for compounds capable of specifically inhibiting protein kinases. Indeed, therapeutic success of several kinase inhibitors that had been approved for the treatment of particular cancer type(s) during recent years established protein kinases as an important class of novel drug targets. Among the 518 human genes encoding protein kinases, cyclin-dependent protein kinases (CDKs) have originally attracted attention because of their frequent deregulation in cancer.[1,2] Cyclin-dependent kinases, listing at least 13 members in humans, are serine/threonine kinases that participate mainly in processes of cell cycle control, transcription, and postranscriptional modifications but also in cell differentiation and cell death.[3,4]

During the past decade many CDK inhibitors have been developed and characterized. Some of the most efficient ones entered clinical trials as candidate drugs against various types of cancer[5,6] and/or advanced to preclinical evaluation of potential value in treatment of other diseases linked with CDK deregulation, such as neurodegenerative and cardiac disorders, viral and protozoan infections, glomerulonephritis or other types of chronic inflammation.[3,7−10] The purine heterocycle became one of the first systematically investigated scaffolds of CDK inhibitors (due to a possibility of variable substitutions mainly at positions 2, 6, and 9), leading to the discoveries of olomoucine and roscovitine.[11−13] Roscovitine is a pan-selective CDK

Received: January 21, 2011

   dx.doi.org/10.1021/jm200064p | *J. Med. Chem.* XXXX, XXX, 000–000

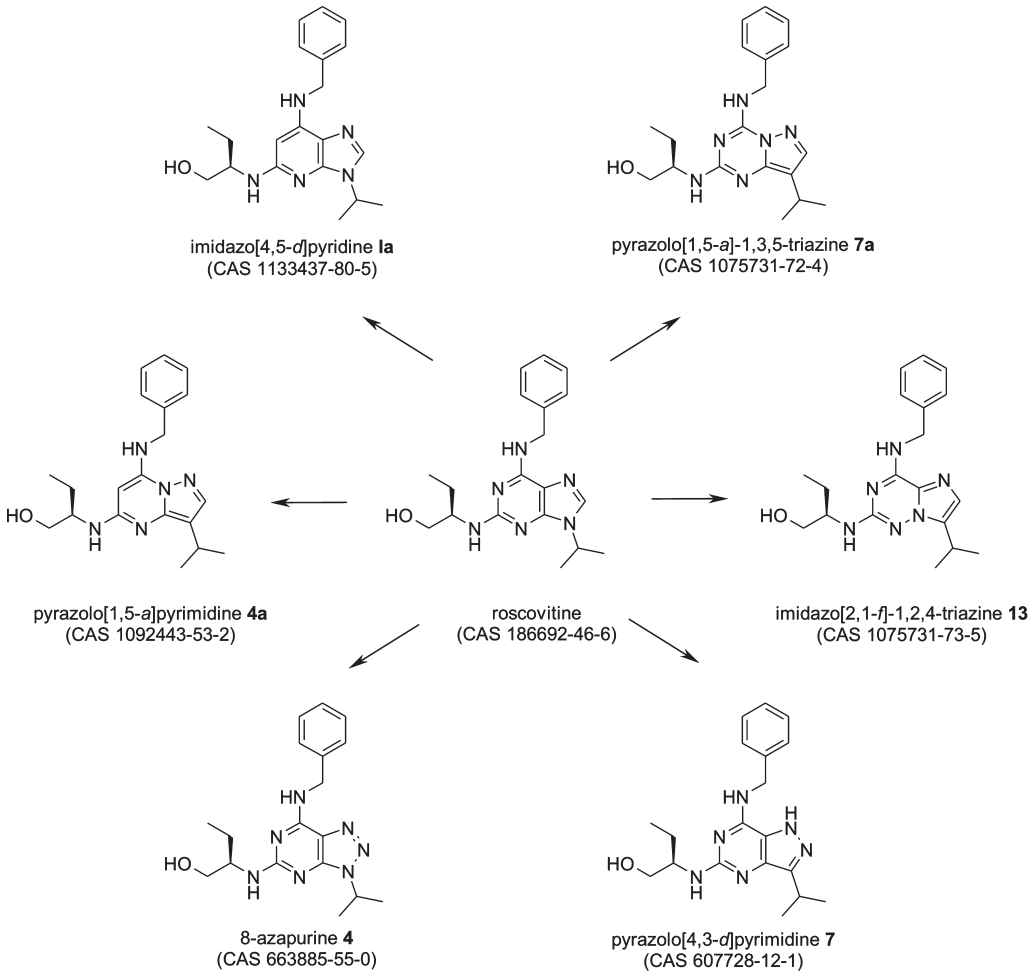


Figure 1. Structure of roscovitine and its known bioisosteres.[26,30,39,42] CAS numbers indicate Chemical Abstracts Service compound identifiers.

inhibitor with multiple effects on cell proliferation, cell cycle progression, p53 expression, and p53-dependent transcription and/or induction of apoptosis in cancer cells.[14] Because of these effects, (*R*)-roscovitine was among the first CDK inhibitors that entered clinical trials.

Inspired by the success of roscovitine, further exploration of purine-derived CDK inhibitors has been mainly oriented toward either modifications of the roscovitine molecule in its substitutable positions or, more recently, redistribution of nitrogen atoms of the purine scaffold. Both these directions have led not only to the large number of purine inhibitors derived from roscovitine[15−22] but also to libraries of compounds with alternative core heterocyclic skeleton structure with side chain types of roscovitine: pyrazolo[3,4-*d*]pyrimidines,[23,24] pyrazolo[1,5-*a*]-1,3,5-triazines,[25−27] imidazo[2,1-*f*]-1,2,4-triazines,[25,26] pyrazolo-[1,5-*a*]pyrimidines,[28−34] imidazo[1,2-*a*]pyrazines,[35,36] triazolo-[1,5-*a*]pyrimidines,[37,38] imidazo[4,5-*d*]pyridines[39] and pyrolo-[3,2-*d*]pyrimidines.[40]

In this study, we describe synthesis of a novel bioisostere of roscovitine with the pyrazolo[4,3-*d*]pyrimidine core. The 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines were previously described as CDK inhibitors,[41] and we have found that introduction of the third substituent to position 5 led to development of a new class of potent purine-related CDK inhibitors. The representative 3,5,7-trisubstituted pyrazolo[4,3-*d*]pyrimidine **7** has been evaluated in diverse biological assays in comparison with roscovitine as a reference molecule. Our results demonstrate that a change in a position of a single nitrogen atom can alter CDK inhibitory properties of this class of compounds, analogous to some other purine bioisosteres (Figure 1).[23,25,26,33,39,42]

RESULTS AND DISCUSSION

Chemistry. We have previously described synthesis of 5,7-di(4-methoxybenzyl)amino-3-isopropyl-1(2)*H*-pyrazolo[4,3-*d*]pyrimidine that was prepared as an analogue of microtubule-interfering drug myoseverin.[43] A key assumed intermediate of the synthetic route was 5,7-dichloro-3-isopropyl-1(2)*H*-pyrazolo[4,3-*d*]pyrimidine. However the intermediate was not isolated and characterized. An attempt to synthesize pyrazolo-[4,3-*d*]pyrimidine analogue of roscovitine, i.e., the compound with different 5,7-substituents in contrast to 5,7-disubstituted myoseverin derivative, via the same synthetic approach was not successful. Therefore, we developed a completely new synthetic route outlined in Scheme 1. This synthetic approach is simple, and all intermediates are easily detectable and isolatable, with the exception of the final compound **7**. The last reaction step gives only poor yield of the desired compound even in a complex reaction mixture, and therefore, compound **7** has to be isolated by a column chromatography.

dx.doi.org/10.1021/jm200064p |*J. Med. Chem.* XXXX, XXX, 000–000

Scheme 1

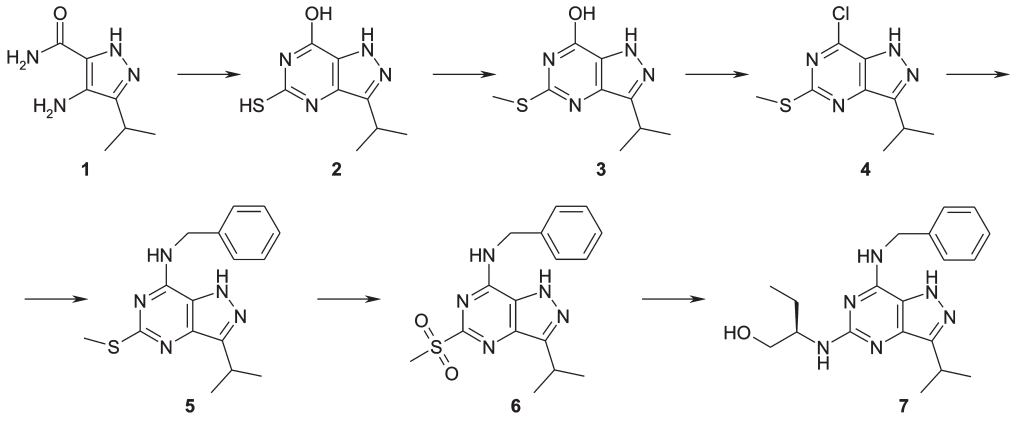


Table 1. Inhibition of Proliferation in a Panel of Human Cancer Cell Lines by Compound 7

human tumor cell lines		
origin	type	IC_{50} (μM),[a] 7
breast	MCF-7	7.5 ± 2.3
	HBL-100	11.3 ± 2.4
	BT-474	8.5 ± 0.5
colon	HT-29	6.6 ± 1.9
	HCT-116	11.0 ± 1.8
multiple myeloma	RPMI-8226	3.6 ± 0.3
	U266	4.9 ± 0.1
leukemia	K-562	7.7 ± 0.8
	CEM	3.8 ± 0.8
	HL-60	7.1 ± 0.5
osteosarcoma	HOS	7.5 ± 1.9
melanoma	G-361	4.8 ± 1.6
cervix	HeLa	6.9 ± 1.7
lung	A-549	7.1 ± 2.1
epidermis	A-431	7.7 ± 0.3

[a] Average ± SD values from three determinations.

Anticancer Cytotoxicity. For a comparison of pyrazolo[4,3-*d*]-pyrimidines with purines, the antiproliferative activity of compound 7 was tested on a panel of human cell lines representing a range of tumor types (Table 1). Determination of proliferation clearly showed stronger activity of compound 7 over roscovitine. This result was further confirmed through testing on the NCI60 panel (Figure S2 in Supporting Information),[44] where compound 7 showed a higher activity than both racemic *R,S*-roscovitine and *R*-roscovitine in all three assay end points (GI_{50}, TGI, LC_{50}) (Table 2). All the observed differences in the activity are statistically highly significant ($p < 10^{-7}$, one-sided Wilcoxon paired test). In contrast to roscovitine, compound 7 showed not only cytostatic (GI_{50}, TGI) but also significant cytotoxic (LC_{50}) effect against the majority (74.6%) of the NCI60 cell lines in the concentration range applied. The observed comparable median activities of compound 7 toward the cell lines with either the wild-type or mutant p53 (7.5 vs 10.4, 23.2 vs 26.4, and 55.8 vs 66.5 μM for GI_{50}, TGI, and LC_{50}, respectively) are consistent with the accepted opinion that p53 status does not play a major role in resistance to CDK inhibitors.[45−47]

In order to identify compounds with similar effects on the NCI60 cell lines, we calculated Pearson correlation coefficients (r) of the activity patterns (GI_{50} values for the individual cell lines) of compound 7 and 16 533 other compounds tested on the NCI60 panel (see Experimental Section for the criteria of compound selection). Calculations were carried out on a log−log scale. The analysis identified 2*H*-pyrazolo[3,4-*d*]-pyrimidine CGP-57380[48] (r = 0.66, rank 1, $p = 1.0 \times 10^{-8}$), an inhibitor of MNK1. MNK1 is a positive regulator of the eukaryotic initiation factor 4E (eIF4E), which besides its role in translation also regulates distribution of cyclin D1 mRNA.[49] Treatment with MNK1 inhibitor leads to a decrease of the cellular content of cyclin D1.[49,50] A strong correlation was observed for the cells with both the wild type (N = 16, r = 0.71, p = 0.0022) and mutant p53 gene (N = 43, r = 0.63, $p = 7.0 \times 10^{-6}$). It is tempting to speculate that the observed similarity in the activity patterns of the two compounds might stem from an effect on CDK activity, directly in the case of compound 7 and indirectly in the case of MNK1 inhibitor. On the other hand, comparison with experimentally validated (IC_{50} < 100 μM) inhibitors of cyclin-dependent kinases 1 and 2 and related kinases (CDKs 4, 5, 7, 8, 9 and/or glycogen synthase kinase-3β) included in BindingDB (33 compounds, 37 activity patterns)[51,52] shows that GI_{50} pattern of compound 7 is distinctly different. This observation suggests that other factors beyond the known shared molecular target(s) influence the resulting biological activity. Figure 2 shows signed coefficients of determination r^2 (measure of variability explained by a regression line) calculated separately for the cell lines with the wild-type and mutant p53 gene. The cumulation of the data points along the x axis indicates various degrees of similarity in the activity of compound 7 and the individual CDK/GSK3B inhibitors against the cell lines expressing wild-type p53. On the other hand, the activity pattern of compound 7 on the p53 mutant cell lines was distinctly different (low signed r^2). A possible explanation of this difference might be a generally high sensitivity of certain p53 wild type cell lines to chemical insults. Similar results were obtained when rank sum correlation was used instead of Pearson's correlation for calculation of signed coefficient of determination (not shown). We conclude that growth inhibitory activity of compound 7 differs

dx.doi.org/10.1021/jm200064p |*J. Med. Chem.* XXXX, XXX, 000–000

Table 2. Cytostatic and Cytotoxic Effects of Compound 7 and *R,S*- and *R*-Roscovitine against NCI60 Panel[a]

	GI_{50} (μM)		TGI (μM)		LC_{50} (μM)	
	median	range	median	range	median	range
7	10.2	3.0−23.8 (0/59)	25.9	10.6−60.8 (0/59)	65.3	39.1 to >100 (15/59)
R,S-roscovitine	17.9	4.6−57.8 (0/59)	58.1	20.9 to >100 (14/59)	>100	51.9 to >100 (39/59)
R-roscovitine	19.3	4.9 to >100 (1/52)	>100	14 to >100 (36/53)	>100	82.0 to >100 (50/53)

[a] Given are median and range of GI_{50}, TGI, and LC_{50} together with proportion of the cell lines for which the end point was not reached at the highest concentration tested (100 μM) (data in parentheses).

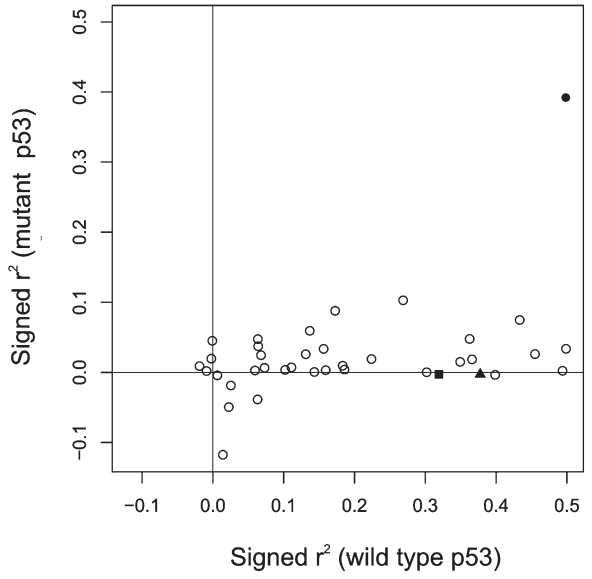


Figure 2. Effect of p53 status on relation (expressed as signed coefficient of determination r^2) between GI_{50} patterns of compound 7 and individual inhibitors of CDKs and/or GSK3B. Black circle designates MNK-1 inhibitor.[48] Black triangle designates *R*-roscovitine, and black square designates racemic roscovitine.

from those of other structurally diverse CDK inhibitors, including its isomer roscovitine, and this difference is typically more pronounced when only the cell lines harboring mutant p53 are considered.

Kinase Selectivity of Compound 7. The kinase selectivity of compound 7 was determined in enzyme assays with recombinant CDK2/cyclin E, CDK5/p35, CDK7/cyclin H/MAT1, and CDK9/cyclin T1. The IC_{50} values for compound 7 were comparable to that of roscovitine (Table 3), but isomer 7 showed generally better efficiency in CDK inhibition with IC_{50} values in the submicromolar range (Figure S1). In the following experiment the selectivity of compound 7 was tested against a panel of purified recombinant human protein kinases at a single concentration of 10 μM. The assays confirmed that compound 7 inhibits preferentially CDKs (Table 3). In addition to potent inhibition of CDKs, moderate inhibition was observed for GSK3B, stress-activated protein kinases PRAK and MSK1, and mitogen-activated kinase MEK, and these activities may contribute to the observed antiproliferative effects of compound 7.

Crystal Structure of Compound 7 with CDK2. The crystal structure of compound 7 in complex with CDK2 has been determined at 1.96 Å resolution. Compound 7 binds in the narrow cleft between the N- and C-terminal domains of CDK2.

Table 3. Kinase Selectivity Profile for Compound 7 and Its Purine Analogue Roscovitine

	kinase inhibition (%)[a]		IC_{50} (μM)[b]	
protein kinase	7	roscovitine	7	roscovitine
CDK1/cyclin B	84	70	nd	nd
CDK2/cyclin A	97	94	nd	nd
CDK2/cyclin E	98	95	0.04	0.22
CDK5/p35	95	85	0.20	0.94
CDK7/cyclin H/MAT1	96	87	0.16	0.48
CDK9/cyclin T1	90	87	1.00	1.77
CK1	7	1	nd	nd
CK2	19	10	nd	nd
GSK3A	32	13	nd	nd
GSK3B	54	25	nd	nd
MEK1	58	38	nd	nd
PRAK	30	28	nd	nd
MSK1	39	29	nd	nd

[a] In the presence of 100 μM ATP with 10 μM compound. [b] nd: not determined.

The electron density for the inhibitor is excellent with all its atoms being well-defined in density and allowing the unambiguous positioning of the inhibitor in the binding cleft (Figure 3A). The binding mode of compound 7 in the active site of CDK2 is near identical in the positioning of the pyrazolo[4,3-*d*]pyrimidine core when compared with other homologous ligand structures (Protein Data Bank codes 2A0C, 1G5S, 1W0X, 1CKP, 2A4L, 3DDP, 3NS9) with the pyrazolo[4,3-*d*]pyrimidine being sandwiched between the side chains of Leu134 and Ile10. Similar to other homologous ligand structures, compound 7 forms a conserved hydrogen bond pattern to the backbone carbonyl of Leu83 and to the backbone NH of this same residue. An additional conserved hydrogen bond is found at the backbone carbonyl of Glu81, yet this interaction is considerably shorter when compared to other similar ligand structures (2.77 Å compared to 3.18 Å for the roscovitine complex, PDB code 2A4L) (Figure 3A). Some variation between homologous structures is found in the positioning of the phenyl ring of the benzylamino group. In general this group is sandwiched between the side chain of Ile10 and the backbone of His83. This is also the case with compound 7, although the interaction of the His84 backbone carbonyl at the ortho position of the ring is longer in this structure (Figure 3A). A further difference in the binding mode is apparent when comparing the position of the hydroxymethylpropyl group. This group takes up a similar orientation to that found in the olomoucine II structure (PDB code 2A0C) and is rotated in the opposite direction to that found in the structure containing

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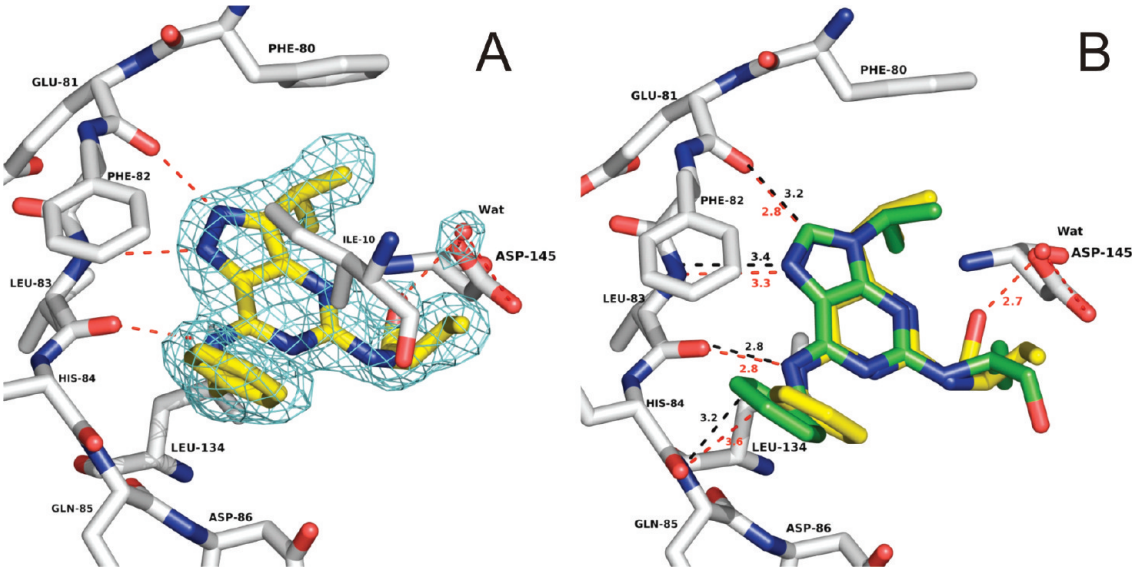

Figure 3. (A) Refined electron density for ligand compound **7** within the active site of CDK2. $2|F_o| - |F_c|$ density is colored cyan and is contoured at 1σ shown around the ligand and an interacting water molecule. Significant protein−ligand interactions are indicated by dashed red lines. (B) Overlay of compound **7** and roscovitine within the active site of CDK2.

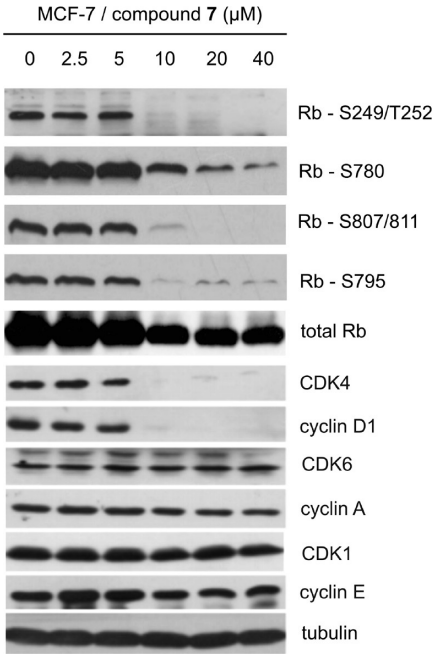

Figure 4. Immunoblot analysis of Rb phosphorylation and some cell cycle regulators. Asynchronous MCF-7 cells were exposed for 24 h to the indicated concentrations of compound **7**. Tubulin levels are included as a control for equal protein loading.

roscovitine (PDB code 2A4L, Figure 3B). The hydroxyl atom of this group makes a strong hydrogen bond to a water molecule (water 71), which in turn forms strong interactions to the side chain of Asp145 (Figure 3). This bridging interaction is not observed in any of the homologous ligand/CDK2 complex structures. Therefore, although ligand compound **7** is reminiscent of other roscovitine-like ligands, these are significant differences in their respective binding modes that make compound **7** distinct.

Cellular Effects of Compound 7. When exponentially growing human MCF-7 breast cancer cells were treated with compound **7** for 24 h, a dose-dependent inhibition of retinoblastoma protein (Rb) phosphorylation at Ser249/Thr252, Ser807/811, Ser780, and Ser795 became apparent on immunoblots of total cellular proteins probed with phosphospecific antibodies. These results demonstrate the ability of compound **7** to affect the activities of CDK4 and CDK2 in proliferating cells. These CDKs play critical positive roles at the G_1/S transition by phosphorylating the Rb protein. Inhibition of cellular CDK activity and consequent Rb dephosphorylation causes cell cycle arrest in the G_1 phase. Similar observations have been published not only with roscovitine[53−56] but also with roscovitine isomers imidazo[2,1-*f*]-1,2,4-triazine **13**[25] and pyrazolo-[1,5-*a*]-1,3,5-triazine **7a**.[25]

Moreover, changes of protein abundance for some cell cycle regulators upstream of the Rb protein were monitored in MCF-7 cells treated with compound **7**, compared with mock-treated controls. A significant reduction in CDK4 protein level was observed, and the abundance of cyclin D1, a positive regulator of CDK4, also diminished. In contrast, no changes in protein levels of CDK1, CDK6, and cyclins E and A were seen (Figure 4). This inhibitory pattern, consistent with the cell-cycle effects of compound **7**, was observed in MCF-7 cells also upon treatment with roscovitine[46] and pyrazolo[1,5-*a*]pyrimidine BS-181.[28] On the other hand, human HT-29 colon cancer cells responded to roscovitine treatment by a decrease in cyclin A protein level, while the abundance of CDK4 remained unchanged.[54]

Cell Cycle Analysis. The antiproliferative activity of compound **7** was verified by flow cytometry analysis of asynchronously growing MCF-7 cells and the multiple myeloma RPMI-8226 cell line, through double staining with propidium iodide and 5-bromo-2′-deoxyuridine (BrdU). As shown in Figure 5, treatment with both compound **7** and roscovitine arrested the cell cycle progression in the G_2/M phase and resulted in decreased S-phase populations in the two cell lines. Upon treatment with higher concentrations of roscovitine and

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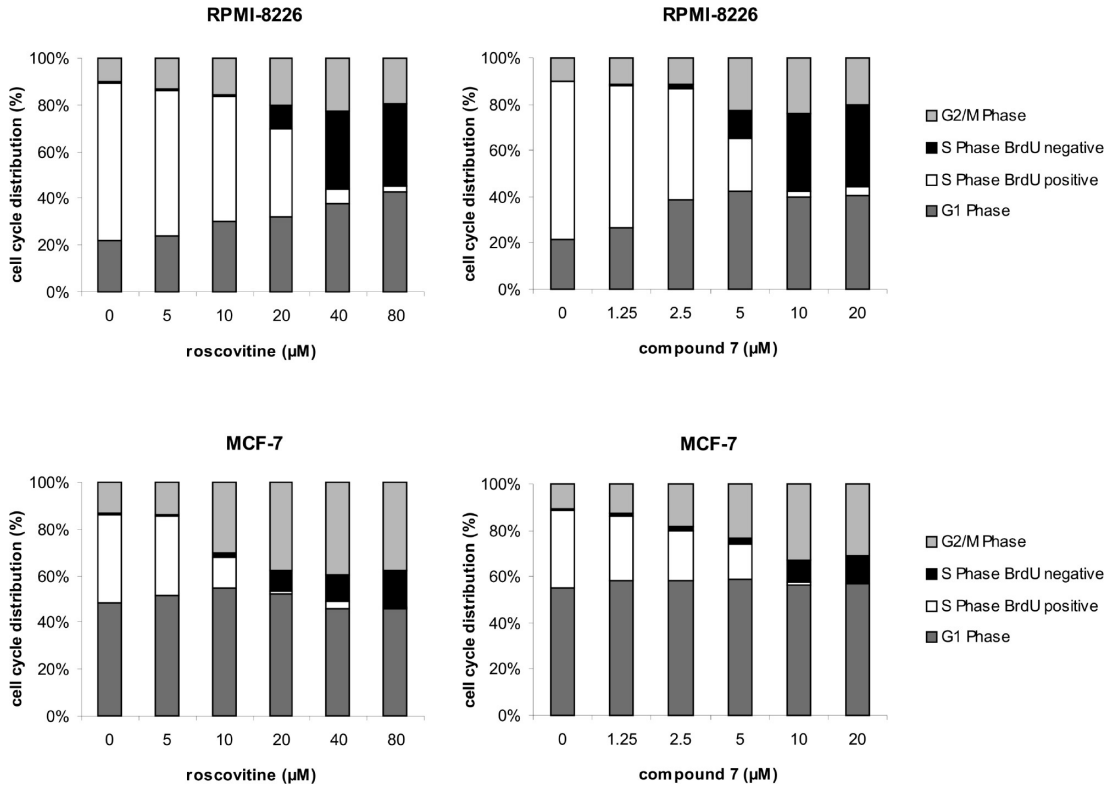

Figure 5. Compound 7 arrests cells at various stages of the cell cycle: flow cytometric analysis of BrdU and propidium iodide incorporation in RPMI-8226 and MCF-7 cells treated for 24 h with roscovitine and compound 7, respectively.

compound 7 the populations of cells actively replicating DNA (i.e., BrdU-positive cells) also decreased markedly. In addition, accumulation of RPMI-8226 cells in G_1 was found, an outcome not observed in MCF-7 cells. In summary, compound 7 arrested the cell cycle of two human cancer cell lines more efficiently than roscovitine, but both compounds displayed similar patterns of the cell-cycle blockade.

Besides cell cycle changes, increases of $subG_1$ population (indicative of apoptosis) upon treatment with the two compounds were also observed (Figure 5). Notably, compound 7 triggered apoptosis already after 24 h of treatment at compound concentrations above 10 μM in MCF-7, U266, and RPMI-8226 cells (Figure S3). These results correlate well with data on induction of apoptosis obtained by other apoptotic assays (see below and Figures 6 and S4).

Induction of Apoptosis. Most CDK inhibitors, including roscovitine, semisynthetic flavone flavopiridol,[57−59] and 3-substituted indolinone compound SU9516,[60,61] exert a strong proapoptotic effect on multiple myeloma cells through down-regulation of Mcl-1 protein.[53,62−64] Therefore, we studied induction of apoptosis in multiple myeloma cell lines in more detail. Compound 7 induced apoptosis in the multiple myeloma cell line RPMI-8226, as documented by detection of a cleaved fragment of caspase-3 and its enhanced enzymatic activity, by fragmentation of poly(ADP ribose)polymerase 1 (PARP) and by down-regulation of antiapoptotic protein Mcl-1 (Figure 6C). As shown in Figure 6A, treatment with 20 μM compound 7 induced strong activation of caspases 3 and 7 as quantified by a biochemical assay. This result correlates well not only with the immunoblotting analysis, where the cleavage of caspase-3 zymogene was observed under the same experimental conditions, but also with the flow cytometric detection of the caspase-3 fragment using the anticleaved caspase-3 (Asp175) antibody (Figure 6B,C). Monitoring of the cleavage of PARP, a nuclear target of caspase-3, further confirmed the above results. Taken together, our results clearly showed that compound 7 induces apoptosis in the RPMI-8226 multiple myeloma cell line in a concentration-dependent manner. Furthermore, consistent results were found also for another multiple myeloma cell line, U266 (Figure S5).

Induction of p53-Dependent Transcription. Treatment of cells harboring wild-type p53 with CDK inhibitors leads to accumulation of p53 and to an increase of p53-dependent transcription, as shown with roscovitine.[65,66] A strong nuclear immunofluorescence signal of p53 was also evident in MCF-7 cells following treatment with compound 7 (Figure 7A). These results were then confirmed and extended by immunoblotting analyses of the levels of p53 and its targets, $p21^{WAF1}$ and Mdm-2 (Figure 7C). We found that after 24 h incubations of proliferating MCF-7 cells with 20 μM roscovitine and 10 μM compound 7 the level of p53 was strongly increased. Moreover, the accumulated p53 was transcriptionally active, as indicated by the enhanced expression of the cell cycle inhibitory protein $p21^{WAF1}$, a well-established transcriptional target of p53.

Next, the effect of compound 7 on p53-dependent transcriptional activity was determined by the β-galactosidase activity measurement in the human melanoma cell line ARN8.[66] Compound 7 showed a dose-dependent activity effect on p53-regulated transcription, with the maximum impact observed between 10 and 20 μM (Figure 7B), while the maximal effect of roscovitine was observed at an approximately 1.5-fold higher concentration (data not shown).

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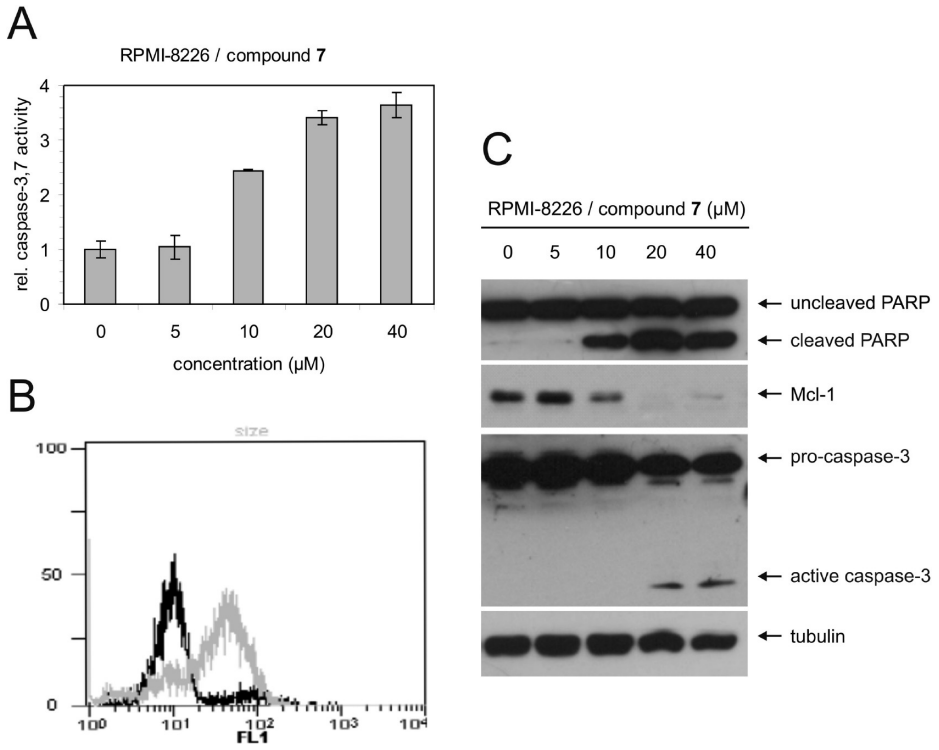

Figure 6. Compound 7 induces apoptosis in RPMI-8226 cell line after continuous 24 h treatment. (A) The activities of caspases 3 and 7 measured using a fluorogenic substrate Ac-DEVD-AMC in lysates of cells treated with increasing doses of compound 7. (B) Active fragment of caspase-3 (gray line) detected by flow cytometry using specific anticleaved caspase-3 (Asp175) antibody in cells treated with 20 μM compound 7. Black and gray lines indicate untreated and treated cells, respectively. (C) Fragmentation of PARP and caspase-3 and down-regulation of Mcl-1 detected by immunoblot analysis. Tubulin levels were monitored to verify equal protein loading.

Compound 7 Inhibits DNA Repair via Homologous Recombination Independently of RAD51 Protein Abundance. Homologous recombination (HR) is important for DNA double strand break (DSB) repair, and its proper function is required for the maintenance of genomic stability and cell survival.[67] HR repair seems to be restricted only to S and G2 phases of the cell cycle, where homologous sequences are available, consistent with regulation of HR activity via CDK dependent mechanism(s). Indeed, in yeast, Sae2 protein phosphorylation by CDK was identified as an important HR regulator[68] and a similar CDK-dependent mechanism was proposed also for mammalian cells. However, in mammalian cells the identification of CDK(s) responsible for regulation of HR is rather problematic because studies with roscovitine or flavopiridol also showed rapid down-regulation of a core HR-pathway protein Rad51,[69,70] the effect which may mask any other potential regulatory impact of CDK inhibition on HR. Moreover, Rad51 down-regulation seems unlikely to represent a physiological mode of HR regulation because Rad51 abundance is not markedly altered throughout G1, S, and G2 phases of the cell cycle.[71] Thus, we examined whether compound 7 could be used as an alternative CDK inhibitor, possibly capable of modulating the HR process without affecting the Rad51 status. We selected the highest concentrations of roscovitine, flavopiridol, and compound 7 which do not yet affect the cell cycle progression (Figure S6) and assessed HR using an assay in which HR efficiency to repair DSB within a reporter plasmid in human cells is quantified through measurement of the repair-generated GFP fluorescence signal by flow cytometry.[72] In parallel we monitored the RAD51 protein level by immunoblotting. In cells treated with compound 7 and flavopiridol, the HR efficiency was reduced to 66% and 62% of control values, respectively (Figure 8A). Notably, at the drug concentrations used for the HR assay only flavopiridol caused a significant RAD51 decrease (Figure 8B). Treatment with roscovitine at a concentration not affecting the cell cycle did not influence HR significantly nor did it affect the RAD51 level (Figure 8). On the basis of these results, obtained particularly because of compound 7, we conclude that CDK inhibition can affect HR efficiency independently of effects on RAD51 protein abundance.

■ CONCLUSION

Compound 7 was prepared and characterized as a representative of a new group of CDK inhibitors, trisubstituted pyrazolo[4,3-*d*]pyrimidines. In several biochemical and biological assays, the effects of compound 7 were compared with those of its bioisostere, roscovitine. These analyses showed similar kinase selectivity profiles of roscovitine and compound 7, yet apparently higher efficiency of the latter compound. The overall molecular and cellular effects of compound 7 were consistent with its ability to inhibit CDKs and, furthermore, revealed evidence for a role of CDKs in regulation of DNA repair by HR. The data suggest that blocking HR-mediated repair by compound 7 and perhaps also by other CDK inhibitors could potentially be exploited in cancer therapy in at least three scenarios: (i) to sensitize cancer cells to therapeutically used clastogens including ionizing radiation; (ii) as single agents to affect cancer cells preferentially, because of the tumor-specific oncogene-evoked replication stress and the ensuing constitutive DNA damage[73−75] whose repair requires HR;

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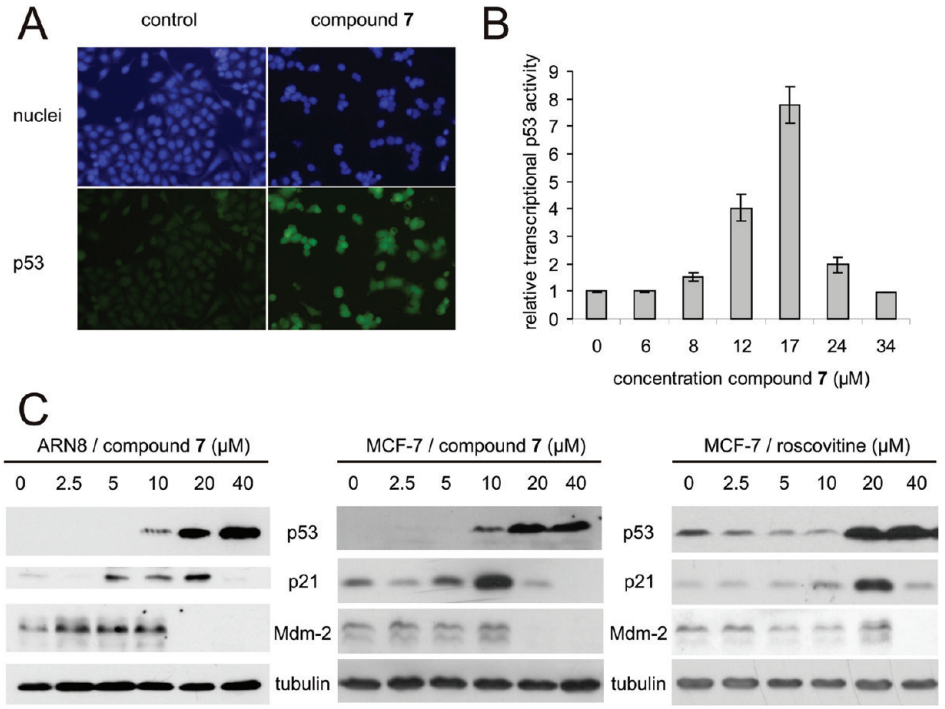

Figure 7. Induction of p53 in cells by compound 7. (A) Double labeling of asynchronous MCF-7 cells, treated with 10 μM compound 7 for 24 h, with anti-p53 antibody and DAPI. (B) Dose-dependent effect of 7 on p53-dependent transcription in ARN8 cells stably transfected with a p53-responsive β-galactosidase reporter construct and treated with 7 for 24 h. Fluorescence of cleaved product of 4-methylumbelliferon-β-D-galactopyranoside (MUG) was determined in lysed cells. Results represent the average ± SD for three independent experiments. (C) Immunoblotting analysis of p53 and its targets, $p21^{WAF1}$ and Mdm-2, in MCF-7 or ARN8 cells treated for 24 h with the indicated concentration of compound 7 and roscovitine, respectively. Tubulin is included as controls for equal protein loading.

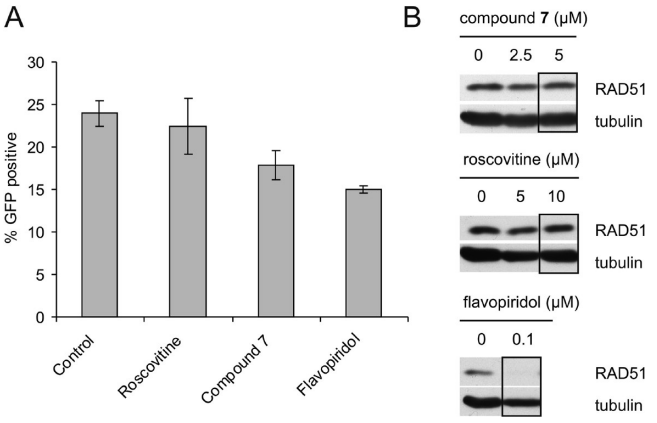

Figure 8. Compound 7 reduces homologous recombination. DR-U2OS-GFP cells expressing I-SceI nuclease to produce DSBs within the HR-reporter sequences were used to measure the GFP product as a readout for effects of CDK inhibitors on HR efficiency. (A) GFP level and its fluorescence intensity in DR-U2OS-GFP cells reflect the degree of successful HR-mediated recombination that was subject to modulation by roscovitine (10 μM), compound 7 (5 μM), or flavopiridol (0.1 μM) for 56 h. (B) Protein levels of RAD51 were analyzed by immunoblotting in DR-U2OS-GFP cells exposed to increasing concentrations of the drugs, as indicated. Frames highlight scenarios with drug concentrations used in the HR assay.

(iii) to selectively kill cancer cells that often harbor defects in DNA damage response pathways, taking advantage of the synthetic lethality principle.[67] Altogether, our present characterization of this novel trisubstituted pyrazolo[4,3-*d*]pyrimidine warrants further evaluation of these purine-derived bioisosteres as potential new candidate anticancer drugs.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined on a Kofler block and are uncorrected. NMR spectra were measured on a Bruker AVANCE III 400 MHz spectrometer (400.13 MHz for ^{1}H and 100.61 MHz for ^{13}C) and a Bruker AVANCE III 600 MHz spectrometer (600.23 MHz for ^{1}H and 150.93 MHz for ^{13}C) and a Varian Geminy 300 (300.1 MHz for ^{1}H and 75 MHz for ^{13}C) in DMSO-d_6 or $CDCl_3$ at 303 K. The residual solvent signal was used as an internal standard (δ_H 2.500 and δ_C 39.60 for DMSO-d_6 or δ_H 7.265 and δ_C 77.00 for $CDCl_3$). ^{1}H NMR, ^{13}C NMR, COSY, HSQC, and HMBC results were obtained using standard manufacturers' software (Topspin 2.1, Bruker Biospin GmbH, Rheinstetten Germany). Chemical shifts are given in δ scale [ppm] and coupling constants in Hz. Digital resolution enabled us to report chemical shifts of protons to 3 and coupling constants to 1 and carbon chemical shifts to 2 decimal places. ESI or APCI mass spectra were determined using a Waters Micromass ZMD mass spectrometer (direct inlet, coin voltage 20 V). IR spectra were recorded on an FT-IR Nicolet 200 instrument with KBr tablet. Compound purity was determined by elemental analyses (0.4%) or LC−MS analysis and was confirmed to be >95% for all compounds. Merck silica gel Kieselgel 60 (230−400 mesh) was used for column chromatography.

Prepared Compounds. *4-Amino-5-isopropyl-1(2)H-pyrazole-3-carboxamide (**1**).* **1** was prepared according to the published synthesis.[43]

*3-Isopropyl-5-sulfanyl-1(2)H-pyrazolo[4,3-d]pyrimidin-7-ol (**2**).* A mixture of amide **1** (1.5 g, 9 mmol) and thiourea (3.8 g, 46 mmol) was fused (195 °C) for 30 min under argon atmosphere. After cooling, the reaction melt was suspended in water (20 mL) and the solution was

dx.doi.org/10.1021/jm200064p |J. Med. Chem. XXXX, XXX, 000–000

alkalized at 0 °C by 2 M NaOH solution to pH 12.3. The resulting dark solution was decolorized by carboraffin. The filtered solution was acidified by HCl to pH 5.7−6.0. After 1 h the product was filtered off, washed with cooled water, and dried at 80 °C/20 Torr. Yield 82%, mp >305 °C (dec). MS ESI$^-$: $[M - H]^-$ = 209.3. ^{1}H NMR (400 MHz, 303.1 K, DMSO-d_6) δ 1.225 (d, J = 6.9 Hz, 6H, $(CH_3)_2$), 3.370 (sept, J = 6.9 Hz, 1H, CH), 12.116 (br s, 1H), 12.651 (br s, 1H), 13.842 (br s, 1H). ^{13}C NMR (100 MHz, 303.1 K, DMSO-d_6) δ 21.91 (q, $(CH_3)_2$), 24.08 (d, CH), 124.04 (s), 126.91 (s), 134.08 (s, C-9), 143.13 (s, C-9), 152.57 (s), 155.34 (s). Anal. ($C_8H_{10}N_4OS$) C, H, N, S.

*3-Isopropyl-5-methylsulfanyl-1(2)H-pyrazolo[4,3-d]pyrimidin-7-ol (**3**).* Thiol **2** (16 g, 70 mmol) was added to a mixture of 50 mL of EtOH and 170 mL of water at 35 °C. The pH of the solution was adjusted to 9.3 by adding a water solution of 30% NaOH, and thus, thiol **2** was dissolved. During vigorous agitation dimethylsulfate (6.6 mL, 70 mmol) was added at 20 °C to the reaction mixture. The crystallized product was filtered off, washed (2×) with a mixture of EtOH/H_2O (1/2.5, 20 mL), and water. The product was dried at 80 °C/20 Torr. Yield 90%, mp 213−216 °C. MS ESI$^-$: $[M - H]^-$ = 223.3. ^{1}H NMR (600 MHz, 303.1 K, DMSO-d_6) δ 1.349 (d, J = 7.0 Hz, 6H, $(CH_3)_2$), 2.531 (s, 3H, CH_3S), 3.216 (sept, J = 7.0 Hz, 1H, CH), 12.398 (s, 1H, NH or OH), 13.513 (s, 1H, NH or OH). ^{13}C NMR (150 MHz, 303.1 K, DMSO-d_6) δ 12,94 (q, CH_3S), 21.69 (q, $(CH_3)_2$), 26.32 (d, CH), 125.08 (s), 136.91 (s, C-4), 149.48 (s, C-9), 151.94 (s, C-2), 153.51 (s). Tautomer: ^{1}H NMR (600 MHz, 303.1 K, DMSO-d_6) δ 1.369 (d, J = 7.0 Hz, 6H, $(CH_3)_2$), 2.50* (s, 3H, CH_3S), 3.285 (sept, J = 7.0 Hz, 1H, CH), 11.959 (s, 1H, NH or OH), 13.770 (s, 1H, NH or OH). ^{13}C NMR (150 MHz, 303.1 K, DMSO-d_6) δ 12,86 (q, CH_3S), 21.69 (q, $(CH_3)_2$), 24.74 (d, CH), 133.60 (s), 134.69 (s), 140.34 (s, C-9), 151.21 (s, C-2), 157.35 (s). HSQC readout was done.

*7-Chloro-3-isopropyl-5-methylsulfanyl-1(2)H-pyrazolo[4,3-d]pyrimidine (**4**).* Dimethylaniline (30 mL) was dropped under nitrogen atmosphere into a mixture of thioether **3** (18 g, 80 mmol) and $POCl_3$ (120 mL) during 30 min. Then the reaction mixture was refluxed for 5 h (bath temperature of 108 °C). After cooling to room temperature, the reaction mixture was concentrated under vacuum (the excess of $POCl_3$ was removed). Methyl *tert*-butyl ether (MTBE) (140 mL) was added, and during agitation water (50 mL) was added. The organic phase was separated off, and the water phase was extracted once more by MTBE. Combined organic phases were diluted by 70 mL of water and alkalized by solid $NaHCO_3$ (2 g) to pH 7.5. The mixture was then agitated for 2 h. The organic phase was separated by carboraffin trituration and dried by $MgSO_4$. Crystallization from MTBE−heptane afforded (after drying at 70 °C/20 Torr) 16 g of a yellow product. Yield 80%, mp >170 °C (dec). MS ESI$^-$: $[M - H]^-$ = 241.3 (100%), 243 (30%). ^{1}H NMR (400 MHz, 303.1 K, $CDCl_3$) δ 1.500 (d, J = 7.0 Hz, 6H, $(CH_3)_2$), 2.644 (s, 3H, CH_3S), 3.488 (sept, J = 7.0 Hz, 1H, CH). ^{13}C NMR (100 MHz, 303.1 K, $CDCl_3$) δ 14.59 (q, CH_3S), 21.34 (q, $(CH_3)_2$), 27.12 (d, CH), 128.77 (s, C-6), 144.21 (s, C-5), 144.51 (s, C-4), 151.96 (s, C-9), 163.60 (s, C-2). Anal. ($C_9H_{11}ClN_4S$) C, H, N, Cl.

*7-Benzylamino-3-isopropyl-5-methylsulfanyl-1(2)H-pyrazolo[4,3-d]pyrimidine Hydrochloride (**5**).* 7-Chloro derivative (8.5 g, 35 mmol), benzylamine (9 mL, 85 mmol), and triethylamine (23 mL, 230 mmol) in 50 mL of 1-butanol were heated with stirring at 100 °C for 3 h. The solution was evaporated to dryness in vacuum, and the residue was partitioned between H_2O and EtOAc. The combined organic phase was purified by carboraffin and dried with magnesium sulfate and evaporated. Product was dissolved in a mixture of 30 mL of MTBE, 10 mL of H_2O, and 4 mL of isopropanol. After acidification by 5 N HCl to pH 0.5, hydrochloride **5** crystallized. Product was filtered off, washed twice with a mixture of MTBE/2-propanol (7/3, 10 mL), and dried at 70 °C/20 Torr. Yield 11 g, 88%, mp 197−204 °C (after recrystallization from boiling EtOH, mp 205−210 °C). MS ESI$^-$: $[M - H]^-$ = 312.3 (100%), 348 (50%, M + Cl$^-$). MS ESI$^+$: 314.3 (100%, M + H$^+$). IR (cm^{-1}): 1618,1581,1532, 1353, 1246, 1181, 1062, 924, 698. ^{1}H NMR (400 MHz, 303.1 K, $CDCl_3$) δ 1.340 (d, J = 7.0 Hz, 6H, $(CH_3)_2$), 2.541 (s, 3H, CH_3S), 3.300 (sept, J = 7.0 Hz, 1H, CH), 4.762 (br s, 2H, NHC**H**$_2$), 6.658 (br s, 1H, N**H**CH_2), 7.213−7.277 (5H, m, H-ortho, H-meta, H-para), 11.615 (br s, H-7 or H-8). ^{13}C NMR (100 MHz, 303.1 K, $CDCl_3$) δ 14.42 (q, CH_3S), 21.51 (q, $(CH_3)_2$), 26.01 (d, CH), 44.65 (t, $NHCH_2$), 127.53 (d, C-para), 127.79 (d, C-ortho), 128.62 (d, C-meta), 137.96 (s, C-ipso), 138.77 (s), 151.24 (s, C-9), 163.34 (s, C-2). Anal. ($C_{16}H_{19}N_5S$) C, H, N.

*7-Benzylamino-3-isopropyl-5-methylsulfonyl-1(2)H-pyrazolo[4,3-d]pyrimidin (**6**).* Thioether **5** (liberated base, 8 g, 26 mmol) was dissolved in 40 mL of ethanol, and solution of 20 g of Oxone in 50 mL of H_2O was added at 45−50 °C. Oxygenation was checked by TLC (silica gel, MeOH/toluene, 1/9) and was completed in 30 min. Water (130 mL) and ethyl acetate (50 mL) were added. Product was extracted in the organic phase once more with 20 mL of ethyl acetate. The combined organic phase was purified by carboraffin and dried with magnesium sulfate and evaporated. Product was crystallized from methanol. Yield 7.5 g, 83%, mp 96 °C. MS ESI$^-$: $[M - H]^-$ = 344.3 (100%, M − H$^+$). MS ESI$^+$: 346.3 (100%, M + H$^+$). IR (cm^{-1}): 1626, 1534, 1453, 1359, 1297, 1128 (SO_2), 1060, 926, 753. ^{1}H NMR (400 MHz, 303.1 K, $CDCl_3$) δ 1.325 (d, J = 5.0 Hz, 6H, $(CH_3)_2$), 3.222 (s, 3H, CH_3SO_2), 3.349 (br s, 1H, CH), 4.761 (br s, 2H, NHC**H**$_2$), 7.259−7.185 (3H, m, H-meta, H-para), 7.310 (2H, m, H-ortho). ^{13}C NMR (100 MHz, 303.1 K, $CDCl_3$) δ 21.56 (q, $(CH_3)_2$), 26.14 (d, CH), 39.13 (q, CH_3SO_2), 45.17 (t, N**H**CH_2), 124.79 (s), 127.64 (d, C-para), 128.01 (d, C-ortho), 128.64 (d, C-meta), 137.27 (s, C-ipso), 150.68 (s), 151.39 (s), 157.79 (s, C-2). Anal. ($C_{16}H_{19}N_5O_2S$) C, H, N.

*7-Benzylamino-5(R)-[2-(hydroxymethyl)propyl]amino-3-isopropyl-1(2)H-pyrazolo[4,3-d]pyrimidine (**7**).* Methylsulfone **6** (0.2 g, 0.58 mmol) and *R*-(−)-2-amino-1-butanol (2 mL, 23 mmol) were heated in sealed ampule for 3 h to 160 °C. Excess of the amine was evaporated at a temperature below 70 °C, and the residue was partitioned in $CHCl_3$/H_2O. The combined organic phases were dried with magnesium sulfate and evaporated. Product was purified by column flash chromatography on silica gel stepwise with 1%, 2%, 4%, and 6% MeOH in $CHCl_3$. Product was obtained in noncrystallizable amorphous colorless glass form. Yield 54 mg, 25%, $[\alpha]_D$ +53 (c 1.35, $CHCl_3$). MS ESI$^+$: $[M + H]^+$ = 355.4 (100). MS ESI$^-$: $[M - H]^-$ = 353.3 (100). ^{1}H NMR (300 MHz, DMSO-d_6) 0.85 (t, J = 7,5 Hz, 3H, C**H**$_3$CH$_2$), 1.32 (d, J = 7.0 Hz, 6H, (C**H**$_3$)$_2$CH), 1.39−1.68 (m, 2H, C**H**$_2$CH$_3$), 3,16 (sept, J = 7,0 Hz, 1H, C**H**$(CH_3)_2$), 3.37 −3.51 (m, 2H, C**H**$_2$OH), 3.78 (m, 1H, C**H**NH), 4.68 (br s, 2H, CH_2Ph), 5.74 (br s, 1H, NH), 7.26 (m, 1H, H-para), 7.34 (m, 2H, H-meta), 7.39 (m, 2H, H-ortho), 11.79 bs ($^1/_2$ H, NH), 13,28 bs ($^1/_2$ H, NH). ^{13}C NMR (75 MHz, DMSO-d_6) δ 10.6, 21.5, 21.6, 23.8, 25.9, 43.0, 54.2, 63.4, 126.9, 127.5, 128.3, 139.3, 147.1, 157.6 . Anal. ($C_{19}H_{26}N_6O$) C, H, N.

Enzyme Inhibition Assay. CDK2/cyclin E kinase was produced in Sf9 insect cells via baculoviral infection and purified on a NiNTA column (Qiagen). CDK5/p35, CDK7cyclin H/MAT1, and CDK9/cyclin T1 were purchased from ProQinase GmbH. The kinase reactions were assayed with 1 mg/mL histone H1 (for CDK2 and CDK5) or $(YSPTSPS)_2KK$ peptide (for CDK7 and CDK9) in the presence of 15/0.15/1.5/1.5 μM ATP (for CDK2/CDK5/CDK7/CDK9), 0.05 μCi [γ-^{33}P]ATP, and the test compound in a final volume of 10 μL, all in a reaction buffer (60 mM HEPES−NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 μM sodium orthovanadate, 1.2 mM DTT, 2.5 μg/50 μL $PEG_{20.000}$). The reactions were stopped by adding 5 μL of 3% aqueous H_3PO_4. Aliquots were spotted onto P-81 phosphocellulose (Whatman), washed 3× with 0.5% aqueous H_3PO_4, and finally air-dried. Kinase inhibition was quantified using digital image analyzer FLA-7000 (Fujifilm). The concentration of the test compounds required to decrease the CDK by 50% was determined from dose-response curves and designated IC_{50}.

dx.doi.org/10.1021/jm200064p |*J. Med. Chem.* XXXX, XXX, 000–000

Kinase Selectivity. All kinase assays were carried out by the SelectScreen Kinase Profiling Service in the presence of 100 μM ATP and 10 μM compound and performed according to the standard protocol of Invitrogen.

Crystallization and Structure Determination. Human recombinant CDK2 was purchased from ProQinase GmbH, and crystals were grown following the protocol of the supplier. The compound **7**/CDK2 complex was prepared by transferring a coverslip containing a drop of native CDK2 crystals over a well solution of 35% PEG 6000 and equilibrating at 17 °C for 24 h. A single crystal of CDK2 was transferred from this drop into a 1 μL drop of 35% PEG 6000, 100 mM Na HEPES buffer (pH 7.5), 1 mM compound **7**, and 5 mM DMSO and placed over a well of the same solution. Crystals were left to soak for 2 days. The crystal of about 0.1 mm in length was mounted in a 0.1−0.2 mm cryoloop (Hampton Research) and was flash-frozen in liquid nitrogen. The soaking solution acted as a cryoprotectant. All diffraction data were collected at 100 K (Cryostream) using a Rigaku Micro7 rotating anode generator and a Mar345 detector (MarResearch). Data processing was carried out using the programs MOSFLM and SCALA.[76] Initial structure solution was performed using the program PHASER[77] using an available CDK2 structure (PDB code 2A0C). The programs REFMAC[78] and PHENIX[79] were used for refinement, with manual refinement and waterfitting being performed by the program COOT.[80] Crystallographic processing and refinement statistics are summarized in Supporting Information (Table S1). Atomic coordinates have been deposited in the Brookhaven Protein Data Bank under the accession code 3PJ8.

Cell Maintenance and Cytotoxicity Assay. The cytotoxicity of the studied compounds was determined with cell lines of different histological origin. The cells, cultured in DMEM (supplemented with 10% fetal calf serum, 4 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin) in a humidified CO_2 incubator at 37 °C, were redistributed into 96-well microtiter plates at appropriate densities for their respective cell sizes and growth rates. After preincubation, test compounds in 3-fold dilutions were added in triplicates. Treatment lasted for 72 h and then calcein AM solution was added. The fluorescence of the live cells was measured at 485 nm/538 nm (excitation/emission) with a Fluoroskan Ascent microplate reader (Labsystems). IC_{50} values, the drug concentrations reducing number of viable cells to 50%, were determined from the dose-response curves.

Immunoblotting. For direct immunoblotting, total cellular protein lysates were prepared by harvesting treated cells in Laemmli sample buffer. Proteins were separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membrane. The blotted membranes were stained with 0.2% Ponceau-S in 1% aqueous acetic acid to verify equal protein loading, destained, and blocked in PBS and 0.1% Tween 20 (PBS-T) with 5% low fat milk or 3% bovine serum albumin (BSA). The membranes were then incubated with specific antibodies overnight at 4 °C. After being washed three times in PBS-T, the membranes were incubated with a 1:2000 dilution of peroxidase-conjugated secondary antibodies. After another three washes in PBS-T, peroxidase activity was detected using ECL+ reagents (AP Biotech) according to the manufacturer's instructions.

Antibodies. Specific antibodies were purchased from Cell Signaling Technology (antitotal pRb, clone 4H1, and anti-pRb antibodies phosphorylated at S780, S795, and S807/811), Sigma-Aldrich (anti-pRb phosphorylated at Ser249/Thr252, anti-α-tubulin, clone DM1A, peroxidase-labeled secondary antibodies), Santa Cruz Biotechnology (anti-Mcl-1, clone S-19, anti-PARP, clone F-2, anti-Mdm-2, clone SMP14, anti-RAD51, clone H-92, anti CDK1, clone B-6; anti-cyclin E, clone HE12), DAKO Cytomation (anti-caspase-3), Roche Applied Science (anti-5-bromo-2′-deoxyuridine-fluorescein, clone BMC 9318), Jackson ImmunoResearch Laboratory (fluorescein-conjugated Goat Anti-Mouse IgG), Beckman Coulter (anti-cleaved caspase-3) or were a generous gift from Dr. B. Vojtěšek (anti-p53, clone DO-1, anti-$p21^{WAF1}$, clone 118, anti-CDK4, anti-cyclin D1, anti-CDK6, anti-cyclin A).

BrdU Incorporation and Cell Cycle Analysis. Subconfluent MCF-7 cells were treated with compound **7** or roscovitine at different concentrations for 24 h. The cultures were fed and pulse-labeled with 10 μM 5-bromo-2′-deoxyuridine (BrdU) for 30 min at 37 °C before harvesting. The cells were trypsinized, washed by PBS containing 1% BSA (PBS/BSA), fixed with ice-cold 70% ethanol, incubated on ice for 30 min, washed with PBS/BSA again, and resuspended in 2 M HCl for 30 min at room temperature in order to denature their DNA. Following neutralization with 0.1 M $Na_2B_4O_7$, the cells were harvested by centrifugation and washed with PBS/BSA containing 0.5% Tween-20. They were then stained with anti-BrdU fluorescein-labeled antibody (1:50) for 30 min at room temperature in the dark. The cells were then washed with PBS, incubated with propidium iodide (0.1 mg/mL) and RNase A (0.5 mg/mL) for 1 h at room temperature in the dark and finally analyzed by flow cytometry using a 488 nm laser (Cell Lab Quanta SC, Beckman Coulter).

p53-Dependent Transcriptional Activity. To measure p53-dependent transcriptional activity, β-galactosidase activity was determined in the human melanoma cell line ARN-8, stably transfected with a p53-responsive reporter construct pRGCΔfoslacZ.[66] After 24 h of incubation with the inhibitors the cells were permeabilized with 0.3% Triton X-100 for 15 min, and then 4-methylumbelliferon-β-D-galactopyranoside was added as a substrate to a final concentration of 80 μM. After 1 h the fluorescence was measured at 355 nm/460 nm (excitation/emission) with a Fluoroskan Ascent microplate reader (Labsystems).

Immunofluorescence. MCF-7 cells grown on coverslips were treated with increasing concentrations of compounds for 24 h. Slips were then rinsed in PBS, and cells were fixed in methanol/acetone (1:1) at −20 °C for at least 1 h. The coverslips were then rehydrated in PBS for 10−20 min, rinsed with 10% fetal bovine serum, and incubated with the mouse monoclonal anti-p53 antibody (DO-1) for 1 h at room temperature. The samples were then washed three times with PBS before being incubated for 1 h with a secondary fluorescein isothiocyanate-conjugated anti-mouse IgG antibody. After incubation the coverslips were rinsed three times in PBS and then the nuclei were stained by DAPI (MP Biomedicals). After the final wash by water the coverslips were mounted on microscope slides using Mowiol mounting medium (Calbiochem) and observed using a fluorescence microscope (Olympus BX50) coupled with a digital camera (Olympus DP71).

Caspase-3/7 Assay. The cells were harvested by centrifugation and homogenized in an extraction buffer (10 mM KCl, 5 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.2% CHAPS, inhibitors of proteases, pH 7.4) on ice for 20 min. The homogenates were clarified by centrifugation at 10000*g* for 30 min at 4 °C. The proteins were quantified by the Bradford method and diluted to equal concentrations. Lysates were then incubated for 1 h with 100 μM Ac-DEVD-AMC as substrate in the assay buffer (25 mM PIPES, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, pH 7.3). For negative controls, the lysates were supplemented with 100 μM Ac-DEVD-CHO as a caspase-3/7 inhibitor. The fluorescence of the product was measured using a Fluoroskan Ascent microplate reader (Labsystems) at 355 nm /460 nm (excitation/emission).

Flow Cytometry Analysis of Cleaved Caspase-3. RPMI-8226 cells were collected by centrifugation, and the pellets were resuspended in 4% formaldehyde solution for 10 min at 37 °C. The cells were then permeabilized by adding ice-cold methanol to a final concentration of 90% and incubated for 30 min on ice. Subsequently the cells were rinsed in BSA/PBS, pelleted, resuspended in a few drops of BSA/PBS, and incubated for 10 min at room temperature. Then anticleaved caspase-3 antibody conjugated with Alexa Fluor 488 (Beckman Coulter) was added, and the cells were incubated for 1 h in the dark at room temperature. Finally the cells were washed in BSA/PBS, resuspended

dx.doi.org/10.1021/jm200064p |*J. Med. Chem.* XXXX, XXX, 000–000

again, and analyzed by flow cytometry using a 488 nm laser (Cell Lab Quanta SC, Beckman Coulter).

Homologous Recombination Assay. A U2OS clone containing a single complete copy of the integrated HR reporter hprt-DR-GFP was obtained from Pierce et al.[81] DR-U2OS-GFP cells (3.3×10^5 per well) were seeded in 100 mm dishes and transfected with 1 μg of pCKA-I-SceI plasmid using Fugene 6 reagent. Sixteen hours later, the medium was replaced with fresh medium containing tested compounds (5 μM for compound **7**, 10 μM for roscovitine, 0.1 μM for flavopiridol). Cells were harvested after 2 days for flow cytometric analysis on a Cell Lab Quanta SC cytometer (Beckman Coulter).

NCI60 Cytotoxicity Assay. Tests of toxicity on NCI60, a set of 59 human cancer cell lines derived from nine tissue types, were performed at the Developmental Therapeutics Program of the National Cancer Institute (Bethesda, MD, U.S.). The cytotoxicity of compound **7** was evaluated by measuring total cell protein using the sulforhodamine B method according to the standard protocol at time 0 and after 48 h. The highest concentration tested was 100 μM. GI_{50}, TGI, and LC_{50} (concentration of a drug inducing 50% reduction of growth, total growth inhibition, and 50% reduction of initial cell population, respectively) were estimated from the dose-response curves.

Correlation Analysis of NCI60 Activity. The activity pattern (GI_{50} values for individual NCI60 cell lines) of compound **7** was correlated with the drug activity patterns in the DTP cancer screening data set, May 2009 release (http://dtp.nci.nih.gov/docs/cancer/cancer_data.html). Pearson correlation coefficients (r) were calculated on a log−log scale. Signed version of coefficient of determination is defined as $[r/\mathrm{abs}(r)]r^2$. Only the activity patterns fulfilling following criteria were analyzed: (1) GI_{50} values for at least 50 cell lines, (2) GI_{50} reached against more than 50% of the cell lines tested, and (3) GI_{50} for the most sensitive cell line at least 5 times lower than GI_{50} for the most resistant cell line. Experimentally validated low molecular inhibitors of CDKs 1, 2, 4, 5, 7, 8, 9 and glycogen synthase kinase-3β with $IC_{50} < 100$ μM were extracted from BindingDB.[51,52] SDfile with the data was downloaded on November 17, 2009. Pubchem ID was used for conversion of BindingDB and NCI60 identifiers. Data manipulation and analysis was done in R 2.8.1.

■ ASSOCIATED CONTENT

Supporting Information. Crystallographic data collection and refinement statistics, kinase inhibition curves, NCI60 screening of compound **7**, cell cycle analysis of K562, U266, and DR-U2OS-GFP cells upon treatment with roscovitine, compound **7**, and flavopiridol, and the influence of compound **7** on subG1 cell population in different cell lines. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

[†]PDB code 3PJ8.

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■ ACKNOWLEDGMENT

The authors thank J. Hudcová and D. Parobková for their technical assistance. Dr. B. Vojtěšek is acknowledged for the gift of antibodies. This work was supported by GACR Grants 204/08/511, 301/11/P554, and 301/02/0475, GAAV Grant IAA50-1370902, IGAMZ Grants NS10282-3/2009 and NT/11065-5/2010, the European Commission (Projects Infla-Care and CZ.1.05/2.1.00/01.0030), and Grant MSM6198959216 and Grant ED0007/01/01 of Centre of the Region Haná for Biotechnological and Agricultural Research. Cytotoxicity testing on NCI60 panel was done at the Developmental Therapeutics Program (DTP) of the National Cancer Institute (Bethesda, MD, U.S.).

■ ABBREVIATIONS USED

BrdU, 5-bromo-2′-deoxyuridine; CDK, cyclin-dependent kinase; CK, casein kinase; DSB, double strand breaks; SAR, structure−activity relationship; GI_{50}, growth inhibition 50%; GSK, glycogen synthase kinase; HR, homologous recombination; LC_{50}, reduction of initial cell population 50%; MEK1, MAPK/ERK kinase 1; MNK-1, MAPK-interacting kinase 1; MSK, mitogen- and stress-activated protein kinase; MTBE, methyl *tert*-butyl ether; MUG, 4-methylumbelliferon-β-D-galactopyranoside; NCI, National Cancer Institute; PARP, poly(ADP ribose)polymerase 1; PCNA, proliferating cell nuclear antigen; PDB, Protein Data Bank; PRAK, p38-regulated-activated protein kinase; Rb, retinoblastoma protein; TGI, total growth inhibition

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PŘÍLOHA V.

Řezníčková E, **Jorda R**, Kryštof V, Havlíček L, Strnad M: A novel pyrazolo[4,3-*d*]pyrimidine inhibitor of cyclin-dependent kinases: antiproliferative and proapoptotic effects. *22nd EORTC-NCI-AACR symposium on Molecular targets and Cancer Therapeutics*, Nov 16-18, 2010, Berlin, Germany, *European Assoc Canc Res*; EJC SUPPLEMENTS Vol 8: pp 160-160.

A NOVEL PYRAZOLO[4,3-*d*]PYRIMIDINE INHIBITOR OF CYCLIN DEPENDENT KINASES: antiproliferative and proapoptotic effects

Poster No. 500

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BACKGROUND

Cyclin-dependent kinases (CDK) are a group of enzymes involved in many cellular processes including regulation of the cell cycle and transcription. Deregulation of the cell cycle connected with CDK hyperactivity is a common feature of the tumor cells and provides a rationale for the development of specific CDK inhibitors. We have recently prepared a novel class of purine bioisostere CDK inhibitors based on the pyrazolo[4,3-*d*]pyrimidine skeleton. This work is focused on the biological and biochemical characterization of new 3,5,7-trisubstituted pyrazolo[4,3-*d*]pyrimidine, LGR1492 (Figure 1).

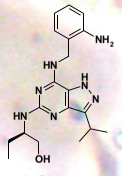


Figure 1: Chemical structure of cyclin-dependent kinase inhibitor LGR1492.

PROTEIN KINASE SELECTIVITY

Preliminary kinetic measurements suggested that LGR1492 is a potent inhibitor of CDK2/cyclinE (IC_{50} = 10 nM) and clearly exceeds the IC_{50} of correspondingly substituted bioisostere with the purine skeleton (Figure 2). Then we profiled it on a panel covering 70 enzymes across all protein kinase families at two doses of the compound (1 and 10 μM). The data confirmed the preference of LGR1492 for CDK2 (residual activity 1% at 1 μM). No other protein kinases are inhibited in the same concentration range, only four kinases (CK1, ERK1/2, ERK8 and PAK4) are inhibited in submicromolar range (6 - 15% residual kinase activity at 10 μM).

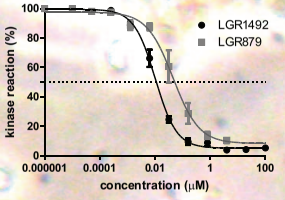


Figure 2: Inhibition of recombinant human CDK2/cyclin E by LGR1492 (black circles) and its purine analogue LGR879 (grey squares).

ANTIPROLIFERATIVE ACTIVITY OF LGR1492

Human tumour cell lines	IC_{50} (μM)
	Compound 1492
MCF-7	2.81 ± 0.42
MDA-MB-468	4.00 ± 0.31
HT-29	9.75 ± 3.63
HCT-116	3.48 ± 0.27
RPMI-8226	4.41 ± 0.19
U266	3.01 ± 0.26
K-562	9.24 ± 1.45
CEM	3.46 ± 0.09
HOS	5.53 ± 0.41
G-361	4.15 ± 0.43
HeLa	4.84 ± 1.78
Average cytotoxicity	4.68

Antiproliferative activity of LGR1492 was tested against selected human cancer cell lines of different histological origins. The results are summarized in Table 1 and they clearly show strong anticancer activity of LGR1492.

Table 1: Inhibition of different human cancer cell line proliferation in Calcein AM assay. (Average values from three determinations ± SD).

CELL CYCLE ANALYSIS: DNA CONTENT

The antiproliferative activity of LGR1492 was verified by flow cytometry analysis of asynchronously growing colon carcinoma cell line HCT-116 stained with propidium iodide. As shown in Table 2 inhibitor LGR1492 potently decreases population of cells in G1 phase of cell cycle and arrests cancer cell line in late S and G2/M phases. Additionally we can also observe the increase of apoptotic cells upon treatment with 10 and 20 μM of LGR1492.

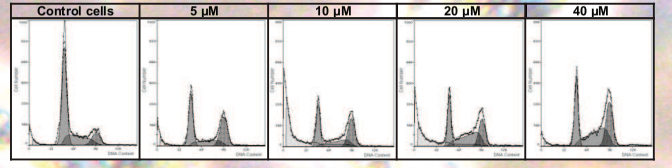
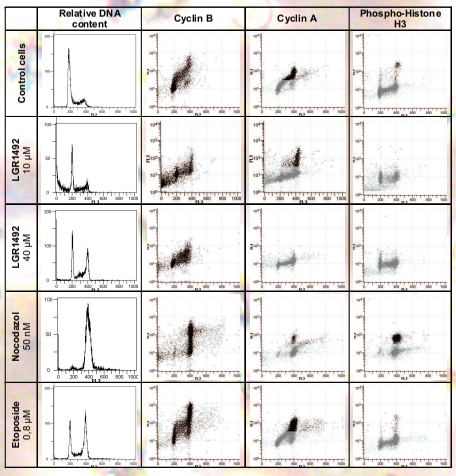


Table 2: Flow cytometry analysis of cell cycle after propidium iodide staining in HCT-116 cell line treated with LGR1492 for 24 hours.

CELL CYCLE ANALYSIS: PROTEIN EXPRESSION



For an accurate determination of cell cycle redistribution in HCT-116 cells upon treatment of LGR1492 we used the multi-parameter flow cytometry analysis of important cell cycle regulators (cyclins A and B) and mitotic marker phospho-histone H3 and etoposide and nocodazole as standard molecules whose effects on redistribution of cell are well known.

It is clear now that effect of pyrazolo[4,3-*d*]pyrimidine LGR1492 (10 μM or 40 μM) has not correlated with neither of used standards. At the dose of 10 μM the cell population showed the expression of cyclin A, but not cyclin B, which was undetectable in both tested concentrations. Absence of phospho-histone H3 was observed in LGR1492-treated cells and this indicates that the compound arrests the cell cycle before the mitosis. Both etoposide and LGR1492 (40 μM) influence relative DNA content similarly (see DNA histograms in Table 3) with significant G2/M peaks, but the expression of cyclins is totally different. Therefore we can speculate assumably for cell cycle arrest in late S, supported by the higher expression of cyclin A in comparison to cyclin B. The effects of higher concentrations of LGR1492 (40 μM) on cells probably reflect other biological impacts, e.g. inhibition of global trancription.

Table 3: Multi-parameter flow cytometry analysis of cyclin B, A and phospho-histone H3 in HCT-116 cell line treated with LGR1492 (10 μM or 40 μM) for 24 hours. Etoposide or nocodazole were used as standard samples.

INDUCTION OF APOPTOSIS

For the evaluation of apoptotic effects of LGR1492 in HCT-116 cells we used fluorimetric-based caspase-3,7 activity assay and immunoblot analyses of some apoptotic markers (Figure 4).

We determined the fragmentation of caspase-3 and its substrate poly(ADP-ribose)polymerase (PARP) as frequently markers of apoptosis. Treatment with LGR1492 lead to cleavage of caspase-3 at 10 and 20 μM LGR1492, surprisingly not at the highest 40 μM concentration. Consequently we confirmed these results by the monitoring of the cleavage of PARP. Its fragment (89 kDa) was detected only in cells treated within the concentration range 5 - 20 μM. The previous results were complemented by caspase-3,7 enzyme activity assay. LGR1492 induced activation of caspases-3,7 with result corresponding with immunoblot analysis where the cleavage of zymogene of caspase-3 was observed at the same concentration (Figure 4B). The ability of LGR1492 to induce apoptosis was also tested in multiple myeloma RPMI-8226 cells. In comparison with results established in HCT-116 cells, the LGR1492 strongly activate apoptosis in a concentration-dependent manner (data not shown).

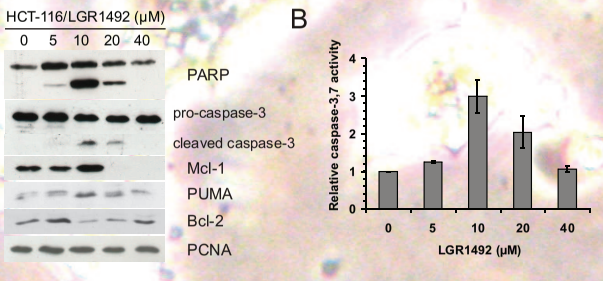


Figure 4: Induction of apoptosis. (A) Immunoblot analysis of PARP, caspase-3, Mcl-1, PUMA and Bcl-2 in HCT-116 treated with LGR1492 for 24 h. PCNA levels were detected to verify equal protein loading. (B) Fluorimetric caspase-3,7 enzyme activity assay based on cleavage of specific Ac-DEVD-AMC peptide substrate.

EFFECT ON REPLICATION AND TRANSCRIPTION

The effect of LGR1492 on replication was verified by flow cytometry analysis of HCT-116 cells doubly stained with propidium iodide and 5-bromo-2'-deoxyuridine (BrdU). As shown in Figure 5, LGR1492 rapidly decreased the population of cells actively replicating DNA (BrdU-positive) in a dose-dependent manner.

For the evaluation of transcription inhibition we pulse-labelled RNA and DNA (using [^{3}H]uridine and [^{14}C]thymidine) to determine the effect of LGR1492 on the synthesis of both mRNA and total RNA in HCT-116 cells. A dose-dependent effect of LGR1492 on the level of newly synthesized RNA was detected after 24 h of treatment. Already at the dose of 5 μM of LGR1492 we observed the significant decrease of transcription to 30 % value of control; higher concentrations then caused total block of transcription.

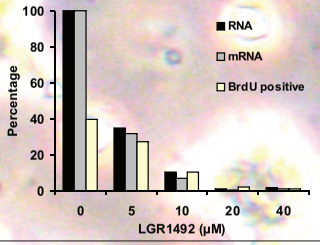


Figure 5: Effect of LGR1492 on transcription and replication of HCT-116 cell line.

ACCUMULATION OF p53 AND p53-ACTIVATED TRANSCRIPTION

Treatment of cells harboring wt-p53 with CDK inhibitors usually lead to the accumulation of p53 and an increase of p53-dependent transcription. Therefore we evaluated these effects upon treatment of LGR1492 in colorectal carcinoma HCT-116 cells. LGR1492 rapidly increased the expression of p53 starting from 10 μM concentration (Figure 3B). Accumulation of p53 was accompanied with decreased expression of Mdm-2, which is a negative regulator of p53. The induction of $p21^{WAF1}$, a direct p53 transcriptional target, was also analyzed.

The effect of LGR1492 on p53-dependent transcriptional activity was confirmed by ß-galactosidase reporter assay with using stable transfected human melanoma cell line Arn-8. Pyrazolo[4,3-*d*]pyrimidine LGR1492 exerted dose-dependent effect on transcriptional activation, with maximum at 9.8 μM concentration (Figure 3C).

Both results were complemented by immunofluorescence visualization of nuclear accumulation of p53; strong nuclear signal of p53 in comparison to control cells was observed after LGR1492 treatment (Figure 3A).

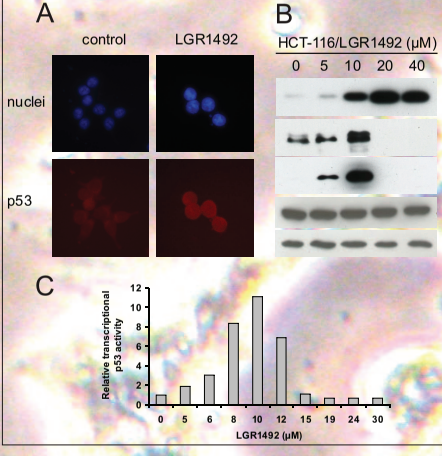


Figure 3. Analysis of p53 activation. (A) Immunofluorescence visualization of p53 in HCT-116 treated with 40 μM LGR1492 (red - p53, blue - DNA). (B) Immunoblot analysis of p53, $p21^{WAF1}$ and Mdm-2 in HCT-116 treated with LGR1492 for 24 h. Tubulin and PCNA levels were detected to verify equal protein loading. (C) Analysis of p53-dependent transcriptional activity by ß-galactosidase reporter assay in Arn-8 cell line treated with LGR1492.

EORTC NATIONAL CANCER INSTITUTE AACR American Association for Cancer Research

22nd EORTC-NCI-AACR symposium on "Molecular targets and Cancer Therapeutics"
Berlin, Germany, 16 - 19 November 2010

PŘÍLOHA VI.

Kryštof V, Chamrád I, **Jorda R**, Kohoutek J. Pharmacological targeting of CDK9 in cardiac hypertrophy, *Med Res Rev*. 2010 Jul;30(4):646-66.

Pharmacological Targeting of CDK9 in Cardiac Hypertrophy

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Published online 15 September 2009 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/med.20172

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Abstract: Cardiac hypertrophy allows the heart to adapt to workload, but persistent or unphysiological stimulus can result in pump failure. Cardiac hypertrophy is characterized by an increase in the size of differentiated cardiac myocytes. At the molecular level, growth of cells is linked to intensive transcription and translation. Several cyclin-dependent kinases (CDKs) have been identified as principal regulators of transcription, and among these CDK9 is directly associated with cardiac hypertrophy. CDK9 phosphorylates the *C*-terminal domain of RNA polymerase II and thus stimulates the elongation phase of transcription. Chronic activation of CDK9 causes not only cardiac myocyte enlargement but also confers predisposition to heart failure. Due to the long interest of molecular oncologists and medicinal chemists in CDKs as potential targets of anticancer drugs, a portfolio of small-molecule inhibitors of CDK9 is available. Recent determination of CDK9's crystal structure now allows the development of selective inhibitors and their further optimization in terms of biochemical potency and selectivity. CDK9 may therefore constitute a novel target for drugs against cardiac hypertrophy. Med Res Rev, 30, No. 4, 646–666, 2010

Key words: P-TEFb; cardiac myocyte; cardiac hypertrophy; protein kinase; inhibitor

1. INTRODUCTION

Cardiovascular disease is today the main cause of mortality worldwide, representing 30% of all deaths, and its incidence is still on the rise. The term cardiovascular disease comprises a broad spectrum of cardiac and circulatory pathologies. One of the most frequent is heart failure, which can be defined as the inability of the heart to pump enough blood to the

Contract grant sponsor: The Ministry of Education, Youth and Sports of the Czech Republic; *Contract grant number:* MSM6198959216; *Contract grant sponsor:* Czech Ministry of Agriculture; *Contract grant number:* MZE0002716202; *Contract grant sponsor:* The Czech Science Foundation; *Contract grant numbers:* 204/08/0511; 301/09/1832.

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body.[1,2] This syndrome has many clinical types and its initial causes can be wide-ranging, e.g. myocardial infarction or other forms of ischemic heart disease, hypertension, coronary artery diseases, valvular diseases, congenital defects, infectious diseases, cardiotoxic substances, or cardiac hypertrophy.[3,4]

The last mentioned disease, cardiac hypertrophy, is characterized by an increase in the size of differentiated cardiac myocytes. This typically, but not always, necessitates enlargement of the entire myocardium. Cardiac hypertrophy can arise as a response of the heart to increased hemodynamic demand. If this process is associated with normal or even improved cardiac myocyte contractile function, it can be seen as adaptive hypertrophy. This type of cardiac hypertrophy can occur in healthy individuals following pregnancy or exercise and is traditionally classified by cardiologists as physiological. However, persistence of this compensatory mechanism for a longer period can lead to cardiac dysfunction and heart failure. In the case of cardiac hypertrophy accompanied by impaired contractile function of cardiac myocytes and subsequent heart dysfunction, the terms maladaptive or pathological can be used.[5,6] Interestingly, hypertrophy also plays a role in cardiac development soon after birth. It has been found that cardiac myocytes of newborns and individuals afflicted with pathological cardiac hypertrophy share some similarities in the gene expression.[7] Due to its occurrence in neonatal cardiac myocytes, this gene expression profile is referred to as the fetal gene program.

Over the past decade, a great diversity of stimuli has been identified triggering cardiac hypertrophy. Our knowledge of diverse regulatory circuits, such as transduction and intracellular signaling pathways, specific transcription factors and their co-factors delineating resurrection of the fetal gene program in the onset of cardiac hypertrophy, has emerged from numerous specialized studies.[8–17] Nevertheless, until recently, central molecular mechanisms underlying pathological growth of cardiac myocytes have not been convincingly elaborated. This review therefore focuses on cyclin-dependent kinase 9 (CDK9), an important regulatory molecule that could act as a linking node of various signaling mechanisms participating in the initiation and progress of cardiac hypertrophy. Its structure, biological function, and especially involvement in hypertrophic growth are discussed in detail, and separate sections are devoted to CDK9 low-molecular inhibitors and their potential in pharmacological modulation of this insidious disease.

2. MOLECULAR BASIS OF CARDIAC HYPERTROPHY

As it had been known that enlargement of cardiac myocytes could be attributed to overall increase of protein content due to elevated RNA production,[18] it came as no surprise that RNA polymerase II (RNAPII), responsible for transcription of coding RNA species, was identified as a limiting factor of hypertrophic growth. In particular, phosphorylation of its *C*-terminal domain (CTD) on serine 2 (Ser2) in a highly multimerized heptapeptide motif (Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7) indicative of fully elongating RNAPII was found to be associated with the hallmark of cardiac hypertrophy.[19,20] The prototypical kinase catalyzing Ser2 phosphorylation is positive transcription elongation factor b (P-TEFb).[21,22] In addition to phosphorylating Ser2, P-TEFb is also responsible for initiating the elongation phase of transcription by overcoming inhibitory effects of negative elongation factors on RNAPII at the proximal promoter regions HSP70, JunB, MAP kinase phosphatase-1 genes, and others.[23–27]

P-TEFb is typically found in two functionally distinct complexes in cells. Active P-TEFb consists of CDK9 and cyclin T1, T2a, or T2b.[21] Inactive P-TEFb is inhibited in a "large" complex by cooperative association with 7SK small nuclear RNA (7SK snRNA),

hexamethylene-bisacetamide inducible protein 1 (Hexim1), 7SK snRNA methylphosphate capping enzyme (MEPCE), and La-related protein 7 (LARP7).[28–34]

Most importantly, the crucial role of P-TEFb for the onset and pathology of cardiac hypertrophy has been demonstrated at multiple levels. First and foremost, genetic ablation of cardiac lineage protein 1 (Clp-1), the mouse homolog of Hexim1, was shown to cause fetal lethality in mouse due to pathological enlargement of the heart with the complex phenotype recapitulating all characteristics of cardiac hypertrophy.[35] In addition, depletion of 7SK snRNA by a small interference RNA approach in primary rat cardiac myocytes has been shown to be sufficient to trigger the myocytes' abnormal growth.[20] By contrast, two- and six-fold increase of cyclin T1 levels in adult heart in transgenic mouse models resulted in concentric hypertrophy with 20 and 40% heart weight/body weight ratios and without detectably reinitiating the fetal gene program.[20] This rather surprising loss of induction of the fetal gene program in these mice can now be elegantly explained by recent observations that provide important evidence that elongation control, directed by P-TEFb, might orchestrate a unique pattern of gene expression during development.[25,36] Thus, P-TEFb most likely plays two equally important roles in the pathophysiology of cardiac hypertrophy (Fig. 1). First, enhanced activity of P-TEFb, elevated by ectopic expression of cyclin T1 in the adult heart, will support transcription of already transcribed genes in the heart. Secondly, as do diverse hypertrophic stimuli, P-TEFb promotes de novo expression of specific genes earlier recognized as susceptible to developmental or environmental cues.[37]

Last but not least, all tested hypertrophic stimuli led to disruption of the large P-TEFb complex and release of fully active P-TEFb (Fig. 1).[20,38] Still, the molecular mechanisms involved in P-TEFb liberation are not yet characterized, except recent observation made by Espinoza-Derout who had shown Jak/STAT signal transduction pathway to be involved in the release of P-TEFb from large complex in cardiac myocytes.[38] We can only speculate that

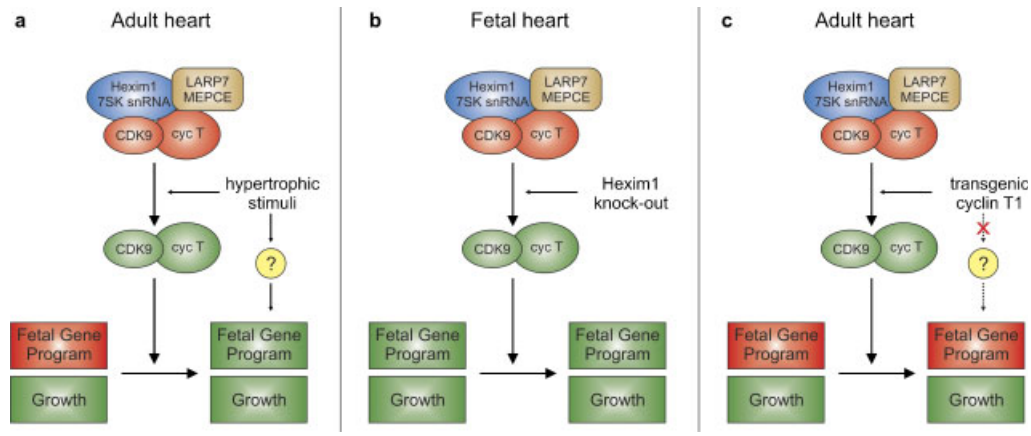

Figure 1. Involvement of P-TEFb (CDK9/cyclin T) in regulating transcription of the fetal gene program and growth-promoting genes during cardiac hypertrophy. Diverse stress stimuli trigger the expression of genes associated with the fetal gene program and of growth-promoting genes. Importantly, participation of the intracellular signaling pathways induced in parallel by these stimuli is absolutely necessary for reactivation of fetal gene program genes through the yet unknown factor(s) represented by the question mark (**a**). Genetic depletion of Hexim1 in the fetal heart results in the expression of growth-promoting genes, as well as of genes of the fetal gene program, even without the activation of intracellular signaling pathways, because both sets of these genes are already actively transcribed. The same hypertrophic phenotype would be most likely seen, too, when cyclin T1 was ectopically expressed in the fetal heart (**b**). In the adult heart, genes of the fetal gene program are silenced. Thus, ectopic expression of cyclin T1 in the adult heart leads to increased transcription of mostly growth-promoting genes without activating fetal gene program genes. It is possible to assume that depletion of Hexim1 in the adult heart could have the same effect as ectopic expression of cyclin T1 (**c**). Green and red rectangles depict active and suppressed transcription of the fetal gene program and growth-promoting genes, respectively. Green and red CDK9/cyclin T ovals signify inactive and active P-TEFb complexes, respectively.

there are either one or more unknown factors sensing hypertrophic stimuli and causing direct disassembly of the large P-TEFb complex or post-translational modification(s) of its subunits leading to the same outcome. The second scenario could be supported, at least in part, by numerous publications defining the relevance of post-translational modifications of CDK9. In the case of CDK9, only modifications so far found to either activate or inhibit CDK9 (P-TEFb) activity are relevant to manifestation of cardiac hypertrophy. Phosphorylation of Ser and Thr residues at the *C*-terminus tail and Thr 186 in the T-loop positively affect CDK9 (P-TEFb) kinase activity by inducing conformational changes to either bind cyclin T1 or allow kinase recognition of the Ser/Thr-Pro substrate motif, respectively.[39–42] In sharp contrast, phosphorylation on Thr29 found within the HIV transcription initiation complex is inhibitory.[43] Dephosphorylation of Thr 186 blocks CDK9 activity, while by contrast dephosphorylation of Thr29 relieves its inhibition.[44,45] In addition, acetylation of Lys44 in the ATP-binding domain by p300/CBP increases its kinase activity but acetylation of Lys44 and Lys48 by P/CAF and GCN5 inhibits its kinase activity.[46,47] Interestingly, acetylations on cyclin T1 support the Hexim1-free active form of P-TEFb. Moreover, functional connection may exist between p300-mediated acetylation of CDK9 and cyclin T1.[48]

It is now clear that in the search for potent and specific inhibitors of CDK9, one must consider processes governing the balance between inactive and active complexes of P-TEFb and post-translational modification that will participate in the final picture of P-TEFb's involvement in adaptive or pathophysiological hypertrophy of cardiac myocytes.

3. SMALL-MOLECULE INHIBITORS OF CDK9

Because CDKs had been originally discovered as key components of the cell cycle regulation machinery, small-molecule CDK inhibitors were initially studied for their antiproliferative effects as compounds with promising potential in cancer therapy.[49,50] Recently, novel functions of some CDKs have been described, including in regulating transcription.[51–53] These findings have suggested brand new indications for possible use of CDK inhibitors and stimulated their further development. These facts, along with findings about up-regulated expression and enhanced activity of CDK9 in cardiac hypertrophy mentioned above, have also led to proposing CDK9 inhibitors as possible therapeutics for this disease.[54] To date, about 30 inhibitors of CDK9 have been identified. Examples of the most potent and selective of these are listed in Table I and illustrated by Figure 2.

One of the first compounds reported to inhibit CDK9 was 5,6-dichloro-1-β-ribofuranosyl-benzimidazole (DRB). Its ability to inhibit transcription had long been known,[55–63] but its exact molecular target remained unknown. Chodosh et al. introduced the idea that DRB might interact with an elongation factor,[55] and this theory was proven after studies by Marshall and Price. They described a P-TEFb complex and showed that it is the limiting factor in the production of long mRNA transcripts.[64] The final breakthrough came with the discovery that DRB could inactivate *Drosophila* P-TEFb transcriptional activity.[65,66] One year later, PITALRE kinase, now called CDK9, was found to be the catalytic subunit of P-TEFb[67] and at the same time DRB was finally shown to block its kinase activity.[68] Although the first information was published more than 10 years ago, further knowledge about the kinase selectivity of this nucleoside analogue has remained poor.

The potential of flavopiridol, an important multi-selective kinase inhibitor, to inhibit CDK9 was suggested by its ability to change levels of different mRNAs in *Saccharomyces cerevisiae*.[69] This possibility was originally confirmed biochemically, and flavopiridol was characterized as an uncompetitive inhibitor of P-TEFb.[70] Although this kind of kinetic behavior was quite surprising because flavopiridol had been known to compete with ATP on

Table I. Selectivity of the Most Effective Cyclin-Dependent Kinase-9 Inhibitors, Selection of Inhibitors with IC_{50}(CDK9) $<0.1\,\mu M$ (underlined values are K_i)

	CDK IC50 (μM)										
Compound	1/B	2/E	2/A	3/E	4/D	5/p25	6/D	7/H	9/T	References	PDB code
Flavopiridol	0.03–0.4	0.1	0.1	n.a.	0.02–0.04	n.a.	0.06	0.11–0.3	0.006	[70,71,73,139]	3BLR[d]
P276-00	0.079	0.224	n.a.	n.a.	0.063	n.a.	0.396	2.870	0.020	[111]	n.a.
Olomoucine II	7.6	0.1	n.a.	n.a.	19.8	n.a.	n.a.	0.45	0.06	[86]	2A0C[c]
Pyrazolotriazine (compound 7a)	0.073	0.026	0.04	n.a.	n.a.	0.07	n.a.	0.5	0.043	[91,92]	3DOG[c]
Meriolin 3	0.17	n.a.	0.011	~0.1	>0.1	0.17	>0.1	>0.1	0.006	[98]	3BHT[c]
Variolin B	0.06	n.a.	0.08	~1	>10	0.09	~10	>1	0.026	[98]	3BHV[c]
Thiazolyl-pyrimidine (compound 1)	0.024	0.014[b]	0.014[b]	n.a.	0.0042	0.034[b]	n.a.	0.020	0.0025	[108]	n.a.
Thiazolyl-pyrimidine (compound 32)	<u>0.08</u>	<u>0.002</u>	n.a.	n.a.	<u>0.053</u>	n.a.	n.a.	<u>0.07</u>	<u>0.004</u>	[107]	1PXO[c]
Indirubin-3′-monoxime	0.18	0.5	0.44	n.a.	3.33	0.10	n.a.	>4	0.05	[104,140]	1E9H[e]
ZK 304709	0.05	0.004	n.a.	n.a.	0.061	n.a.	n.a.	0.085	0.005	[103]	n.a.
SNS-032	0.48	n.a.	0.038	n.a.	0.925	0.34[a]	>1	0.062	0.004	[105,141]	n.a.
AG-012986	<u>0.044</u>	<u>0.094</u>	n.a.	n.a.	<u>0.0092</u>	0.022[a]	n.a.	n.a.	0.004	[109]	n.a.
AT7519	0.190	0.510	0.044	n.a.	0.067	0.018	0.660	2.8	<0.1	[114]	2VU3[c]
R547	0.0002	0.0004	0.0001	0.0008	<u>0.001</u>	0.0001[a]	0.004	0.171	0.013	[106,142,143]	2FVD[e]
RGB-286638	0.002	0.003	n.a.	0.005	0.004	0.005[a]	0.055	0.044	0.001	[110]	n.a.
Imidazole pyrimidine (compound 7d)	<0.001	0.002[b]	0.002[b]	n.a.	n.a.	n.a.	n.a.	n.a.	0.003	[112]	2W17[f]
EXEL-3700	0.199	0.167[b]	0.167[b]	n.a.	0.643	n.a.	n.a.	1.718	0.0102	[115]	n.a.
EXEL-8647	>3.6	>10[b]	>10[b]	n.a.	>10	n.a.	n.a.	>10	0.029	[115]	n.a.

[a]Compounds were tested on CDK5/p35 complex.

[b]Accurate binding partner in kinase assay is not known.

[c]Solved crystal structure in complex with CDK2.

[d]Solved crystal structure in complex with CDK9.

[e]Solved crystal structure in complex with CDK2/cyclinA with related derivative indirubin-5-sulfonate.

[f]Solved crystal structure in complex with CDK2 with related imidazole pyrimidine (S)-8B.

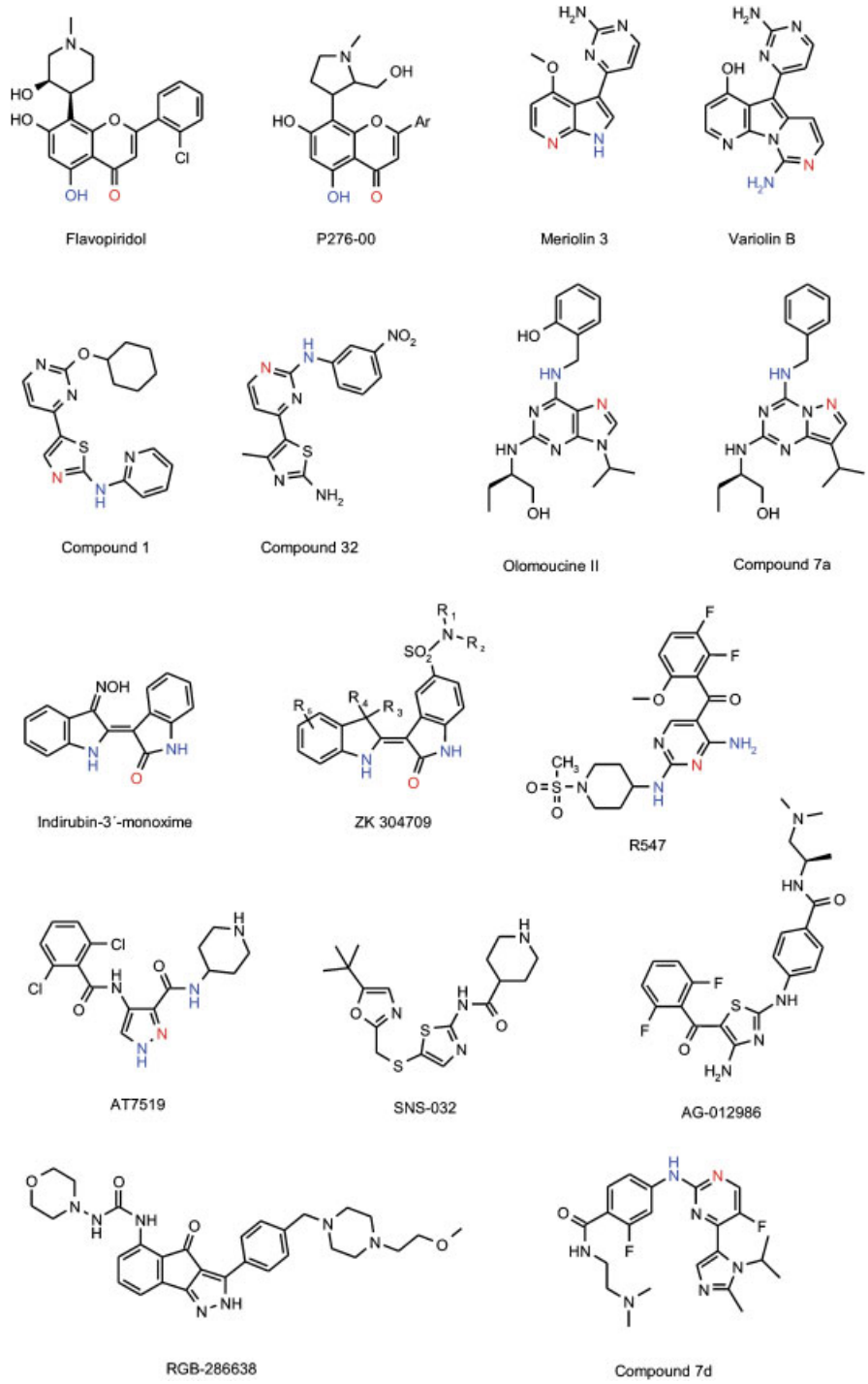

Figure 2. Chemical structures of some CDK9 inhibitors stated in Table I. Color marked atoms of compounds (red/blue–acceptor/donor of hydrogen bond) indicate published or predicted binding motifs with the backbone of residues Leu83 and Glu81 in CDK2 or corresponding residues in other CDKs, respectively.

CDK1, 2 and 4,[71–73] similar results were obtained by other research groups and flavopiridol was presented to be an allosteric inhibitor of CDK9.[74] While there had been doubts about these conclusions,[70,75–77] direct evidence for competitive inhibition has recently been provided. In the last year, the structure of the CDK9/cyclinT1 complex co-crystallized with flavopiridol was described. It is now absolutely clear that flavopiridol binds at the ATP-binding site of CDK9.[78] Its orientation is similar to the binding mode of its des-chloro analogue to inactive monomeric CDK2,[72] with the molecule almost entirely buried in CDK9. The tight-binding mode also sheds light on the strange kinetic behavior of flavopiridol, because it is known that tight-binding inhibitors do not follow Michaelis-Menten kinetics.[79,80] Flavopiridol has been tested primarily in cancer models since its discovery, but some reports indicate its possible use as a drug for cardiovascular diseases. It has been demonstrated that flavopiridol could be considered a pharmacological candidate for prevention and treatment of smooth muscle cell-rich vascular lesions following vascular injury in humans, such as in-stent restenosis.[81,82] Flavopiridol was also effective in a rat model of focal ischemia.[83]

Soon after the discovery of the action of flavopiridol, another well-known pan-specific CDK inhibitor, roscovitine, was also reported to significantly suppress mRNA production.[84] Especially CDKs 7 and 9 that both phosphorylate the *C*-terminal domain of RNAPII are sensitive to roscovitine.[85,86] Importantly, roscovitine is the only CDK inhibitor tested in a cellular model of cardiac hypertrophy.[87] Roscovitine was shown to significantly reduce hypertrophic growth of cardiac myocytes caused by treatment with angiotensin II. Moreover, it also efficiently repressed protein synthesis, E2F-dependent transcription, DNA synthesis, and endoreduplication.[87] This antihypertrophic activity obviously was not connected with the inhibition of CDK2, as expression of the nonfunctional CDK2 mutant had no effect on hypertrophic cells. Because the molecular basis of roscovitine's effects was not fully explained by the authors, it is tempting to speculate that hypertrophy was suppressed through the CDK9 inhibition-dependent mechanism, at least in angiotensin II-stimulated cardiac hypertrophy.

Even though DRB, flavopiridol and roscovitine were demonstrated to inhibit CDK9, their effects on transcription differ slightly. All three compounds especially affect transcripts with short half-lives, but DRB and flavopiridol have similar profiles of suppressing genes and reducing mRNA levels in a global manner.[88] In contrast, roscovitine does not induce a global loss of gene expression and the number of genes responding to roscovitine treatment by increased expression is nearly the same as the number of genes whose expression decreases.[89]

DRB, flavopiridol, and roscovitine are undoubtedly the most-studied inhibitors of CDK9, but, as stated in the introduction to this section, there are many other compounds sharing the same kinase target. Employing Tat-dependent transcription assay, the compounds T172298 and TRB bearing the same benzimidazole scaffold as DRB were discovered to inhibit CDK9. In the same screen, inhibitory effects toward CDK9 of isoxazole T276339 and isoquinoline sulphonamide H7 were identified as well.[68] Each of these compounds can be interpreted as a mild CDK9 inhibitor with an IC_{50} ranging from 0.9 to 9 μM.[68]

Structural modifications to roscovitine have led to the discovery of CDK9 inhibitors such as olomoucine II,[86,90] pyrazolo[1,5-*a*]-1,3,5-triazine 7a,[91,92] imidazo[2,1-*f*]-1,2,4-triazine 13,[91,92] and CR8.[93] CDK selectivity of these purine analogues subtly differs, but it can be summarized that the inhibitory ability regarding CDK9 was fairly improved.

Meridianins and variolins are natural products originated from marine invertebrates and which display CDK9 inhibitory activity and cytotoxicity against several human cancer cell lines.[94–97] As a consequence of its abilities, deoxyvariolin B (PM01218) is now being investigated as a potential antitumor drug.[98] Recently, the chemical similarities between meridianins and variolins have been utilized to synthesize their hybrid structure, meriolins.[98]

In comparison with meridianins and variolins, these new chemical compounds have enhanced specificity toward CDK with marked potency on CDK2 and 9.[98,99] SU9516,[100,101] B5,[102] ZK 304709,[103] and indirubin-3′-monoxime[104] also belong to the group of CDK9 inhibitors sharing an indole skeleton. The CDK9 IC_{50} values of these compounds vary from micromolar (4.3 μM for B5 and 0.1 μM for SU9516) to nanomolar (indirubin-3′-monoxime and especially ZK 304709, Table I).

In recent years, several other compounds have been developed with broad CDK specificity but that inhibit CDK9 at nanomolar concentrations. SNS-032,[105] R547,[106] thiazolylpyrimidines (compounds 32 and 1),[107,108] AG-012986,[109] RGB-286638,[110] P276–00,[111] and imidazole pyrimidine amides (compounds 6d, 7d, (*S*)-8b and 9b)[112] can be included in this category. The additional compounds AZ703 and AT7519 have been characterized as highly selective for CDK9 and their IC_{50} measures are under 1 μM.[113,114] All these compounds have undergone thorough preclinical evaluation, and some of them have already entered clinical trials as anticancer agents.[49]

EXEL-8647 is a CDK9 inhibitor identified in a high-throughput screen carried out by Exelixis, Inc.[115] This compound has a very interesting CDK selectivity profile, because its CDK9 inhibitory potential is more than 100-fold greater than that of other CDKs.[115] Another compound showing significant CDK9 specificity is CAN508, the most potent competitive CDK9 inhibitor from the series of 4-arylazo-3,5-diamino-1*H*-pyrazoles.[116] Based on these facts, EXEL-8647 and CAN508 are the only inhibitors that could be referred to as CDK9 specific.

To complete the summary, the nucleoside analogue 4-amino-6-hydrazino-7-ß-D-ribofuranosyl-7*H*-pyrrolo[2,3-*d*]-pyrimidine-5-carboxamide (ARC) and pyrazolopyrimidone-related RGB-286147 should not be omitted. The inhibitory effect of these compounds on CDK9 is clear, but unfortunately there is no information as to their CDK9 inhibition potency.[117,118]

4. THE STRUCTURE OF CDK9/CYCLIN T1

In order to obtain maximal efficiency of CDK9 inhibitors in all possible therapeutic indications, molecules with high selectivity for CDK9 are required. Rational design of such selective compounds is now facilitated by determination of the kinase's crystal structure in complex with cyclin T1.[78] The structure of the CDK9 complex is similar to all previously determined structures for other CDKs. The enzyme adopts the bilobal fold typical of most protein kinases, with the smaller *N*-terminal domain consisting predominantly of β-sheet structure and the larger *C*-terminal domain consisting primarily of α-helices (Fig. 3a). The most obvious structural difference between CDK9/cyclin T1 and the well-characterized CDK2/cyclin A related to the cell cycle is in the size of the CDK/cyclin interface. The orientation of cyclin T1 with respect to CDK9 is rotated by about 26°, which results in a reduced number of mutual contacts. The buried molecular surface area of the CDK9/cyclin T1 complex is just 60% of the molecular surface area that is buried on the CDK2/cyclin A complex.[78] In this regard, the structure of CDK9/cyclin T1 is reminiscent of the recently solved CDK4/cyclin D that also has a relatively small interface.[119,120] This structural feature of both CDK9 and CDK4 influences their physiological regulation and also is probably linked to the relatively strict substrate specificity of these two kinases.

As with CDK2, full activity of CDK9 depends not only on cyclin binding but also on phosphorylation of Thr186 within the activation segment.[78] In contrast to active CDK2, where the activation segment attaches directly to cyclin, the phosphorylated Thr186 of CDK9 does not interact with cyclin T1. Nevertheless, this phosphorylation repositions the

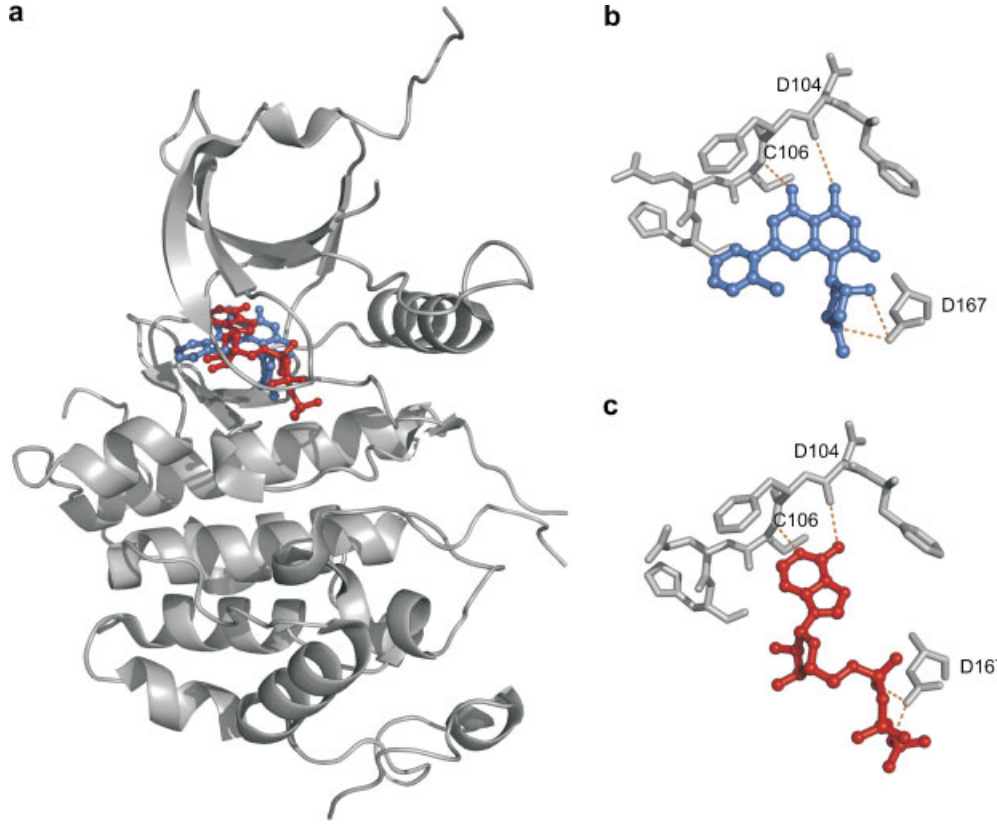

Figure 3. Structure of CDK9 in a complex with flavopiridol (blue) or ATP (red) localized within its active site (**a**). Detailed views on interactions of CDK9 with flavopiridol (**b**) and ATP (**c**) with selected amino acid residues forming the active site of CDK9 (b). Prepared with PyMol (http://pymol.sourceforge.net/) using the PDB entries 3BLR and 3BLQ.

activation loop and allows the kinase to recognize the substrate Ser/Thr-Pro motif. Another residue important for substrate recognition is Arg188 located in one of the basic clusters, which is able to interact with CDK7-phosphorylated Ser5 within the CTD. Recent experiments suggest that phosphorylated Ser5 is not a prerequisite for efficient recognition by CDK9 and phosphorylation at Ser2.[121] In the transcription complex, however, phosphorylations of Ser5 by CDK7 release the CTD from the DNA and make it available for CDK9.[121]

The active site, where ATP as well as all small-molecule inhibitors bind, is located in the deep hydrophobic cleft between the two lobes that are connected by the hinge region (residues 104–107 in CDK9, residues 81–84 in CDK2). This region contains a set of hydrogen bond donor and acceptor sites that are used for binding the adenine of ATP. Moreover, the adenine ring is fixed between the *N*- and *C*-terminal domains through hydrophobic contacts with a number of residues, including Ile25, Val33, Ala46, Val79, Phe105, and Leu156 (in CDK9) that are identical in almost all CDKs (Fig. 4a).

5. INTERACTIONS OF CDK9 WITH INHIBITORS

The majority of small-molecule inhibitors of CDKs are ATP-competitive and bind in the deep cleft between the two domains.[122,123] Most of the contacts are hydrophobic, but the inhibitors complexed with CDK2 also accept a hydrogen bond from the backbone nitrogen of

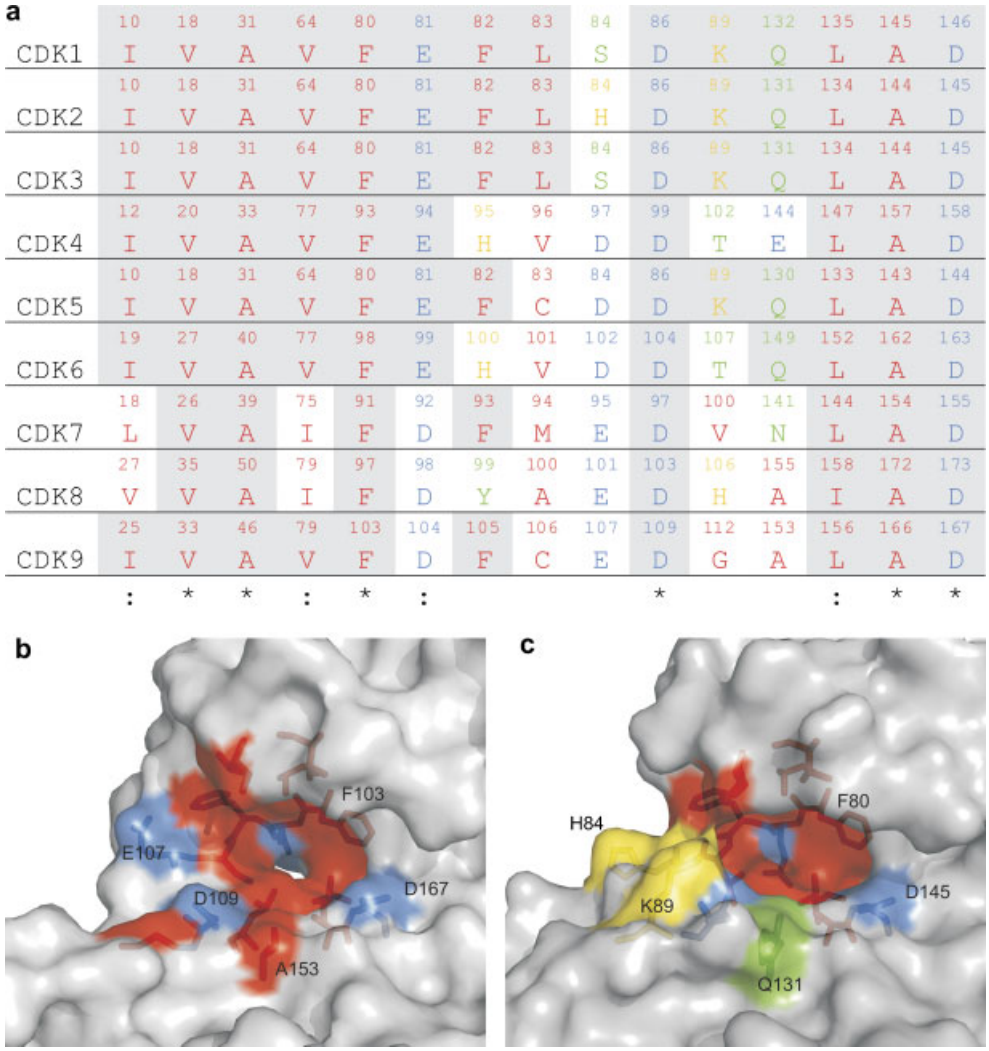

Figure 4. Comparison of human CDK9 and CDK2 primary and tertiary structures. Alignment of amino acid residues that form the active site in human CDK1–9 (**a**). Amino acids are colored according to their physicochemical properties: red–hydrophobic, blue–acidic, yellow–basic, green–polar; amino acids identical in most CDKs are highlighted gray; (*) identical; (:) conserved. Comparison of the active site in CDK9 (**b**) and CDK2 (**c**), color-coded as in the alignment. All structural figures were prepared with PyMol (http://pymol.sourceforge.net/) using the PDB entries 3BLR and 1GY3.

Leu83 and donate another hydrogen bond to the backbone carbonyl of Glu81. A third hydrogen bond to the backbone carbonyl group of Leu83 has been observed for some inhibitors. Besides bonding to these three amino acids, inhibitors interact also with the ribosylphosphate-binding site of CDKs. Until the structure of CDK9 was solved experimentally, a structural model of CDK9 had been used to study the interactions between CDK9 and flavopiridol or CAN508, respectively, both well established and potent inhibitors of CDK9.[76,116] Similar interaction patterns were also suggested for binding of these inhibitors.

In 2008, flavopiridol was co-crystallized with CDK9/cyclin T1 and the complex structure was finally solved.[78] As expected from the modeling studies, flavopiridol binds to CDK9 in a manner similar to the binding of des-chloro-flavopiridol to CDK2.[72] The compound forms conserved hydrogen bonds to the hinge residues Asp104 and Cys106 and an additional

hydrogen bond to Asp167 through its piperidine ring (Fig. 3b). Interestingly, flavopiridol induces a conformation change in the glycine-rich loop of CDK9, closing tightly the active site into an inactive conformation that excludes ATP binding. A similar change in conformation has been observed in other protein kinases upon interaction with inhibitors, e.g. in the Abl complex with imatinib,[124] but among CDKs such change has been described for the first time.

In the recently reported complex of CDK9 with EXEL-8647, the inhibitor is also localized in the active site, but no details have been disclosed other than a note about an observed salt bridge, probably between Lys35 and Glu107.[115] This salt bridge has been predicted by modeling studies as a reason for increased affinity of flavopiridol and CAN508 to CDK9, as it should bring the residues from two different lobes of the kinase closer together. Therefore it may be the cause for stronger interactions.[76,116]

6. STRUCTURE-BASED INHIBITOR DESIGN

Solving the structure of CDK2 by X-ray analysis has driven the development of a large number of various ATP-competitive inhibitors. Specificity profiles of several known CDK inhibitors suggest that truly CDK9-specific compounds can be prepared (e.g. EXEL-8647 or CAN508, see Table I).[115,116] Nevertheless, the high degree of sequence similarity among the phylogenetically conserved CDK family members complicates reaching reasonable selectivity for individual CDKs. Successful design of such inhibitors should be facilitated by the known crystal structures of approximately half the family, including CDK2,[125] CDK4,[119,120] CDK5,[126,127] CDK6,[128] CDK7,[129] and especially CDK9.[78] Limited information on other CDKs that still resist crystallizing has been generated by indirect procedures including in silico modeling and genetic engineering. For example, CDK1 and CDK3 were homology modeled based on the crystal structures of related CDK2 and CDK6.[130–132] Alternatively, when the crystal structure of CDK4 was not available, its active site was modeled from crystallographic analysis of the CDK2 variant containing amino acids forming the active site of CDK4.[133] Both experimental and modeled structures of individual CDKs should allow for determining the specific residues that comprise the key differences between their active sites and identifying the interactions that could be exploited in the design of highly selective CDK9 inhibitors.

For the purposes of inhibitor design, the active site can be parceled out into several elements that may be approached independently. The hinge region of CDKs, utilized by all known inhibitors by means of its set of hydrogen bonds, is identical in CDKs 1–3 but is less conserved in other CDKs (see Fig. 4a). As demonstrated by Lu and Schulze-Gahmen, the differences in the hinge regions of CDK2 and CDK6 are responsible for a pyrido[2,3-*d*]-pyrimidin-7-one inhibitor specificity as they induce changes in the inhibitor orientation that may lead to steric clashes in only some CDKs.[128] This raises the possibility that the hinge conformation is kinase specific and may be important for the specificity. In contrast, other authors surmise that as these residues provide only contacts through the backbone atoms they would not be expected significantly to impact inhibitor binding.[131]

A much more interesting area is located around the entry to the active site, and is sometimes referred to as the specificity surface. For example, local sequence differences are not only the reason for the specificity of purine-based inhibitors toward CDK2 over CDK4.[69] Conversely, they also account for the specificity of some anilinopyrimidines for CDK4.[131] Especially in the case of the latter compounds, the reason for such specificity lies in the changes of ionizable amino acids: in CDK4, an acidic residue (Glu144) replaces Gln131 of CDK2, and a neutral residue Thr102 of CDK4 is substituted for the positively charged Lys89

in CDK2. In CDK9, these two residues are replaced with the much smaller Ala153 and Gly112, respectively (Fig. 4b). As shown in the crystal structures of CDK9,[78] this area is therefore markedly both less crowded and polar and should be able to host relatively bulky substituents of potential inhibitors.

The structurally adjacent ribosylphosphate-binding site of CDKs is covered by a flexible glycine-rich loop that significantly changes conformation upon cyclin binding and activating phosphorylation. Despite the fact that the dynamic nature of this region complicates efforts to rationally improve affinity of the inhibitors, many of them have been shown to interact through hydrogen bonds with the amino acids Gln131, Asp86,[69,72,134] Lys33, and Asp145 of CDK2. As demonstrated in the CDK9-flavopiridol co-crystal, the inhibitor induces a change in conformation of the glycine loop that subsequently moves the side chain of Phe30 closer to the bound ligand and stabilizes it through additional van der Waals contacts while CDK9 adopts an inactive conformation that excludes ATP binding. A similar change in conformation has been observed also in CDK2 upon binding of NU2058, an inhibitor with a hydrophobic side chain occupying the ribose site that is reminiscent of a conformation seen in inactive CDK2.[135]

In summary, there is a lot of space around the active site of CDKs to be explored for the design of truly monospecific CDK9 inhibitors. The conformation of the glycine-rich loop and the possibility to stabilize CDK9 in its inactive conformation, in particular, provide attractive initial points for the rational design of inhibitors possessing both high affinity and specificity to CDK9.

7. *PERSPECTIVES*

Despite advances in the development of CDK inhibitors, no truly monospecific inhibitors have been described. However, many selective CDK inhibitor scaffolds discovered to date offer various motifs for optimization in terms of their selectivity through analogue synthesis guided at least partially by knowledge of the active site of CDK9. Alternatively, the structure of CDK9 now can be also used to find novel inhibitors by virtual screening methods. This provides the advantage of filtering out unselective compounds according to their interactions with other CDKs. On the other hand, one of the most disputable questions about pharmacological applications of CDK inhibitors concerns relationships between selectivity of the inhibitors and their potential therapeutic applications. Although studied for more than a decade, no proof has yet been established regarding the concept of using CDK inhibition for anticancer therapy and no such therapy has been approved. Meanwhile, the possible application of CDK inhibitors in cardiac hypertrophy has been far less studied and discussed. Would it be better to use (if any are to be used at all) highly selective CDK9 inhibitors, or molecules with combined effects on multiple CDKs? Although CDK9 inhibition with monospecific compounds could be sufficient to repress hypertrophic growth, less selective CDK inhibitors are not out of the game, either, as demonstrated by roscovitine that targets not only CDK9 but also other CDKs.[87] Then again, more promiscuous inhibitors targeting simultaneously CDK9 and some protein kinases involved in signaling pathways that participate in hypertrophic response may provide additional area for designing and optimizing the most suitable selectivity profile and to find the best therapeutic molecules.[11,12,14–16]

Besides CDK inhibitors alone, it might be worth trying their combined use with histone deacetylase (HDAC) inhibitors in therapeutic management of cardiac hypertrophy. A rationale for their application is based on observations from cell culture experiments and in vivo models. Use of HDAC inhibitors on cultured cardiac myocytes prevented hypertrophy and activation of the fetal gene program classically induced by hypertrophic agonists.

Moreover, administration of apicidin (a specific class I HDAC inhibitor) or Trichostatin A significantly decreased myocardial hypertrophy and fetal gene expression after induction of cardiac hypertrophy in mice by thoracic aortic banding.[136,137]

While on the subject of combined applications, it is extremely interesting that the classical CDK inhibitor roscovitine has recently been shown to inhibit and slow activation and enhance inactivation of L-type Ca^{2+} channels in cardiac myocytes.[138] As L-type Ca^{2+} channel blockers are classic cardiovascular drugs, these extra properties make roscovitine a very attractive and promising antihypertrophic compound, and especially when considering its CDK9 inhibitory competence. In our noble attempts to find a systematic approach for treating, or at least attenuating, the pathology of cardiac hypertrophy, one must also think of combining strategies aimed at different processes closely involved in the hallmark of cardiac hypertrophy. Therefore, targeting the P-TEFb complex, and especially its kinase subunit CDK9, by specific regimes seems to be a truly promising point of departure.

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PŘÍLOHA VII.

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Elsevier Editorial System(tm) for Bioorganic & Medicinal Chemistry Letters
Manuscript Draft

Manuscript Number: BMCL-D-11-00535

Title: Antileishmanial activity of disubstituted purines and related pyrazolo[4,3-d]pyrimidines

Article Type: Article

Keywords: leishmania, cyclin-dependent kinase, inhibitor, therapy

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Abstract: We report here results of screening directed to finding new antileishmanial drugs among 2,6-disubstituted purines and corresponding 3,7-disubstituted pyrazolo[4,3-d]pyrimidines. These compounds have previously been shown to moderately inhibit human cyclin-dependent kinases. Since some compounds reduced viability of axenic amastigotes of Leishmania donovani, we screened them for interaction with recombinant leishmanial cdc-2 related protein kinase (CRK3/CYC6), an important cell cycle regulator of the parasitic protozoan. Eighteen pairs of corresponding isomers were tested for viability of amastigotes and for inhibition of CRK3/CYC6 kinase activity. Some compounds (9A, 12A, 13A) show activity against amastigotes with EC50 in a range 1.5 to 12.4 μM. Structure-activity relationships for the tested compounds are discussed and related to the lipophilicity of the compounds.

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Title

Antileishmanial activity of disubstituted purines and related pyrazolo[4,3-*d*]pyrimidines

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Abstract

We report here results of screening directed to finding new antileishmanial drugs among 2,6-disubstituted purines and corresponding 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines. These compounds have previously been shown to moderately inhibit human cyclin-dependent kinases. Since some compounds reduced viability of axenic amastigotes of *Leishmania donovani*, we screened them for interaction with recombinant leishmanial cdc-2 related protein kinase (CRK3/CYC6), an important cell cycle regulator of the parasitic protozoan. Eighteen pairs of corresponding isomers were tested for viability of amastigotes and for inhibition of CRK3/CYC6 kinase activity. Some compounds (**9A, 12A, 13A**) show activity against amastigotes with EC_{50} in a range 1.5 to 12.4 μM. Structure-activity relationships for the tested compounds are discussed and related to the lipophilicity of the compounds.

Abbreviations

CDK, cyclin-dependent kinase; CRK, cdc-2 related protein kinase; CYC, cyclin

Text

Leishmaniasis encompasses a spectrum of human diseases caused by protozoan parasites belonging to the genus Leishmania. Designated a "neglected disease" by the World Health Organization, it is found in more than 88 countries worldwide and where an estimated 350 million people are exposed to infection. The main diseases caused by these parasites include (i) cutaneous leishmaniasis, a self-limiting skin disease that leaves scars; (ii) mucocutaneous leishmaniasis, a debilitating, disfiguring, chronic disease of the nasopharynx and mucosal tissue; and (iii) visceral leishmaniasis, a fatal disease of the liver, spleen and bone marrow causing extensive morbidity and mortality. Recently, perhaps due to global warming, the leishmaniases appear to be spreading to regions previously free of these diseases.[1]

Existing chemotherapeutics, such as pentavalent antimony, pentamidine and amphotericin B, show serious limitations and require intravenous injection, clinical supervision and hospitalization due to significant toxicity. Liposomal encapsulated amphotericin B exhibits lower toxicity and is very expensive, although a recent study suggests that costs may be significantly reduced by shortening the therapeutic regime.[2] In India, parasite resistance against pentavalent antimony drugs has become a serious problem, with > 60% of the visceral leishmaniasis patients failing to respond to treatment.[3] Similar problems of parasite resistance appear in HIV/*Leishmania* co-infection patients not receiving highly active antiretroviral therapy, who tend to be refractory to treatment and frequently relapse.[4] Recently, miltefosine, the only oral drug for treating visceral leishmaniasis, was registered in India, Europe and South America[5]. Use of miltefosine in pregnant women is limited, however, due to teratogenic effects, and resistance to the drug develops easily in culture.[5]

Improved treatment protocols, such as combination therapy, are under investigation in an effort to optimize efficacy, reduce costs and prevent parasite resistance, but new drugs are urgently needed to expand the treatment options available for these diseases. Modern approaches are being employed that integrate genomic, proteomic and cellular analyses for developing novel and effective anti-leishmanial drugs. Rational drug design directed against parasite enzymes, such as dihydrofolate reductase, pteridine reductase or malate dehydrogenase, essential for proliferation or survival, has identified specific enzyme inhibitors, including trisubstituted pyrimidines, triazines and paullones.[6-11] Alternatively, parallels between parasites and cancer cells, including unlimited proliferation in the host, independence of exogenous growth factors and resistance to apoptosis, may provide new insights into drug development,[12] suggesting that anti-cancer drugs and compounds originally developed for oncological indications should be screened as potential leishmanicidal agents.[12,13] While such an approach led to the discovery of miltefosine, most anti-cancer drugs studied to date show only moderate anti-parasitic activity and have low selectivity indices, a major parameter in drug-toxicity evaluation.

Drug development for cancer has focused in recent years on protein kinase inhibitors. As parasite protein kinases frequently show limited homology to host enzymes and play important roles in regulating parasite proliferation, differentiation and survival, as well as virulence molecule expression and host protective responses, they have been proposed as targets for drug development. Indeed several leishmanial protein kinase families including cyclin-dependent kinase (CDK),[14,15] glycogen synthase kinase,[15] mitogen activated protein kinase[16] and cAMP dependent protein kinase[17] have been shown to be essential for parasite growth and survival.[7,17-19]

Analysis of the leishmanial kinome has identified 12 cdc-2 related protein kinases (CRKs) belonging to the CDK family and 11 cyclins (CYC).[20] The large number of CRKs and CYCs may be related to the asynchronous replication of the nuclear and kinetoplast DNA during the cell cycle of these protozoan eukaryotes.[20,21] Several protein kinase inhibitors including flavopiridol,[22] substituted purines,[14,15] paullones,[9,14] indirubins[9,14] and staurosporine derivatives,[14] have been screened for anti-leishmanial activity. Many these compounds are also active on parasitic CRKs, and have been shown to block cell cycle and reduce parasite viability. One particular protein kinase, CRK3, is an essential enzyme for *Leishmania mexicana*,[22] and it has been shown, in complex with its binding partner CYC6,[23,24] to regulate the G2/M transition.[19] We describe a library of 6,9-disubsituted purines and corresponding 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines (Fig. 1) that have been previously shown to cause growth arrest, induce apoptosis in cancer cells, and inhibit human CDK1,[25] as leishmanicidal compounds targeting CRK3/CYC6 kinase.

We tested 18 isomeric pairs of purine and corresponding pyrazolo[4,3-*d*]pyrimidine (Table 1.) for their ability to inhibit leishmanial CRK3 protein kinase activity and to kill axenic amastigotes of *Leishmania donovani.* Kinase inhibition assay were performed with CRK3/CYC6 complex and human histone H1.[32,33] Anti-amastigote activity was evaluated using a viability assay based on the reduction of alamarBlue.[27,28] All compounds were initially screened for anti-leishmanial activity at a single concentration (30 μM). The results are summarized in Table 1. Comparison of the activity of purine and pyrazolo[4,3-*d*]pyrimidine derivatives in the single point assay (Fig. 3A) clearly shows that pyrazolo[4,3-*d*]pyrimidines (A series) are markedly more potent inhibitors of CRK3/CYC6 activity than are the corresponding purines. They were always more active with at least 3-fold greater activity observed for 12 compound pairs. More precise information about the CRK3/CYC6 inhibitory activity of the compounds was subsequently obtained from the dose-response kinase activity curves. Median IC_{50} values for pyrazolo[4,3-*d*]pyrimidines and purines were 57.9 and >100 μM, respectively ($p < 0.005$, paired Wilcoxon test, two-sided). Analysis of structure-activity relationships in the A series (pyrazolo[4,3-*d*]pyrimidines) shows that the most potent inhibitors of CRK3/CYC6 either are highly lipophilic (adamantyl derivative **9A**, IC_{50} = 1.8 μM; halogenophenyl derivatives **11A-14A**, IC_{50} = 6.8-16.1 μM) or have 2-hydroxybenzyl group at the N^7 position (**2A,** IC_{50} = 11.9 μM) (Fig. 4). The positive inhibitory effect of *ortho*-substitution of benzyl groups on human CDK2 has been reported previously and explained by the stabilization of the active conformation of the inhibitor by an intramolecular hydrogen bond between *o*-hydroxy with the nitrogen in position 1 of the purine ring.[34] In contrast, activity of 2-aminobenzyl derivative (**8A**) was lower that of 2-hydroxybenzyl derivative (**2A**), but equlled to unsubstituted compound **1A**. On the other hand, most of the compounds showing no or limited activity in the kinase assay (IC_{50} > 100 μM) belong to the most polar compounds in the set. These observations suggest that interaction between the N^7 (or N^6 for purines) substituent and the active site of CRK3 might be stabilized by hydrophobic interactions. In series B (purines), only adamantyl purine **9B** (IC_{50} = 12.3 μM) and fluorophenyl derivatives **11B** and **12B** (IC_{50} = 49.9 and 22.8 μM, respectively) had IC_{50} values lower than the maximum concentration tested (100 μM). The observation that certain substitutions increase kinase inhibitory potency in both compound types suggests that pyrazolo[4,3-*d*]pyrimidines and purines might share a similar mode of binding to the CRK3/CYC6 complex.

Several studies using other human CDK inhibitors show that these compounds can block CRK activity and that this inhibition reduces parasite proliferation and viability.[14, 15, 22] Therefore, we screened all the compounds also for their ability to kill *Leishmania donovani* amastigotes, the form of the parasite responsible for disease. Anti-amastigote activity of the compounds are summarized in Table 1 and Figure 3, which clearly show that leishmanicidal activity reflects inhibition of the CRK3/CYC6 kinase. Average inhibitory effect of pyrazolo[4,3-*d*]pyrimidines at the single concentration of 30 micromoles was always higher than that of corresponding purine derivatives. Activity at least 3 fold greater was observed in the case of 9 compound pairs. The most active pyrazolo[4,3-*d*]pyrimidines in terms of EC_{50} were adamantyl (**9A**), halogenophenyl (**12A, 13A**) and 2-OH-benzyl derivatives (**2A**). Since these compounds are also the most potent inhibitors of CRK3/CYC6 in the datasets, the observed effect on parasite viability is probably mediated by CRK3/CYC6 inhibition in agreement with previous reports.[14] A link between the anti-amastigote activity of CDK inhibitors and leishmanial CRK inhibition was first suggested by experiments using flavopiridol, a pan-selective CDK inhibitor. This compound inhibits CRK3 kinase (IC_{50} ~ 100 nM), and it also has been shown to arrest the parasite's cell cycle.[22] Additional experiments have shown that trisubstituted purines and indirubin analogs (all known CDK inhibitors) that are potent CRK3 inhibitors are also effective in killing *Leishmania in vitro*.[14, 15] Nevertheless, the possibility cannot be excluded that inhibition of another target contributes to the effect.

Finally, we note that our dataset demonstrates that the positive association between the activity and polarity in the group of pyrazolo[4,3-*d*]pyrimidines (Fig. 3B) was not limited to the viability assay but was also observed in the *in vitro* kinase assay. Our analysis demonstrates that the relationship between compound polarity and its cellular activity may in certain cases reflect the effect of polarity on the affinity for the binding site of a molecular target rather than its effect on the transport through biological membranes. Similar relationships between lipophilicity and anti-leishmanial activity of many diverse compounds have also been reported previously.[13,36]

In summary, the present study clearly shows that the series of 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines displaying moderate inhibition activity against the leishmanial CRK3/CYC6 protein complex also kills axenic amastigotes, thereby confirming previously published hypothesis.[22] These findings may not only provide chemical tools for basic studies on *Leishmania* biology, but they can also help to develop a new series of related compounds specifically directed against these parasites and having an improved therapeutic index.

Acknowledgments

The work was supported by grants from the Czech Science Foundation (204/08/0511; 301/08/1649), from the Ministry of Education, Youth and Sports of the Czech Republic (MSM 6198959216) and by a grant of the European Commission (Contract No LSHB-CT-2004-503467; CLJ). The Centre for Translational and Chemical Biology and Edinburgh Protein Production Facility (University of Edinburgh) were funded by the Wellcome Trust and the Biotechnology and Biological Sciences Research Council. CLJ holds the Michael and Penny Feiwel Chair in Dermatology. We are very grateful to Dr. Jeremy C. Mottram for contributing constructs of recombinant CRK3 and CYC6.

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Figures captions

Figure 1.

Structures of studied 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines (A) and 6,9-disubstituted purines (B).

Figure 2.

Elution profile of CRK3/CYC6 complex purification on a Co^{2+}-NTA column. (A) 10 µl of each eluted fraction from Co^{2+}-NTA column was separated by SDS-PAGE and stained by a Coomassie Brilliant Blue for visualizing purity of the protein complex. Molecular masses of both proteins correspond to their predicted values (35.6 and 36.2 kDa for CRK3 and CYC6, respectively). (B) Detection of CYC6 by immunoblotting using an anti-His tag antibody. Lane numbering refers to the fraction number.

Figure 3.

(A) Plot showing relationship between leishmanicidal activity and CRK3/CYC6 kinase inhibition (expressed as % inhibition) of studied 2,6-disubstituted purines (white circles) and corresponding 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines (black circles). Both determinations were carried out in at least duplicates using 30 µM of compound (Table 1). (B) Plot showing relationship between leishmanial axenic amastigote viability inhibition and CRK3/CYC6 kinase inhibition (IC_{50}) by 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines (A series). Circles correspond with lipophilicity (log*P*) of each compound, with log*P* values shown as shades of gray (white, 1.65 – 2.29; light gray, 2.29 – 2.94; dark gray, 2.94 – 3.58; black, 3.58 – 4.23). Numbering of the circles correspond with numbers of compounds listed in Table 1. Values of log*P* were calculated using ACD/PhysChem Suite software (version 12.0, ACD/Labs).

Figure 4.

Inhibition of recombinant CRK3/CYC6 by the most effective compounds **9A** (grey curve) and **12A** (black curve).

Table 1.
Antileishmanial activity and CRK3/CYC6 inhibition activity of studied 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines (A series) and 6,9-disubstituted purines (B series).

Comp.	R-substitution	*Leishmania donovani* axenic amastigotes inhibition		CRK3/CYC6 kinase inhibition	
		[%][a]	EC_{50} [μM]	[%][b]	IC_{50} [μM][c]
1A	benzyl	29.6 ± 0.1	68.6	24.5 ± 0.2	54.16
1B		19.9 ± 2.4	n.d.	18.5 ± 1.2	>100
2A	2-hydroxybenzyl	87.4 ± 0.4	35.7	68.0 ± 2.1	11.91
2B		11.7 ± 2.7	n.d.	-2.8 ± 1.3	>100
3A	3-hydroxybenzyl	25.4 ± 3.8	>100	35.8 ± 1.4	83.16
3B		6.5 ± 1.4	n.d.	8.8 ± 0.6	>100
4A	4-methoxybenzyl	31.5 ± 3.3	83.0	36.0 ± 7.5	75.47
4B		15.1 ± 1.6	n.d.	-6.9 ± 9.8	>100
5A	3,4-dimethoxybenzyl	8.4 ± 4.2	>100	19.9 ± 3.0	>100
5B		1.9 ± 0.5	n.d.	-2.3 ± 1.6	>100
6A	3-hydroxy-4-methoxybenzyl	34.3 ± 2.4	94.0	46.8 ± 3.4	28.16
6B		8.8 ± 2.2	n.d.	11.2 ± 2.9	>100
7A	4-hydroxy-3-methoxybenzyl	10.4 ± 2.4	>100	41.0 ± 1.8	100
7B		9.5 ± 1.1	n.d.	11.9 ± 0.1	>100
8A	2-aminobenzyl	41.3 ± 2.2	>100	42.3 ± 0.5	58.64
8B		6.6 ± 4.0	n.d.	11.0 ± 6.3	>100
9A	adamantan-1-yl	73.2 ± 0.0	1.22	93.8 ± 0.3	1.82
9B		72.0 ± 0.2	n.d.	66.1 ± 2.1	12.23
10A	3-methylbut-2-en-1-yl	45.5 ± 2.3	85.0	32.5 ± 2.0	57.18
10B		0.04 ± 5.2	n.d.	3.2 ± 1.4	>100
11A	3-fluorophenyl	66.6 ± 0.1	23.2	70.4 ± 1.8	14.56
11B		16.5 ± 0.7	n.d.	30.4 ± 0.7	49.92
12A	4-fluorophenyl	75.8 ± 1.7	11.6	78.8 ± 0.4	6.8
12B		21.5 ± 1.1	n.d.	53.3 ± 6.1	22.84
13A	3-chlorophenyl	73.3 ± 1.1	12.4	81.3 ± 2.3	9.86
13B		35.1 ± 3.3	n.d.	22.3 ± 4.9	>100
14A	2-bromophenyl	40.7 ± 6.7	18.7	35.6 ± 3.4	16.13
14B		26.2 ± 3.2	n.d.	1.8 ± 5.0	>100
15A	2-aminocyclohexyl	15.8 ± 4.1	>100	2.4 ± 3.6	>100
15B		4.5 ± 3.8	n.d.	-5.4 ± 3.4	>100
16A	4-aminocyclohexyl	3.3 ± 1.4	>100	35.0 ± 1.6	>100
16B		1.0 ± 2.7	n.d.	26.4 ± 3.7	>100
17A	furfuryl	16.6 ± 1.9	>100	28.5 ± 7.8	>100
17B		14.5 ± 3.0	n.d.	3.5 ± 1.7	>100
18A	pentyl	27.3±1.6	54.7	46.5 ± 2.7	>100
18B		22.6 ± 5.3	n.d.	14.8 ± 5.5	>100

n.d. – not determined; all values were determined by duplicate or triplicate assays; [a] in the presence of 30 μM compound; [b] in the presence of 15 μM ATP with 30 μM compound; [c] in the presence of 15 μM ATP

Figure 1.

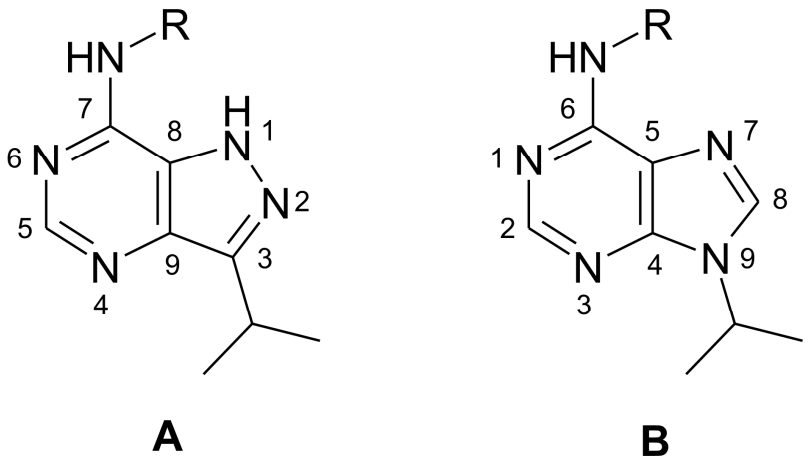


Figure 2.

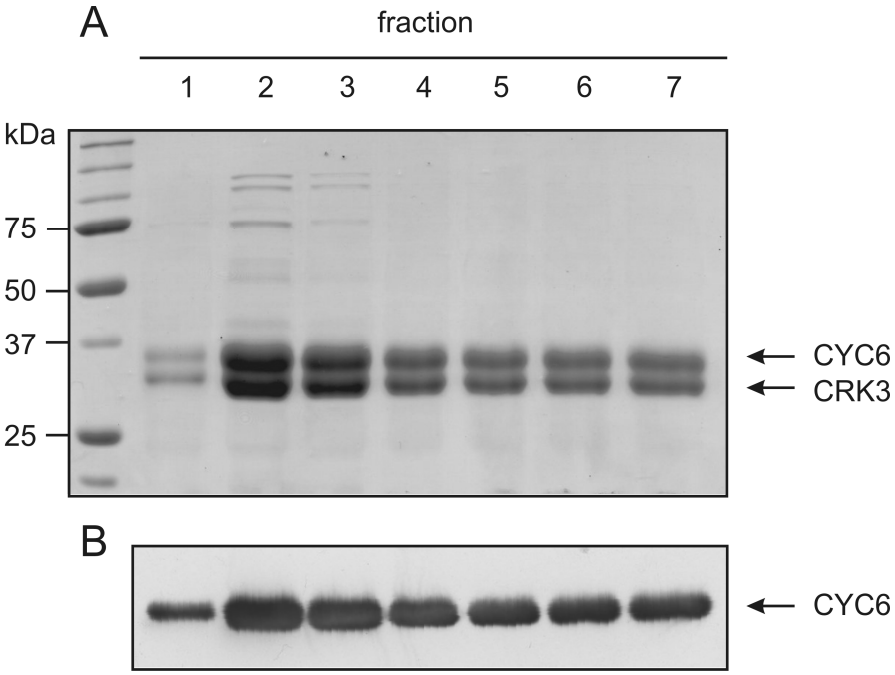


Figure 3.

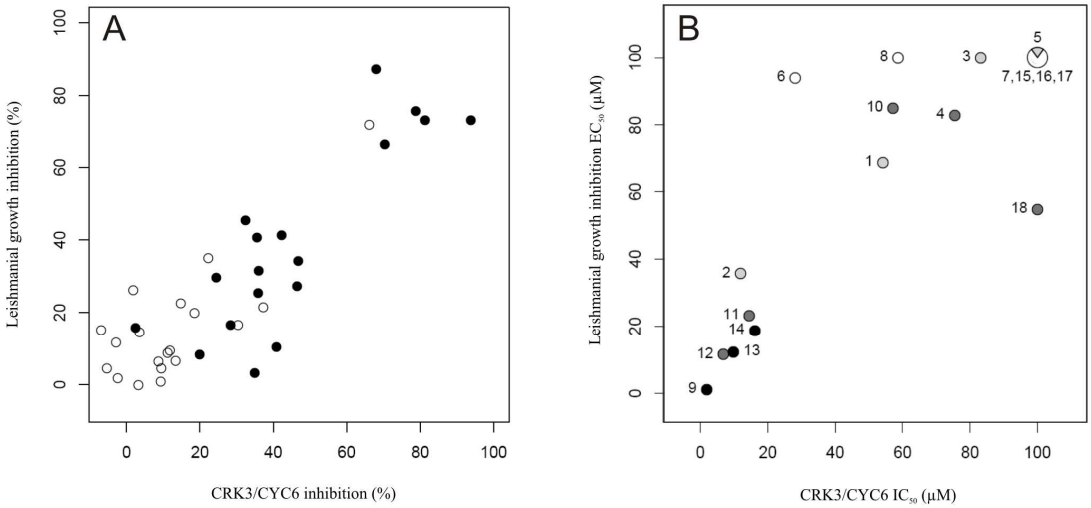
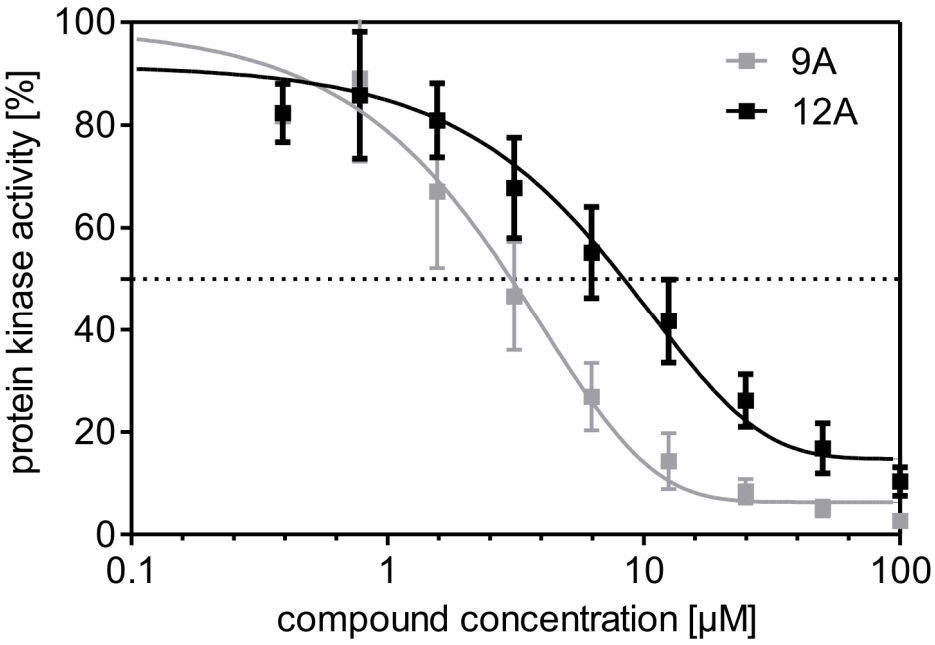


Figure 4.



Supplementary data

Antileishmanial activity of disubstituted purines and related pyrazolo[4,3-*d*]pyrimidines

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Chemistry

The straightforward synthesis of 9-isopropyl-6-substituted purines (series B) (Fig. 1) from 6-chloro-9-isopropylpurine was described previously.[1-3] 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines (series A) (Fig. 1) were prepared according to published procedures[3]. Briefly, 3-isopropyl-4-nitropyrazolecarboxylic acid was esterified to nitroester and reduced to the corresponding aminoester. Substituted pyrazole was then cyclised and chlorinated to 7-chloro derivative which was used for nucleophilic substitution with different amines to get final 7-substituted-3-isopropylpyrazolo[4,3-*d*]pyrimidines. All structural data of the compounds **1A - 4A, 6A, 10A, 13A, 14A, 17A, 18A, 1B - 4B, 6B, 10B, 13B, 14B, 17B, 18B** are described in our previous articles.[1,3] Data of newly synthesized compounds are presented here (see Materials and methods).

Materials and methods

General procedures

Column chromatography was performed on silica gel (Merck Kieselgel 60, 230-400). Melting points were determined on a Kofler block. The ^{1}H NMR spectra (δ, ppm; *J*, Hz) were recorded on a Bruker Avance 300 spectrometer operating at a temperature of 295 K. Mass spectra were measured on an MS Waters/Micromas ZMD-detector using direct inlet electrospray ionization with coin voltage of 15V. Elemental analyses indicated by symbols of the elements were within ± 0.4% of the theoretical value.

Prepared compounds

7-(3,4-Dimethoxybenzylamino)-3-isopropylpyrazolo[4,3-*d*]pyrimidine (5A)

Column chromat.: 2% MeOH in $CHCl_3$; mp 179 °C; MS ESI + : 328.3 (100%, M + H^+); MS ESI - : 326.3 (100%, M - H^+); ^{1}H NMR (300 MHz, $CDCl_3$) δ(ppm): 1.45 (6H, d, *J*=7.0 Hz, ($C\underline{H}_3$)$_2$CH), 3.476 (1H, sept, *J*=7.0 Hz, $C\underline{H}(CH_3)_2$), 3.657 (3H, s, OCH_3), 3.792 (3H, s, OCH_3), 4.65 (2H, bd, *J*=3.5 Hz, $C\underline{H}_2NH$), 6.47 (1H, bs, NH), 6.68-6.72 (2H, m, ArH), 6.834 (1H, d, *J*=8.0 Hz, ArH), 8.499 (1H, s, HC^5). Anal. ($C_{17}H_{21}N_5O_2$) C, H, N.

6-(3,4-Dimethoxybenzylamino)-9-isopropylpurine (5B)

Column chromat.: 3% MeOH in $CHCl_3$; mp 147 - 150 °C; MS ESI + : 328.3 (100%, M + H^+); ^{1}H NMR (300 MHz, DMSO–d_6) δ(ppm): 1.513 (6H, d, *J*=6.7 Hz, ($C\underline{H}_3$)$_2$CH), 3.691 (3H, s, OCH_3), 3.707 (3H, s, OCH_3), 4.61 (2H, bs, $C\underline{H}_2NH$), 4.707 (1H, sept, *J*=6.7 Hz, $C\underline{H}(CH_3)_2$), 6.850 (2H, s, ArH), 7.025 (1H, s, ArH), 8.198 (1H, s, HC^8), 8.242 (1H, s, HC^2). Anal. ($C_{17}H_{21}N_5O_2$) C, H, N.

7-(4-Hydroxy-3-methoxybenzylamino)-3-isopropylpyrazolo[4,3-*d*]pyrimidine (7A)

Column chromat.: 2% MeOH in $CHCl_3$; mp 200 - 206 °C; MS ESI + : 314.3 (100%, M + H^+); MS ESI - : 312.2 (100%, M - H^+), 348.2 (20%, M + Cl^-); ^{1}H NMR(300 MHz, DMSO-d_6) δ(ppm): 1.363 (6H, d, *J*=6.9 Hz, ($C\underline{H}_3$)$_2$CH), 3.312 (1H, sept, *J*=6.9 Hz , $C\underline{H}(CH_3)_2$), 3.753 (3H, s, OCH_3), 4.615 (2H, bd, *J*=5.0 Hz, $C\underline{H}_2NH$), 6.73 - 6.89 (2H, m, ArH), 6.998 (1H, s, ArH), 7.672 (1H, bs, NH), 8.251 (1H, s, HC^5), 8.949 (1H, s, NH), 12.26 (1H, s, OH). Anal. ($C_{16}H_{19}N_5O_2$) C, H, N.

6-(4-Hydroxy-3-methoxybenzylamino)-9-isopropylpurine (7B)

Column chromat.: 2% MeOH in $CHCl_3$; mp 158 - 164 °C; MS ESI + : 314.3 (100%, M + H^+); ^{1}H NMR(300 MHz, DMSO-d_6) δ(ppm): 1.514 (6H, d, *J*=6.8 Hz, ($C\underline{H}_3$)$_2$CH), 3.715 (3H, s, OCH_3), 4.583 (2H, bs, $C\underline{H}_2NH$), 4.704 (1H, sept, *J*=6.8 Hz, $C\underline{H}(CH_3)_2$), 6.62 - 6.98 (2H, m, ArH), 6.983 (1H, s, ArH), 7.672 (1H, bs, NH), 8.13 (1H, bs, NH), 8.238 (1H, s, HC^8), 8.76 (1H, s, HC^2). Anal. ($C_{16}H_{19}N_5O_2$) C, H, N.

7-(2-Aminobenzylamino)-3-isopropylpyrazolo[4,3-*d*]pyrimidine (8A)

Column chromat.: $CHCl_3$ / MeOH / aq. 25% NH_4OH (97 / 2.5 / 0.2); mp 147 - 149 °C. MS ESI + : 283.2 (100%, M + H^+), MS ESI - : 281.2 (100%, M - H^+), 317.2 (25%, M + Cl^-); ^{1}H NMR (300 MHz, MeOD) δ(ppm): 1.41 (6H, d, *J*=7.0 Hz, ($C\underline{H}_3$)$_2$CH); 3.47 (1H, sept, *J*=7.0 Hz, $C\underline{H}(CH_3)_2$), 4.73 (2H, s, $C\underline{H}_2NH$), 6.64 - 6.80 (2H, m, ArH), 7.07 (1H, t, *J*=7.4 Hz, ArH), 7.20 (1H, dd, *J*=7.7 Hz, 1.4, ArH), 8.25 (1H, s, HC^5). Anal. ($C_{15}H_{18}N_6$) C, H, N.

6-(2-Aminobenzylamino)-9-isopropylpurine (8B)

Crystallized from reaction mixture (n-BuOH), recrystallized from n-BuOH; mp 147 - 149 °C. MS ESI + : 283.2 (100%, M + H^+); ^{1}H NMR (300 MHz, DMSO-d_6) δ(ppm): 1.516 (6H, d, *J*=7.0 Hz, ($C\underline{H}_3$)$_2$CH), 4.52 (2H, bs, $C\underline{H}_2NH$), 4.712 (1H, sept, *J*=7.0 Hz, $C\underline{H}(CH_3)_2$), 5.217 (2H, bs, NH_2), 6.463 (1H, dd, *J*=7.5 Hz, 7.3, ArH), 6.586 (1H, d, *J*=7.8 Hz, ArH), 6.917 (1H, dd, *J*=7.2 Hz, 7.0, ArH), 7.131 (1H, d, *J*=7.6 Hz, ArH), 8.12(1H, bs, NH), 8.207 (1H, s, HC^8), 8.247 (1H, s, HC^2). Anal. ($C_{15}H_{18}N_6$) C, H, N.

7-(Adamantan-1-ylamino)-3-isopropylpyrazolo[4,3-*d*]pyrimidine (9A)
Column chromat.: 1% MeOH in $CHCl_3$; syrup-like product crystallized after several days; mp 72 - 98 °C; MS ESI + : 312.3 (100%, M + H^+); MS ESI - : 310.2 (100%, M - H^+), 346.2 (20%, M + Cl^-); ^{1}H NMR (300 MHz, $CDCl_3$) δ(ppm): 1.441 (6H, d, *J*=6.9 Hz, ($C\underline{H}_3$)$_2$CH), 1.738 (6H, bs, adamantyl), 2.14 (3H, bs, adamantyl), 2.253 (6H, bs, adamantyl), 3.486 (1H, sept, *J*=6.9 Hz, $C\underline{H}(CH_3)_2$), 8.410 (1H, s, HC^5). Anal. ($C_{18}H_{25}N_5$) C, H, N.

6-(Adamantan-1-ylamino)-9-isopropylpurine (9B)
Column chromat.: 1% MeOH in $CHCl_3$; mp 133 - 147 °C; MS ESI + : 312.3 (100%, M + H^+); ^{1}H NMR (300 MHz, DMSO-d_6) δ(ppm): 1.512 (6H, d, *J*=6.5 Hz, ($C\underline{H}_3$)$_2$CH), 1.673 (6H, bs, adamantyl), 2.072 (3H, bs, adamantyl), 2.205 (6H, bs, adamantyl), 4.715 (1H, sept, *J*=7.0 Hz, $C\underline{H}(CH_3)_2$), 6.447 (1H, bs, NH), 8.197 (1H, s, HC^8), 8.220 (1H, s, HC^2). Anal. ($C_{18}H_{25}N_5$) C, H, N.

7-(3-Fluoroanilino)-3-isopropylpyrazolo[4,3-*d*]pyrimidine (11A)
Crystallized from reaction mixture ($CHCl_3$), recrystallized from $CHCl_3$; mp 208 - 210 °C; MS ESI + : 272.3 (100%, M + H^+); MS ESI - : 270.2 (100%, M - H^+); ^{1}H NMR (300 MHz, DMSO-d_6) δ(ppm): 1.383 (6H, d, *J*=6.9 Hz, ($C\underline{H}_3$)$_2$CH), 3.515 (1H, sept, *J*=6.9 Hz, $C\underline{H}(CH_3)_2$), 7.130 (1H, dd, *J*=4.3 Hz, 4.2 , ArH), 7.508 (1H, dd, *J*=15.0 Hz, 8.2 ArH), 7.761 (1H, d, *J*=8.2 Hz, ArH), 7.993 (1H, d, *J*=10.7 Hz, ArH), 8.819 (1H, s, HC^5), 12.21 (1H, bs, NH). Anal. ($C_{14}H_{14}FN_5$) C, H, N.

6-(3-Fluoroanilino)-9-isopropylpurine (11B)
Column chromat.: 1% MeOH in $CHCl_3$; mp 94 - 99 °C; MS ESI + : 272.3 (100%, M + H^+); ^{1}H NMR (300 MHz, DMSO-d_6) δ(ppm): 1.577 (6H, d, *J*=6.9 Hz, ($C\underline{H}_3$)$_2$CH), 4.841 (1H, sept, *J*=6.9 Hz, $C\underline{H}(CH_3)_2$), 6.826 (1H, dd, *J*=8.8 Hz, 8.7 , ArH), 7.335 (1H, dd, *J*=15.0 Hz, 8.2, ArH), 7.785 (1H, d, *J*=8.2 Hz, ArH), 8.039 (1H, d, *J*=12.3 Hz, ArH), 8.459 (1H, s, HC^8), 8.220 (1H, s, HC^2). Anal. ($C_{14}H_{14}FN_5$) C, H, N.

7-(4-Fluoroanilino)-3-isopropylpyrazolo[4,3-*d*]pyrimidine (12A)
Column chromat.: 3% MeOH in $CHCl_3$; mp 233 °C; MS ESI + : 272.3 (100%, M + H^+); MS ESI - : 270.2 (100%, M - H^+), 306.4 (15%, M + Cl^-); ^{1}H NMR (300 MHz, DMSO-d_6, 335 K) δ(ppm): 1.432 (6H, d, *J*=7.0 Hz, ($C\underline{H}_3$)$_2$CH), 3.398 (1H, sept, *J*=7.0 Hz, $C\underline{H}(CH_3)_2$), 7.207 (2H, dd, *J*=8.6 Hz, 8.5, ArH), 7.882 (2H, bs, ArH), 8.365 (1H, s, HC^5), 9.39 (1H, bs, NH). Anal. ($C_{14}H_{14}FN_5$) C, H, N.

6-(4-Fluoroanilino)-9-isopropylpurine (12B)
Column chromat.: 2% MeOH in $CHCl_3$; mp 139 - 145 °C; MS ESI + : 272.3 (100%, M + H^+); ^{1}H NMR (300 MHz, DMSO-d_6) δ(ppm): 1.559 (6H, d, *J*=6.7 Hz, ($C\underline{H}_3$)$_2$CH), 4.801 (1H, sept, *J*=6.7 Hz, $C\underline{H}(CH_3)_2$), 7.16 (2H, dd, *J*=6.6 Hz, 6.6, ArH), 7.955 (2H, dd, *J*=9.2 Hz, 5.0, ArH), 8.375 (1H, s, HC^8), 8.421 (1H, s, HC^2), 9.910 (1H, bs, NH). Anal. ($C_{14}H_{14}FN_5$) C, H, N.

7-(2-Aminocyclohexylamino)-3-isopropylpyrazolo[4,3-*d*]pyrimidine (15A)
Column chromat.: $CHCl_3$ / MeOH / aq. 25% NH_4OH (94 / 6 / 0.2); mp 112 - 118 °C. MS ESI + : 275.4 (100%, M + H^+), MS ESI - : 273.2 (100%, M - H^+), 309.2 (5%, M + Cl^-); ^{1}H NMR (300 MHz, MeOD) δ(ppm): 1.20 – 1.39 (8H, m, cyclohexyl), 1.37 (6H, d, *J*=6.7 Hz, ($C\underline{H}_3$)$_2$CH), 1.83 – 2.02 (2H, m, cyclohexyl), 2.92 (1H, bs, NH_2), 3.344 (1H, sept, *J*=6.7 Hz, $C\underline{H}(CH_3)_2$), 8.26 (1H, s, HC^5). Anal. ($C_{14}H_{22}N_6$) C, H, N.

6-(2-Aminocyclohexylamino)-9-isopropylpurine (15B)
Column chromat.: $CHCl_3$ / MeOH / aq. 25% NH_4OH (94 / 6 / 0.2); amorphous glass, mp 98 – 110 °C. MS ESI + : 275.4 (100%, M + H^+); ^{1}H NMR (300 MHz, DMSO-d_6) δ(ppm): 1.21 - 1.40 (8H, m, cyclohexyl), 1.604 (6H, d, *J*=6.8 Hz, ($C\underline{H}_3$)$_2$CH), 1.85 – 2,08 (2H, m, cyclohexyl), 4.789 (1H, sept, *J*=7.0 Hz, $C\underline{H}(CH_3)_2$), 4.29 (1H, bd, NH), 5.42 (2H, bs, NH_2), 8.11 (1H, s, HC^8), 8.381 (1H, s, HC^2). Anal. ($C_{14}H_{22}N_6$) C, H, N.

7-(4-Aminocyclohexylamino)-3-isopropylpyrazolo[4,3-*d*]pyrimidine (16A)
Column chromat.: $CHCl_3$ / MeOH / aq. 25% NH_4OH (94 / 6 / 0.2); amorphous glass, mp 95 – 105 °C. MS ESI + : 275.4 (100%, M + H^+), MS ESI - : 273.2 (100%, M - H^+), 309.2 (10%, M + Cl^-); ^{1}H NMR (300 MHz, MeOD) δ(ppm): 1.18 – 1.38 (8H, m, cyclohexyl), 1.36 (6H, d, *J*=6.9 Hz, ($C\underline{H}_3$)$_2$CH), 1.82 – 2.00 (2H, m, cyclohexyl), 2.97 (1H, bs, NH_2), 3.343 (1H, sept, *J*=6.9 Hz, $C\underline{H}(CH_3)_2$), 8.232 (1H, s, HC^5). Anal. ($C_{14}H_{22}N_6$) C, H, N.

6-(4-Aminocyclohexylamino)-9-isopropylpurine (16B)
Column chromat.: $CHCl_3$ / MeOH / aq. 25% NH_4OH (94 / 6 / 0.2); mp 127 – 133 °C. MS ESI + : 275.4 (100%, M + H^+); ^{1}H NMR (300 MHz, $CDCl_3$) δ(ppm): 1.20 - 1.40 (8H, m, cyclohexyl), 1.604 (6H, d, *J*=7.0 Hz, ($C\underline{H}_3)_2CH$), 1.85 – 2.08 (2H, m, cyclohexyl), 4.789 (1H, sept, *J* = 7.0 Hz, $C\underline{H}(CH_3)_2$), 4.14 (1H, bd, NH), 5.38 (2H, bs, NH_2), 7.819 (1H, s, HC^8), 8.385 (1H, s, HC^2). Anal. ($C_{14}H_{22}N_6$) C, H, N.

Leishmania donovani cell culture
L. donovani (MHOM/SD/1962/1S-Cl2d) was used in this work. Axenic amastigotes were grown in a complete RPMI 1640 medium containing 20% fetal calf serum, pH 5.5, at 37 °C in a 5% CO_2 atmosphere, as described elsewhere.[4]

Axenic amastigote viability assay
Screening of the compounds for leishmanicidal activity was carried out using an alamarBlue (AbD Serotec) viability assay similar to that reported for leishmanial promastigotes.[5] Standardization and optimization of the assay for axenic amastigotes are described elsewhere.[6] Compounds to be assayed were diluted to twice the final concentration in the complete amastigote medium, containing 1% dimethyl sulfoxide (DMSO), and were aliquoted in triplicate (125 µL/well) into 96-well flat-bottom plates (Nunc). Amastigotes (5.0×10^5 cells/mL; 125 µL/well) were added to each well and incubated for 24 h at 37 °C in a 5% CO_2 incubator. The alamarBlue viability indicator was added (25 µL/well) and the plates were incubated for an additional 24 h, at which time the fluorescence (λex = 544 nm; λem = 590 nm) was measured in a microplate reader (Fluoroskan Ascent FL). Complete medium both with and without DMSO was used as negative controls (providing 0% inhibition of amastigote growth). Amphotericin B (Sigma-Aldrich), a drug used to treat visceral leishmaniasis, was included as a positive control on each plate and gave >90% inhibition of parasite growth at 1 µM.

Kinase assays
CDK2-cyclin E kinase was produced in Sf9 insect cells co-infected with appropriate baculoviral constructs, as previously described.[7] The leishmanial full length proteins CYC6 (His-tagged) and CRK3 were co-expressed in RosettaBL21(DE3)pLysS *E. coli* cells, induced with 100 µM IPTG at 20 °C overnight (Fig. 2). Both proteins form a stable complex in bacteria and as such was purified on a Co^{2+}-NTA column accordingly to manufacture's protocol (Qiagen). Purified protein complex was always prepared freshly using one-step affinity purification. Fraction 6 (Fig. 2), showing the highest kinase activity, was used in appropriate dilution for kinase inhibitor screening and assayed with 1 mg/mL histone H1 in the presence of 15 µM ATP, 0.05 µCi [γ-^{33}P]ATP and of the test compound in a final volume of 10 µL, all in a reaction buffer (50 mM HEPES, 10 mM $MgCl_2$, 5 mM EGTA, 10 mM 2-glycerolphosphate, 1 mM NaF, 1 mM DTT, pH 7.4). After 30 min incubation, reactions were stopped by adding 5 µL of 3 % aq H_3PO_4. Aliquots were spotted onto P-81 phosphocellulose (Whatman), which was subsequently washed 3 × with 0.5% aq H_3PO_4 and finally air-dried. To quantify kinase inhibition, a digital image analyzer BAS-1800 (Fujifilm) was employed. Kinase activity was expressed as a percentage of maximum activity. The concentration of the test compounds required to decrease the CDK by 50% was determined from dose-response curves and designated IC_{50}.

Electrophoresis and immunoblotting
Aliquots of each fraction from a CRK3/CYC6 purification were subjected to SDS-polyacrylamide gel electrophoresis (12.5% gel). Purity of the CRK3/CYC6 complex was evaluated by staining with Coomassie Blue G-250 (Sigma-Aldrich). In parallel, proteins separated on a second gel were electrophoretically transferred onto a nitrocellulose membrane, then blocked with PBS containing 0.1% Tween-20 (PBS-T) and 5% low-fat milk. The membrane was then incubated overnight with anti-His probe (clone H3 at 1:500 dilution; Santa Cruz Biotechnology). After washing three times in PBS-T, the membrane was incubated with a 1:1000 dilution of peroxidase-conjugated secondary antibody (Dako Cytomation). After an additional three washes in PBS-T, peroxidase activity was detected using ECL+ reagents (AP Biotech) according to the manufacturer's instructions.

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PŘÍLOHA VIII.

Jorda R, Nowicki M W, Charles Ch L, Havlíček L, Kryštof V, Strnad M, Walkinshaw M D. Antileishmanial activity of disubstituted purines and related pyrazolo[4,3-*d*]pyrimidines. *6th International Conference: INHIBITORS OF PROTEIN KINASES,* June 27-July 1, 2009, Warsaw, Poland (in *Biochimica Polonica*, 56 Supplement 1/2009).

ANTILEISHMANIAL ACTIVITY OF DISUBSTITUTED PURINES AND RELATED PYRAZOLO[4,3-*d*]PYRIMIDINES

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INTRODUCTION

Due to very high identity and similarity in amino acid sequence among mammalian cyclin-dependent kinases (CDKs) and leishmanial cyclin-dependent related kinases (CRKs) (Figure 1) new developed inhibitors with CDK selectivity could also be very effective in treatment of parasite diseases applied to CRK protein kinases. We report here results of screening directed to find new antileishmanial drugs among 6,9-disubstituted purines and structure related 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines. These compounds have been previously shown to moderately inhibit human cyclin-dependent kinases CDK1 or CDK2 [1]. Since some compounds blocked the proliferation of axenic amastigotes of *Leishmania donovani*, we screened them for interaction with recombinant leishmanial kinase CRK3, an important regulator of the cell cycle of the parasitic protozoan leishmanias [2,3], using methods of Thermofluor thermal shift assay and surface plasmon resonance (SPR). Some compounds from this screen showed promising results in all of these tests and could be used as lead structures for a further development of a new potential antileishmanial drugs.

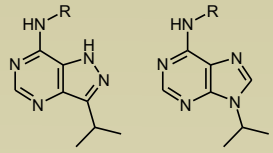

Figure 1: Structures of studied 3,7-disubstituted pyrazolo[4,3-d]pyrimidines (**A**) and 6,9-disubstituted purines (**B**).

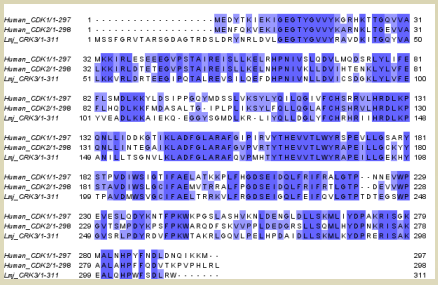

Figure 2: Sequence alignment of human CDK1, CDK2 and CRK3 from *Leishmania major*. Blue boxes indicate 54% and 58% identity in amino acid sequence (light - two sequences; dark - all three sequences), but similarity in amino acids sequence rises up 70% and 75%.

Table I. Antileishmanial activity, CDK inhibition activity and CRK3/ligand interaction data of studied 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines and 6,9-disubstituted purines.

Compound	R-substitution	% inhibition of *Leishmania donovani* axenic amastigotes		CDK1 IC_{50}[μM]	CDK2 IC_{50} [μM]	Thermal shift T_m	SPR K_d [M]
		30 μM	15 μM				
1A	benzyl	29.6±0.1	n.d.	1.2	0.16	-0.7	>100
1B		19.9±2.4	n.d.	3.6	2.69	-1.1	n.b.r.
2A	2-hydroxybenzyl	87.4±0.4	68.1±0.6	0.4	0.18	-0.6	>100
2B		14.2±2.1	n.d.	4.4	6.65	0.3	n.b.r.
3A	3-hydroxybenzyl	20.7±5.4	n.d.	1.7	0.11	0.3	>100
3B		6.5±1.4	n.d.	3.1	0.70	0.4	>100
4A	4-hydroxybenzyl	36.9±20.8	n.d.	1.8	0.09	0.3	>100
4B		18.5±4.9	n.d.	4.0	0.60	-0.2	n.b.r.
5A	4-methoxybenzyl	31.5±3.3	23.7±4.9	2.3	0.32	-0.7	n.b.r.
5B		15.1±1.6	n.d.	3.8	2.12	-0.7	n.b.r.
6A	3,4-dimethoxybenzyl	8.4±4.2	n.d.	n.a.	1.27	-1.6	>100
6B		1.9±0.5	n.d.	n.a.	12.46	-1.6	n.b.r.
7A	2-hydroxy-3-methoxybenzyl	34.3±2.4	25.6±3.1	n.a.	0.10	0.4	77.1
7B		8.8±2.2	n.d.	n.a.	1.81	-0.1	>100
8A	4-hydroxy-3-methoxybenzyl	10.4±2.4	n.d.	n.a.	0.29	-0.6	>100
8B		9.5±1.1	n.d.	n.a.	5.34	-0.7	n.b.r.
9A	2-aminobenzyl	41.3±2.2	26.5±1.1	n.a.	>100	0.4	>100
9B		6.6±4.0	n.d.	n.a.	5.48	-0.6	n.b.r.
10A	4-carboxybenzyl	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
10B		19.4±1.8	n.d.	n.a.	1.28	0.3	n.b.r.
11A	adamantan-1-yl	73.2±0.0	65.9±16.2	n.a.	25.16	6.3	59.2
11B		72.0±0.2	46.8±0.0	n.a.	>100	-1.6	>100
12A	3-methylbut-2-en-1-yl	45.5±2.3	32.8±1.2	4.5	1.14	n.a.	n.a.
12B		0.04±5.2	n.d.	17	37.37	-0.7	n.b.r..
13A	2-fluorophenyl	66.6±0.1	49.8±2.6	n.a.	0.48	0.4	>100
13B		16.5±0.7	n.d.	n.a.	1.98	0.3	n.b.r.
14A	4-fluorophenyl	75.8±1.7	58.3±0.6	n.a.	0.21	0.4	93.4
14B		21.5±1.1	n.d.	n.a.	2.83	-0.6	n.b.r.
15A	3-chlorophenyl	73.3±1.1	52.3±1.6	0.9	0.44	-3.7	>100
15B		35.1±3.3	n.d.	5.9	2.78	-1.1	n.b.r.
16A	2-bromophenyl	52.1±2.1	36.7±2.5	n.a.	0.60	n.a.	>100
16B		26.2±3.2	n.d.	n.a.	3.96	-0.7	>100
17A	2-aminocyclohexyl	15.8±4.1	12.4±1.4	n.a.	>100	0.1	>100
17B		4.5±3.8	n.d.	n.a.	67.45	-0.8	>100
18A	4-aminocyclohexyl	3.3±1.4	n.d.	60	1.05	-0.6	27.8
18B		1.0±2.7	n.d.	n.a.	1.48	-0.6	81.2
19A	furfuryl	16.6±1.9	n.d.	2.5	0.73	-0.6	>100
19B		14.5±3.0	n.d.	9.1	5.02	-0.7	n.b.r.
20A	pentyl	27.3±1.6	n.d.	1.2	0.79	0.4	>100
20B		27.9±4.8	n.d.	6.9	1.02	-0.7	n.b.r.
21A	(2-hydroxy-1-phenyl)ethyl	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
21B		4.5±1.1	n.d.	n.a.	18.39	-0.7	>100

n.a. - not available, n.d. - not determined, n.b.r. - no binding response in one-point concentration screen. All values were determined by duplicate or triplicate assays.

EFFECT ON AXENIC AMASTIGOTES

We have tested in total 19 pairs of purine isomers and corresponding pyrazolo[4,3-d]pyrimidines for antiproliferative effects on axenic amastigotes of *Leishmania donovani* using the method which was described previously [4]. All results are summarized in Table I. Testing of listed compounds showed that all pyrazolo[4,3-d]pyrimidines have better antiproliferative effects than related disubstituted purines. In the first series of isomers we could compare an antiproliferative efficiency of compounds with different substitution on benzyl ring. Addition of a polar amino (9A) or hydroxy (2A) group at ortho or para positions of the benzyl ring dramatically increase the inhibitory activity in comparison to the unsubstituted derivative. Substitution at the meta position (e.g. methoxy) led to a decrease in inhibitory activity. All tested halogen-phenyl pyrazolopyrimidine derivatives (13A, 14A, 15A) display very promising results and exhibit about 70% inhibition at 30 μM and 50% at 15 μM. Only the bromo derivative 16A shows a slight decrease in this inhibition trend. The most potent compound in this series was 14A (4-fluorophenyl substituent) which, together with hydroxy derivative (2A), is the most effective member of this screen. Furthermore, both isomers of compound 11 which contain the bulky hydrophobic adamanthyl group have exhibited very high inhibition. We also tested many compounds with various N6 side chains (17-21), but surprisingly no inhibition was observed.

THERMAL-SHIFT ASSAY

All compound listed in Table I. were tested for CRK3/ligand interaction using a Thermofluor-based thermal shift assay [5]. Many of these compounds have shown destabilizing effects on enzyme stability, which correlates with low inhibition in *in vivo* studies and inhibition through human CDK enzymes as well (e.g. 8A, 8B, 5A, 5B, 18A, 18B). A slight promising result is observed with compounds with a hydroxyl or amino group on aromatic side chain ring (9A). Quite surprising data were determined by halogen derivatives which have shown very high inhibition data on tested axenic amastigotes. Positive thermal shift was assigned only on isomers with fluoro substitution (13A, 14A). However no effect was observed in presence of other halogen derivatives (isomers 15 and 16). The highest stabilizing effect was found for pyrazolo[4,3-d]pyrimidine isomer with adamanthyl group with T_m about 6.3.

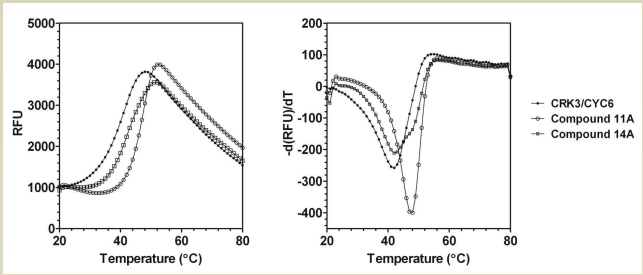

Figure 3. Stabilizing effect of selected compounds on denaturating process of protein complex CRK3/CYC6. Curves represent nonlinear fits as a function of relative fluorescence units RFU (**left**) or first derivative -d(RFU)/dT (**right**) against temperature. Each curve is a mean of three determinations. T_m was determined from the peak minima in the first derivative curves.

SURFACE PLASMON RESONANCE

To complete an interaction study of CRK3/ligand the method surface plasmon resonance was used. Protein-ligand interaction was determined first by one-point concentration screen (200 μM) to guage any binding response. Subsequently, positive hits were tested in the concentration range 49 nM - 50 μM for K_d determination (Table I). Many of the studied ligands showed no binding response against CRK3 kinase (e.g. isomers 5). Determined Kd values of selected positive hits was observed mostly above 100 μM which signified low binding affinity to CRK3. Only a few ligands returned positive hits from both the SPR and previous thermal shift screen; fluoro (14A) and adamanthyl (11A) derivatives, which gave Kd values 93.4 μM and 59.2 μM respectively. The next three strongest binding ligands (18A, 18B, 7A) have Kd values below 100 μM, but they have not shown any significant data through other tests, *in vivo* especially.

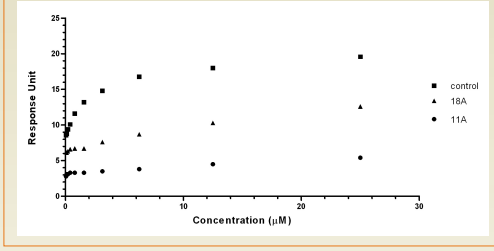

Figure 4. Steady-state affinity analysis of selected ligands using surface plasmon resonance. Indirubin derivative was used as a control (K_d ~ 3 μM).

METHODS

THERMAL-SHIFT ASSAY

Method is based on monitoring ligand effects on temperature-dependent protein unfolding. To monitor protein unfolding, the environmentally sensitive fluorescent dye Sypro Orange was used. The unfolding process exposes the hydrophobic region of proteins and results in a large increase in fluorescence, which is used to monitor the protein-unfolding transition. Interaction of studied ligand with protein expresses as positive shift in transition melting temperature T_m.

Solutions of 25 μl of 500x Sypro Orange, 1 μl of test compound (10 mM in 100% DMSO), 2 μl of protein (1 mg/ml) and protein buffer (50 mM Hepes, 150 mM NaCl, pH 8.0) to the final volume 50 μl were added to the wells of a 96-well thin-wall PCR plate. Protein buffer was added instead of test compound or protein in the control samples to correlate results for background and autofluorescence effect of ligand. The plates were sealed with Microseal Adhesive Sealer and heated in an iCycler iQ Real Time PCR Detection System from 20 °C to 80 °C in increments of 0.5 °C/30 s. Fluorescence changes in the wells were monitored simultaneously with a charge-coupled device camera. The wavelengths for excitation and emission were 485 and 575 nm, respectively.

SURFACE-PLASMON RESONANCE

SPR is an optical method based on measurement of change in refractive index (RI) as a result of interaction immobilized ligand (protein) onto the sensor surface and its binding partner (inhibitor) which is injected under continuous flow through the flow cell.

Real time protein-ligand interaction analysis was performed using Biacore T100 instrument and immobilization of protein CRK3 (200 nM) was performed on NTA sensor chip. All interaction assays were carried out in HBS buffer + 50 μM EDTA, 2% DMSO, pH 7.4 at 25 °C with a flow rate 50 μL/min and the contact time 50 s. Protein-ligand interaction was determined first by one-point concentration screen (200 μM) to determine any binding response, then positive hits were tested in concentration range 49 nM - 50 μM. Data was evaluated for solvent and reference correction and overall binding affinity (K_d) was determined by steady-state affinity analysis.

Acknowledgments

The work was supported by grants GA CR 204/08/0511 and MSM 6198959216. The Centre for Translational and Chemical Biology and Edinburgh Protein Production Facility (University of Edinburgh) were funded by the Wellcome Trust and the BBSRC.

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IPK2009