

Synthesis and Target Identification of Hymenialdisine Analogs

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Summary

Hymenialdisine (HMD) is a sponge-derived natural product kinase inhibitor with nanomolar activity against CDKs, Mek1, GSK3 β , and CK1 and micromolar activity against Chk1. In order to explore the broader application of the pyrrolo[2,3-c]azepine skeleton of HMD as a general kinase inhibitory scaffold, we searched for additional protein targets using affinity chromatography in conjunction with the synthesis of diverse HMD analogs and profiled HMD against a panel of 60 recombinant enzymes. This effort has led to nanomolar to micromolar inhibitors of 11 new targets including p90RSK, KDR, c-Kit, Fes, MAPK1, PAK2, PDK1, PKC θ , PKD2, Rsk1, and SGK. The synthesis of HMD analogs has resulted in the identification of compounds with enhanced and/or dramatically altered selectivities relative to HMD (28n) and in molecules with antiproliferative activities 30-fold higher than HMD (28p).

Introduction

Protein kinases play an essential role in regulating various cellular functions including gene expression, cellular proliferation, differentiation, membrane transport, and apoptosis [1–7]. Aberrant kinase activities have been associated with several diseases including cancer, diabetes, and Alzheimer's disease [8–13]. As a consequence, considerable effort has been directed toward the development of specific and potent small molecule

inhibitors of kinase function. While there are a few examples of non-ATP competitive kinase inhibitors [14, 15], the majority of kinase inhibitors target the ATP binding pocket of the enzyme. These ATP site-directed inhibitors fall into a limited number of families including quinazolines [16], pyrimidines [17–20], indolinones [21, 22], purines [23–28], imidazoles [29–31], flavonoids [32, 33], paullones [34–36], and alkaloids (for example, staurosporine and its derivatives [37–39]). To date, approximately 46 kinase inhibitors have entered clinical trials targeting kinases involved in cancer (EGFR, CDKs, Raf, PKC, PKA, KDR, Mek-1), angiogenesis (KDR), leukemia (bcr-abl, FLT3), inflammation (p38), immune disorder (JNK), and neurodegenerative disorders (JNK).

In this paper, we report the use of affinity chromatography and in vitro enzymatic profiling against a panel of 60 kinases to identify additional potential targets of the natural product Hymenialdisine (HMD 1), as well as synthesis and biological characterization of a variety of analogs. HMD is one member of a family of tricyclic pyrrole compounds that is constructed from a brominated pyrrolo[2,3-c]azepine skeleton and a 5-membered glycoxyamide ring system. It was isolated from marine sponges including *Hymeniacidon aldis*, *Axinella verrucosa*, and *Acanthella aurantiaca* in the early 1980s based on its antiproliferative effects on cultured lymphocytic leukemia cells [40–44]. It was later shown to exhibit strong inhibitory activity against several closely related CDKs, such as CDK1/cyclin B (IC_{50} = 22 nM), CDK2/cyclin A (IC_{50} = 70 nM), CDK2/cyclin E (IC_{50} = 40 nM), and CDK5/p25 (IC_{50} = 28 nM), as well as against more distantly related kinases such as GSK3 β (IC_{50} = 10 nM) and CK1 (IC_{50} = 35 nM) [45]. HMD was also isolated from the sponge *Stylissa massa* as the compound inhibiting the Raf/Mek-1/MAPK cascade and shown to be a potent inhibitor of Mek1 (IC_{50} = 6 nM) [46]. Kinetic analysis has shown that HMD acts in an ATP-competitive fashion [45], and the cocrystal structure with CDK2 revealed that HMD occupies the ATP site and makes many of the hydrogen bonding interactions seen in other CDK-inhibitor complexes [45]. Despite potent activities against these kinases, HMD is relatively selective in vitro and exhibits only moderate to weak activity against many other kinases [45–50].

The activities of HMD as a potential antiproliferative, anti-inflammatory, and antineurodegenerative agent have been investigated in several cellular models. For example, HMD in the micromolar range was shown to have antiproliferative effects against human tumor cell lines, presumably as a result of CDK and Mek inhibitory activity [46]. Using U937 cells, HMD was shown to be a micromolar inhibitor of NF κ B-mediated gene transcription [51–53]. Furthermore, the hyperphosphorylation of tau, a known component of the neurofibrillary plaques characteristic of patients with Alzheimer's disease, is inhibited in vitro by HMD at 50 μ M [45].

In order to identify additional potential intracellular targets of HMD, we performed affinity chromatography with immobilized HMD using brain extracts. Previously,

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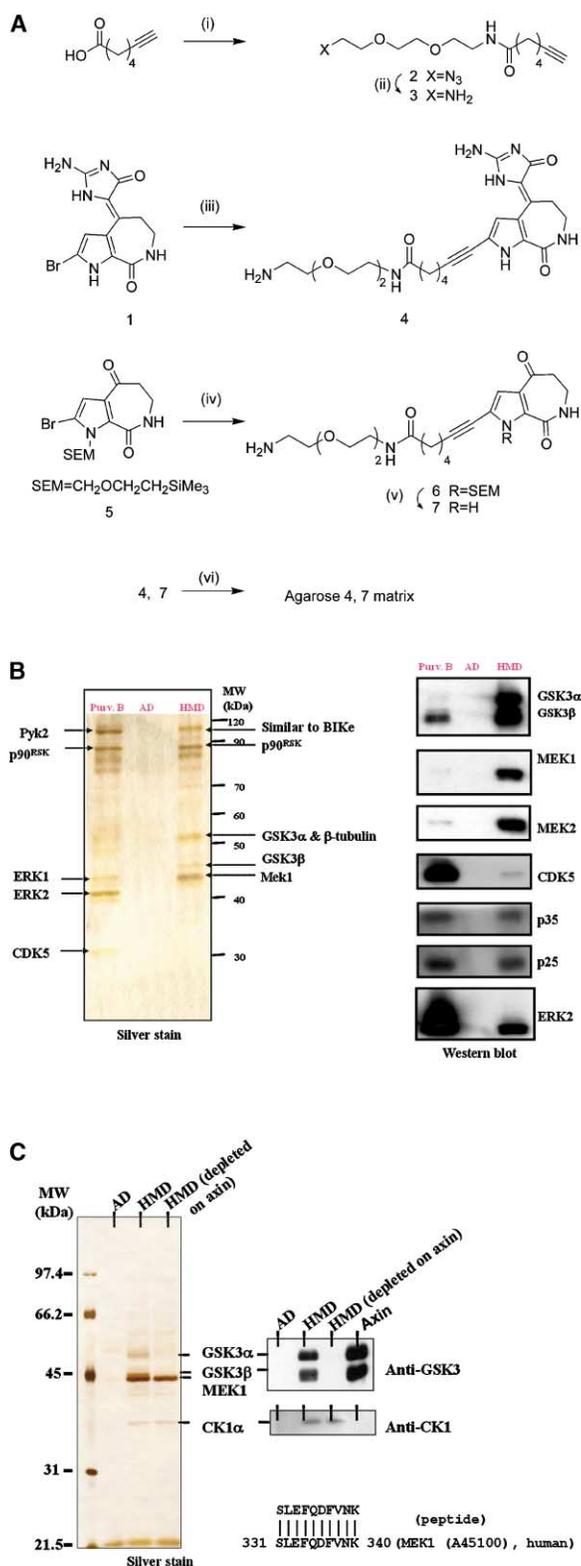


Figure 1. Preparation of Hymenialdisine and Aldisine Control Affinity Resins and Affinity Chromatography Pull-Down Experiment (A) Synthetic scheme for Hymenialdisine (HMD) and aldisine (AD) affinity resins. (i) 6-heptynoic acid, 2-[2-(2-azido-ethoxy)-ethoxy]-ethylamine, 1,3-diisopropylcarbodiimide, CH_2Cl_2 ; (ii) 2, PMe_3 , THF-1% H_2O ; (iii) HMD 1, 3, $PdCl_2(PPh_3)_2$, CuI, PPh_3 , Et_2NH , DMF;

this method has yielded valuable insights into the cellular targets of two other kinase inhibitors: purvalanol B [54] and paullone [55]. To complement this approach, we also tested HMD against a panel of recombinant kinases not previously examined.

Despite the description of the total synthesis of HMD by two research groups [56–58], there have been few reported modifications to the core structure [59, 60]. The second part of this paper describes the synthesis and the structure-activity relationship (SAR) of new HMD analogs with altered kinase selectivity profiles. Of particular interest are selected analogs that retain activity against CDKs or GSK3 β , which are comparable to that of the parent compound but have enhanced selectivity. The cellular activities of these compounds have also been investigated, and selected analogs showed up to 30-fold improvement over HMD.

Results and Discussion

Identifying Additional Substrates of HMD Affinity Chromatography

The crystal structure of CDK2 complexed with HMD revealed that HMD binds in the ATP binding pocket with its bromine pointing toward the outside of the pocket [45]. Based on this observation, we reasoned that addition of a linker moiety at the 2-position of the pyrrole ring might enable us to immobilize the compound without destroying its kinase inhibitory activity. We first synthesized HMD 1 and SEM (2-(trimethylsilyl)ethoxymethyl) protected 2-bromoaldisine 5 [56]. 2-bromoaldisine, an analog lacking the 5-membered glycoacymidine that is crucial for potent kinase inhibitory activity, was used as a negative control for the experiment. A palladium-catalyzed reaction using $Pd(PPh_3)_2Cl_2$ [61] allowed the coupling of 2-bromopyrrolo compounds to an amino-PEG linker containing a terminal alkyne. After removing the SEM group, compounds 4 and 7 were immobilized to an N-hydroxysuccinimide ester-activated agarose matrix through amide bond formation (Figure 1A).

In order to identify HMD-interacting proteins present in neuronal cells, mouse brain extracts were incubated with the HMD or aldisine (AD) control matrix. After washing the matrices to remove unbound/weakly bound pro-

(iv) 2-bromoaldisine 5, 3, $PdCl_2(PPh_3)_2$, CuI, PPh_3 , Et_2NH , DMF; (v) a. MeOH/10% aq. HCl (1:1); b. aq. NH_3 in MeOH/ H_2O (1:1); (vi) 3 mM of 4 or 7 in DMSO, 1 ml of Affi-Gel 10 Gel (Bio-Rad Laboratory), 24 hr.

(B) HMD-affinity pull-down experiment data from mouse brain extracts and intracellular target identification. Purv. B, purvalanol B resin; AD, aldisine control resin; HMD, hymenialdisine resin. Brain extracts were loaded on Purv. B, AD, or HMD matrix. Bound proteins were resolved by SDS-PAGE and visualized by silver staining and by Western blotting with anti-GSK3 α/β , anti-Mek1/2, anti-CDK5, anti-p25, anti-p35, and anti-Erk2.

(C) HMD-affinity pull-down experiment data from porcine brain extracts and intracellular target identification. Brain extracts were loaded on AD or HMD matrix. Bound proteins were resolved by SDS-PAGE and visualized by silver staining and by Western blotting with anti-GSK3 α/β and anti-CK. In order to purify Mek1 for microsequencing, the extracts were also depleted of GSKs using axin beads prior to chromatography with the HMD resin.

ered with enhanced potency and selectivity toward these additional kinase targets.

Synthesis of Analogs Based on HMD Scaffold

The crystal structure of CDK2 complexed with HMD has revealed multiple hydrogen bonds that are important to the interaction of HMD with the enzyme active site (Figure 4A). These include the pyrrole N1 with the carbonyl oxygen of Leu83, the carbonyl oxygen of azepine ring with the backbone amide of Leu83, the amide of the azepine ring N2 with the carbonyl oxygen of Glu81, the guanidine amino group N5 with one of the side chain oxygen atoms of Asp145 and the side chain carbonyl of Asn132, two water-mediated hydrogen bonds between the O2 of HMD with the main chain carbonyl of Asp145, and N5 of HMD with the main chain carbonyl of Gln131 [45]. Since several of these hydrogen bonds are conserved in other inhibitor-CDK complexes, they are likely to contribute to the potency of HMD against CDKs and were therefore mostly retained in our synthetic efforts. The bromine substituent, in contrast, occupies a spacious hydrophobic pocket that is more completely filled by other CDK inhibitors such as olomoucine and purvalanol [45]. In order to optimize the interactions in this region, analogs were prepared where the pyrrole ring was replaced with an indole.

Synthesis of Close Analogs of HMD

To examine the effect of various substituents on the pyrrole ring, we synthesized a series of HMD analogs as shown in Table 2. HMD (**1**), debromohymenialdisine (**8**), 3-bromohymenialdisine (**10**), and 3-bromo-2-debromohymenialdisine (**9**) were prepared according to reported procedures via hymenin (**11b**) [56–58]. As an alternative procedure, alcohol **13b**, obtained from the reduction of azepinedione **12b**, reacts with 2-aminoimidazole in methanesulfonic acid to give **11b** through an in situ generated intermediate alkene **14b** (Figure 2A). Since azepinediones can be used as common building blocks for other structural modifications, we utilized this modified scheme to prepare other HMD analogs including dichlorinated analog **15**, acetamide **16**, N-ethylated analog **17**, and 3-methyl analog **18**. Cyclization of 3-[(4,5-dibromo-1H-pyrrole-2-carbonyl)-amino]-butyric acid in PPA gave the ketone in very poor yield, presumably due to electron deficiency of the dibromopyrrole ring and steric hindrance caused by the methyl group. We therefore cyclized pyrrole-2-carboxylic acid and performed bromination after reduction of the ketone (see Supplemental Figure S1 at <http://www.chembiol.com/cgi/content/full/11/2/247/DC1>).

The synthesis of indole analogs is outlined in Figure 2B. The attempt to synthesize the 6-chloro substituted indole analog from acetal **20b** [58] gave indole dimer as the exclusive product. Consequently, a modified scheme was attempted. Carboxylic acids **23a–g** were cyclized in P₂O₅-methanesulfonic acid [66] to yield azepino [3,4-*b*]indole-1,5-diones **24a–g**. These ketones were then reduced to alcohols and reacted with aminoimidazole in methanesulfonic acid. Subsequent bromination of **26** afforded compound **27** in moderate to good yields. Hydrogenation of **27a**, **27f**, and **27g** furnished compounds **27d**, **27h**, and **27i**, respectively.

Synthesis of Hydrazone and Amide Derivatives

To further probe SAR of HMD analogs, a variety of aromatic and aliphatic groups were introduced to replace the glycoamidine ring of HMD that can be divided into two categories: hydrazones and amides. Hydrazone derivatives **28** of the previously synthesized pyrrolo- and indolo-azepinediones were prepared by reacting ketones with aromatic hydrazines (Figure 2C). Amides **31** and **34** were prepared from carboxylic acids **30** and **33** (Figure 2D). Compound **30** was obtained from the hydrolysis of *t*-Boc protected ester [60]. Double bond migration occurred during ester hydrolysis under basic conditions as a result of the formation of a more stable carbanion intermediate [67, 68]. Amine building blocks chosen include various aliphatic and aromatic amines.

Biological Results and SAR

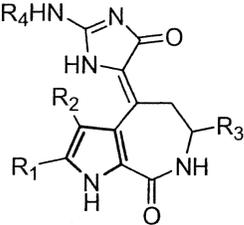
SAR on Kinase Assay

In this study, we focused on investigating the inhibitory properties of HMD analogs against purified CDK5/p25, CDK1/cyclin B, and GSK3 β . The structure activity relationships (SAR) of HMD analogs were examined by measuring the in vitro inhibitory activity of each HMD analog against these three kinases. Table 2 is a summary of the IC₅₀s of close structural analogs of HMD containing the pyrrolo- or indolo-azepine skeleton and the glycoamidine appendage.

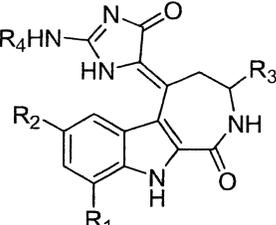
Inhibition of CDK5/p25 by Close Structural Analogs of HMD. As shown, non-, mono-, or dihalogenation at the 2- and 3-position on the pyrrole ring has little effect on the activity of these compounds against CDK5/p25. Simple pyrrole derivatives (entry 1–5) are potent inhibitors, whereas the simple indole analog (entry 9) exhibits reduced activity. Indole substituents such as a halogen at the 7-position (entry 10–12) or a nitro/amino substituent at the 9-position (entry 16 and 17) result in derivatives that are as potent as the nonsubstituted indole analog (entry 9), whereas substitution of a nitro, amino, or methanesulfonyl group at the 7-position on the indole results in reduced potency (entry 13–15).

We next investigated the effect of substitution on the azepine ring (R₃) and glycoamidine ring (R₄). When R₃ is a methyl group, the activity is reduced by approximately 80% for both pyrrole and indole derivatives (entry 6 versus 2 and entry 18 versus 12). This phenomenon suggests that the hydrophobic pocket adjacent to methylenes of the azepine ring is quite shallow. Alkylation or acylation of the glycoamidine ring leads to a dramatic decrease in activity, presumably as a result of steric hindrance and loss of hydrogen bonds (entry 7, 8, and 19).

Inhibition of CDK1/cyclin B by Close Structural Analogs of HMD. HMD appears to be the most potent inhibitor of CDK1/cyclin B among its close structural analogs (entry 1 versus 2–5). The inhibitory activity of these analogs against CDK1/cyclin B appears to be more susceptible to the halogen substitution pattern on the pyrrole ring than that against CDK5/p25. In particular, introducing a bromine at the 3-position on pyrrole (entry 4) has the most negative impact on the CDK1/cyclin B activity. For indole analogs, fluorine, but not chlorine or bromine, substitution at the 7-position increases the potency (entry 10, 11, 12 versus 9).

Table 2. Close Analogs of HMD and Their Biological Activity against CDK5/p25, CDK1/cyclin B, and GSK3 β


Entry	Compound Number	R ₁	R ₂	R ₃	R ₄	IC ₅₀ /nM		
						CDK5/p25	CDK1/cyclin B	GSK3 β
1	1	Br	H	H	H	37	70	73
2	10	Br	Br	H	H	56	530	51
3	8	H	H	H	H	112	250	111
4	9	H	Br	H	H	79	1500	90
5	15	Cl	Cl	H	H	62	220	47
6	18	Br	Br	Me	H	251	500	268
7	16	Br	Br	H	Ac	533	1500	363
8	17	Br	Br	H	Et	9120	>10000	1388



Entry	Compound Number	R ₁	R ₂	R ₃	R ₄	IC ₅₀ /nM		
						CDK5/p25	CDK1/cyclin B	GSK3 β
9	27d	H	H	H	H	177	800	64
10	27c	H	F	H	H	177	550	52
11	27b	H	Cl	H	H	191	2300	51
12	27a	H	Br	H	H	213	1000	47
13	27e	H	SO ₂ Me	H	H	1240	890	120
14	27f	H	NO ₂	H	H	713	3200	710
15	27h	H	NH ₂	H	H	681	1600	400
16	27g	NO ₂	H	H	H	109	1200	48
17	27i	NH ₂	H	H	H	187	1600	163
18	27j	H	Br	Me	H	1010	500	175
19	27k	H	Br	H	Ac	5110	≥10	2700

IC₅₀s were determined by two separate tests and reported as mean values. Assays were conducted at 1.5 μ M ATP for CDK5/p25 and GSK3 β (for SPA procedure, see Experimental Procedures) and 15 μ M for CDK1/cyclin B [45].

Inhibition of GSK3 β by Close Structural Analogs of HMD. When the inhibitory activity of the analogs against GSK3 β was examined, we found that most of the HMD analogs are potent inhibitors. Indole derivatives generally exhibit similar activity compared to pyrrole derivatives. The substitution pattern on the indole ring has little effect on potency against GSK3 β ; however, alkylation or acylation of the glycohydrazide amide reduces activity (entry 7, 8 versus 2 and entry 19 versus 12).

Based on the above observations on the inhibitory properties of HMD analogs, it can be concluded that the R₃ and R₄ substituents are more influential with respect to binding activity than are R₁ and R₂. Although we believe that all these HMD analogs exhibit similar binding orientations in the ATP binding site of all three enzymes, cocrystal structures with each kinase will be needed to rationalize selectivity trends. The slightly dif-

ferent kinase selectivity profile among these compounds indicates the opportunity to achieve different selectivities relative to HMD. For example, the 7-bromoindole analog (entry 12) showed 10-fold improvement of selectivity between GSK3 β and CDKs compared to HMD (entry 1).

Inhibition of CDK5/p25 and GSK3 β by Hydrazones and Amides of HMD Analogs. In order to replace glycohydrazide ring of HMD, a variety of aryl hydrazones and amides were introduced in the context of a dibromopyrroloazepinone skeleton. Hydrazone derivatives exhibit large decreases in inhibitory activity toward CDK5/p25 and GSK3 β (Figure 3, row 1). This is not surprising, as the glycohydrazide ring of HMD is known to contribute multiple hydrogen bonds to the kinase in the ATP binding pocket [45]. Previous results reported in the literature on the inhibition of CDKs, GSK3 β , and CK1 by HMD-

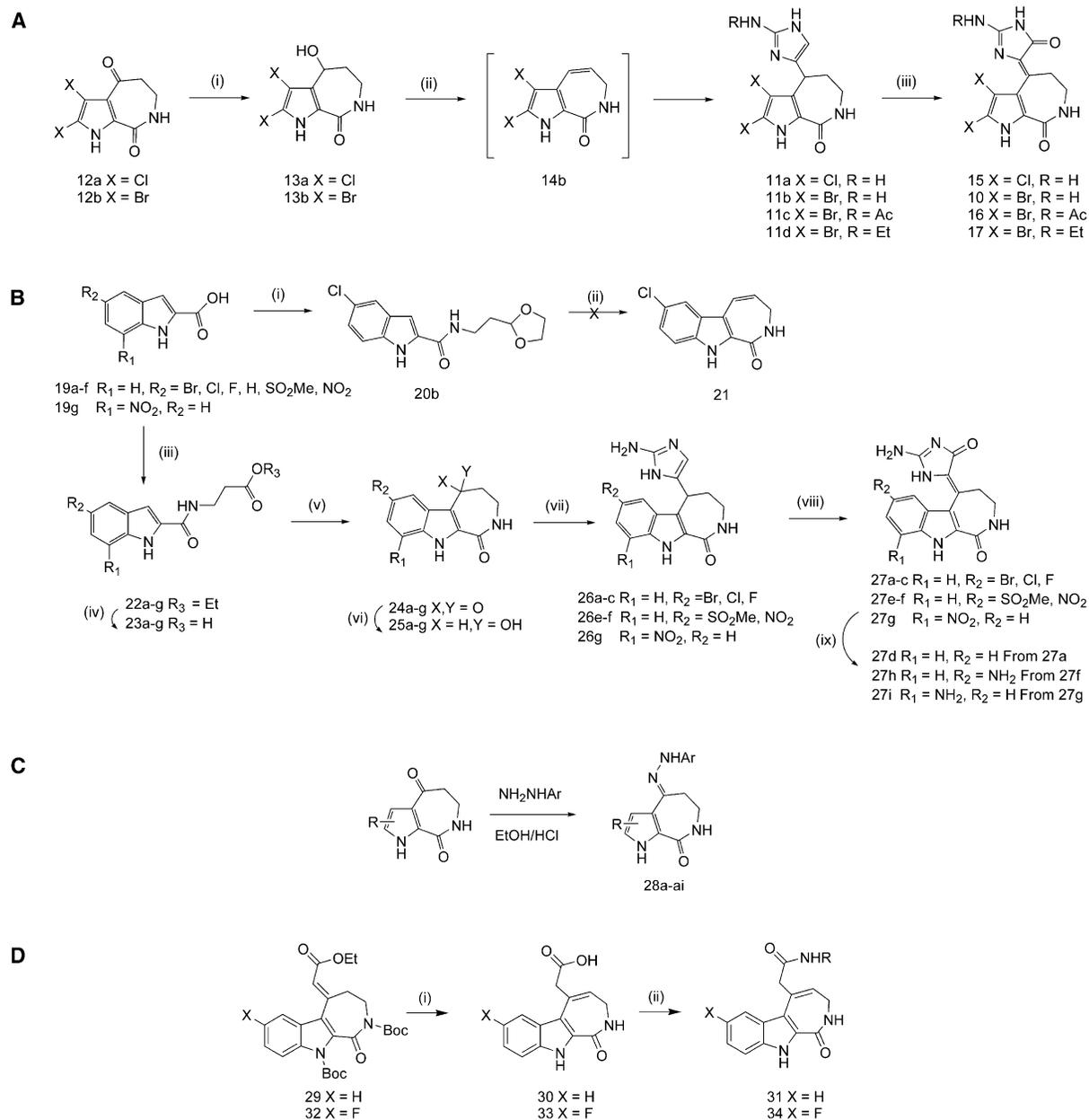


Figure 2. Synthetic Schemes for Close Analogs of HMD and Analogs Bearing Replacements to the Glycocyamidine Ring of HMD
(A) Synthetic scheme for pyrrole analogs of HMD. (i) NaBH₄, EtOH/DMF; (ii) 2-aminoimidazole or substituted 2-aminoimidazole, CH₃SO₃H; (iii) Br₂, NaOAc/HOAc.
(B) Synthetic scheme for indole analogs of HMD. (i) 2-(2-aminoethyl)-1,3-dioxolane, DIC, HOBT, DIEA, CH₂Cl₂; (ii) CH₃SO₃H; (iii) DIC, DIEA, HOBT, β-alanine ethyl ester; (iv) KOH; (v) P₂O₅, CH₃SO₃H; (vi) NaBH₄, EtOH/DMF; (vii) 2-aminoimidazole, CH₃SO₃H; (viii) Br₂, NaOAc/HOAc; (ix) H₂, 10% Pd/C or SnCl₂.
(C) Hydrazone derivatives.
(D) Amide derivatives. (i) a. LiOH, MeOH/H₂O; b. H⁺; (ii) HATU, NH₂R, DMF.

related compounds isolated from marine sponges and few synthetically modified HMD analogs have also shown that slight structural modification of HMD has led to a dramatic drop in activity [45]. Nonetheless, hydrazones with 2-pyridyl, 3-pyridyl, or 4-sulfonamidophenyl groups exhibit CDK5/p25 IC₅₀s in the 10 μM range (compound 28c, 28l, 28m). We subsequently fixed the hydrazone moiety and explored the diversity of different

pyrrolo- or indolo-azepinones (Figure 3, row 2). Indole analogs show at least a 10-fold improvement in activity compared to their pyrrole counterparts. In addition, introducing a halogen at the 7-position of the indole significantly enhances the potency of derivatives against CDK5/p25 and GSK3β (compound 28t). We prepared additional hydrazone derivatives in the context of a 5-chloro or fluoro indoloazepinone skeleton (Figure 3,

CDK5/p25	10	11b	28a	28b	28c	28d	28e	28k	28l	28m	28j
GSK3β	0.056	8.15	>100	82.4	10.4	3.80	12.6	1.88	3.42	12.1	22.5
CDK1/cyclin B	0.53	8.86	ND	>33	4.28	16.6	11.6	5.97	22.3	29.7	4.66
CDK5/p25	10.4	23.2	6.91	0.757	0.081	0.125	0.437	0.741	28i	28i	28i
GSK3β	4.28	9.13	6.99	2.62	0.171	0.399	0.741	0.741	22.5	22.5	22.5
CDK1/cyclin B	ND	ND	ND	ND	1.3	1.0	ND	ND	5.95	5.95	5.95
CDK5/p25	28c	28q	28s	28f	28t	28g	28h	28i	28n	28ad	
GSK3β	10.4	23.2	6.91	0.757	0.081	0.125	0.437	0.741	0.059	0.081	
CDK1/cyclin B	ND	ND	ND	ND	1.0	1.0	ND	ND	1.0	1.02	
CDK5/p25	28g	28ab	28ag	28ah	28p	28y	28aa	28ai	28o	28n	28ad
GSK3β	0.125	0.179	0.869	0.118	0.032	5.31	0.050	0.050	0.433	0.059	0.081
CDK1/cyclin B	1.0	1.40	2.30	0.984	0.790	2.01	0.147	1.43	4.61	1.81	1.02
CDK5/p25	28t	28ac	28af	28w	28x	28z	28v	28ae			
GSK3β	0.081	0.075	0.437	0.012	2.93	0.012	0.063	0.177			
CDK1/cyclin B	1.3	0.613	2.24	0.557	9.59	0.088	3.45	1.99			

Figure 3. HMD Analogs with the Replacement of the Glycohydrazide Ring and Their IC_{50} s in μ M against CDK5/p25, GSK3 β , and CDK1/cyclin B

Assays were conducted at 1.5 μ M ATP for CDK5/p25 and GSK3 β (for SPA procedure, see Experimental Procedures) and 15 μ M for CDK1/cyclin B [45]. Dibromopyrrolo derivatives (row 1); variations to the dibromopyrrolo of the 2-pyridylhydrazones (row 2); and hydrazone variations to 5-chloro and fluoro azepinone skeletons (rows 3 and 4) (ND: not determined).

rows 3 and 4) and observed optimal CDK5/p25 inhibitory activity for 7-fluoro indole analogs with pyridyl or 4-sulfonamidophenyl hydrazone substituents (compound **28t**, **28w**, and **28v**). Pyridin-3-yl-hydrazine derivatives are more potent than the corresponding pyridin-2-yl-hydrazine and pyridin-4-yl-hydrazine ones (compound **28p** versus **28g** and **28o**). Compound **28w** and **28z** even exhibit lower IC_{50} values against CDK5/p25 than HMD. The activity of hydrazone analogs against CDK5/p25 demonstrates that the glycohydrazide ring can be replaced with other heterocycles. Attempts to create hydrazones possessing both the pyridyl and sulfonamide functionality result in no further improvement in enzyme inhibition, indicating that the interactions introduced by this functionality could not be exploited synergistically.

Most of the hydrazones obtained show much reduced potency against GSK3 β compared to the close structural analogs of HMD. This result demonstrates that a favorable selectivity toward CDK5/p25 against GSK3 β can be achieved. For example, hydrazone **28w** is 3-fold more potent than HMD against CDK5/p25 (IC_{50} 12 nM) but eight times less potent against GSK3 β (IC_{50} 557 nM) relative to HMD. As another example, hydrazone **28v**, with 4-sulfonamidophenyl group, has an IC_{50} value twice

that of HMD against CDK5/p25, but 50 times that of HMD against GSK3 β .

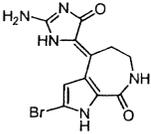
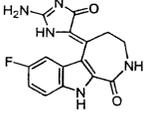
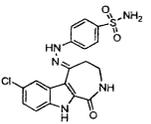
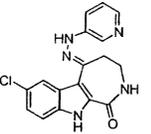
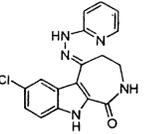
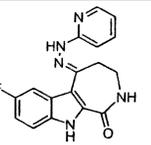
Kinase Selectivity of HMD and Selected Analogs

To investigate how these scaffold modifications alter the specificity against a broader range of kinases, six compounds including HMD were tested against 20 different kinases at a concentration of 10 μ M (Table 3). All six compounds exhibited potent activity against CDK1, and most also inhibit c-src, with the exception of sulfonamide **28n**. In general, most analogs are more selective than HMD, while the in vitro inhibitory profile of the 7-fluoroindole analog **27c** is the closest to HMD. Sulfonamide **28n** is the most selective compound among the six tested, with >95% inhibition of CDK1 and <45% inhibition of the rest of the kinases. Compounds containing amide replacement of the glycohydrazide ring (**31** and **34**) demonstrated very weak activities against CDK5/p25 and GSK3 β . Potential targets of this series are still under investigation.

Docking Results

To study in detail the essential binding interaction of HMD analogs with CDK5, we performed docking studies of the inhibitors using GOLD v2.0 [69]. The CDK5 structure used for docking studies was taken from the crystal

Table 3. 20 Enzyme Inhibition Pattern for HMD and Selected Analogs: Percent Remaining Enzyme Activity at 10 μ M Inhibitor Concentration

						
	1	27c	28n	28p	28g	28t
c-Abl	12	36	62	40	9	14
HER-1	60	81	96	64	60	69
HER-2	52	53	76	44	57	74
KDR	7	30	84	73	52	65
Flt-3	16	37	57	41	28	25
IGF-1R	81	81	70	44	18	24
c-Raf-1	84	77	74	36	53	54
PDGFR- β	16	35	79	3	32	42
Flt-4	36	56	74	78	33	33
c-Kit	4	14	67	53	21	31
Flt-1	21	49	69	72	15	21
Tek	25	61	73	30	27	19
PKA	56	61	73	76	69	62
c-src	9	11	56	22	8	10
CDK1	1	1	5	3	12	2
PKB	47	52	60	49	50	52
PDK1	100	100	100	81	89	100
Ins-R	68	81	73	52	38	46
FGFR-1	18	11	74	70	71	82
c-Met	41	26	93	43	49	88

The results are presented as kinase activity as a percentage of that in control incubations. ATP was present at 10 μ M in all assays. Assays were conducted according to the procedures in [64] and [65].

structure of CDK5 in complex with p25 (PDB code 1H4L) [70]. Water and p25 were extracted from the cocomplex structure and hydrogen atoms were added to the CDK5 protein. There are four residues missing in the glycine-rich loop of CDK5 structure. Since these four residues are not important to the binding of HMD, they were not included in the docking. All atom types and charges were assigned in GOLD, and all docking parameters were taken from the standard default settings. Although the CDK2 in the CDK2-HMD complex is in an inactive conformation while CDK5 in the CDK5-p25 complex is in an active conformation, the binding pockets of HMD in CDK2 and CDK5 are structurally very similar. After superposition of 17 residues in the ATP binding pocket within 5 Å of HMD, the rmsd of C_{α} atoms from CDK2 and CDK5 is only 0.6 Å. We compared the model of HMD bound to active CDK5 with the crystal structure of inactive CDK2 in complex with HMD and found the interaction of HMD with protein in both structures were very similar. Experimentally, crystal structures of indirubin bound to active and inactive CDK2 also showed that a kinase inhibitor bound to active and inactive CDK2 with a similar binding mode [71].

We noticed that for the close structural analogs of HMD (Table 2), indole derivatives **27** exhibit reduced CDK5 versus GSK3 β activity relative to the corresponding pyrrole analogs. One possible explanation for this differing kinase inhibitory profile of pyrrole and indole compounds is the steric interaction between the 4-H of the indole ring and the glycoyamidine amide. While the tricyclic ring system of HMD appears to be coplanar when HMD is bound to CDK2 [45], the steric repulsion

prevents the glycoyamidine-indole analogs from achieving the perfect geometry for binding to CDK2 (see Supplemental Figure S2 at <http://www.chembiol.com/cgi/content/full/11/2/247/DC1>).

In addition to the three commonly observed hydrogen bonds between the pyrrolo- or indolo-azepinones and the backbone Glu81 and Cys83 (Leu83 in the case of CDK2-HMD complex) of the enzyme, the modeling of hydrazone analogs **28v**, **28t**, and **28w** suggested possible hydrogen bonds between the heterocyclic ring appendage and the enzyme (Figures 4C–4E). HMD may form a hydrogen bond between the amino group of the glycoyamidine and the side chain of Asp144, as well as a water-mediated hydrogen bond between the amino group of the glycoyamidine and the main chain amide of Gln130. The pyridyl groups of **28t** and **28w** may form electrostatic interactions with the Asp144 carboxylate. In addition, Gln130 is within 5 Å of the hydrazone proton, making possible a water-mediated hydrogen bond. In order to fit into the ATP pocket, sulfonamide **28v** may adopt a different geometry of the hydrazone double bond from pyridyl compounds **28t** and **28w**. It may be capable of forming a hydrogen bond between the sulfonamide and the Asp144 side chain, as well as a hydrogen bond between the oxygen of the $-SO_2-$ and the Tyr11 NH. The different hydrogen bonding mode could be responsible for compound **28v**'s different kinase selectivity profile. Docking **28t** or **28w** with a different double bond geometry leads to less favorable inhibitor-kinase interactions. As the orientation of heterocyclic appendage of the hydrazones is quite different from that of HMD, we propose that the 10-fold improved potency

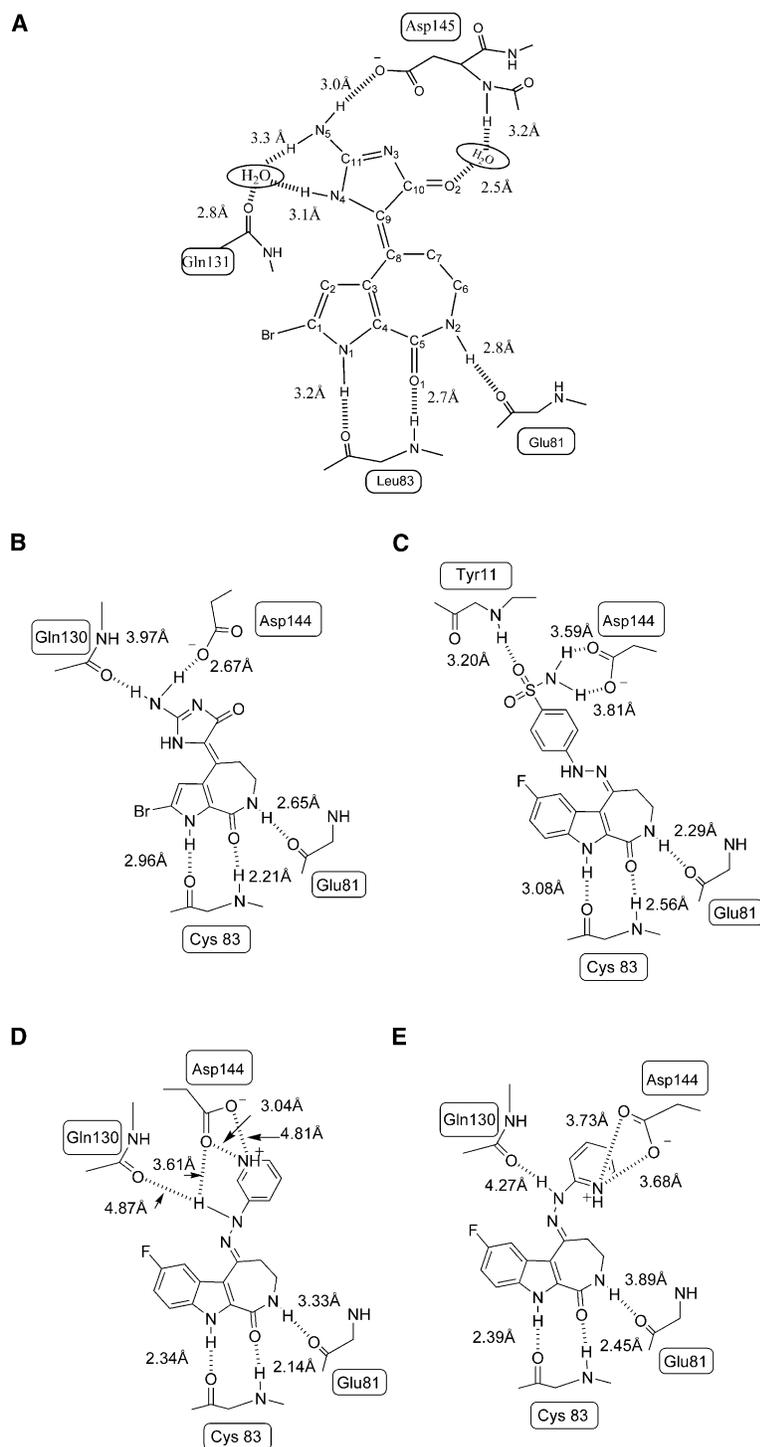


Figure 4. Illustration of Possible Interactions between HMD or Its Analogs with CDK2 or CDK5/p25

Hydrogen bonds and electrostatic interactions are indicated by broken lines.

(A) Cartoon illustration of key hydrogen bonds in HMD and CDK2 complex, reproduced according to [45].

(B) HMD and CDK5/p25.

(C) Hydrazone 28v and CDK5/p25.

(D) Hydrazone 28w and CDK5/p25.

(E) Hydrazone 28t and CDK5/p25.

against CDK5/p25 and GSK3 β of the hydrazone indoles (e.g., compound 28w, Figure 3) versus the glycohydrazide indoles (e.g., 27c, Table 2) results from a more optimal orientation of the hydrazone heterocycle relative to the indole core to relieve strain from the indole 4-H position.

Cellular Effects and Cell Cycle Studies

To assess the efficacy of these compounds as CDK inhibitors in a cellular context, we treated 786-O renal

adenocarcinoma cells with selected compounds and characterized cell proliferation and DNA content redistributions in the population using fluorescence microscopy. The majority of unsynchronized 786-O cells growing in serum are in G1 phase (53.1% \pm 1.6%), with 16.1% \pm 0.9% and 30.8% \pm 1.6% in S and G2/M phases, respectively (see Supplemental Figure S3 and Table S2 at <http://www.chembiol.com/cgi/content/full/11/2/247/DC1>). Inhibition of CDK1/cyclin activity results in G2/M

arrest [3]. Although HMD is an effective inhibitor of CDK1/cyclin B activity in vitro ($IC_{50} = 70$ nM), its cell cycle progression inhibitory effect in 786-O cells is modest. The lowest concentration of HMD tested that results in significant (>40%) G2/M arrest is 100 μ M (Supplemental Table S2). While this discrepancy between enzymatic and cellular activity is large, it is generally consistent with an earlier study that shows HMD inhibition of GSK3 activity at 50 μ M in cells [45].

HMD analogs that were most similar to HMD in structure similarly showed limited cellular activity. Compounds 10, 15, 27a, 27b, 27c, 27d, and 27g had no effect on the percentage of cells in G2/M phase up to 30 μ M and no growth inhibition phenotype up to 15 μ M. A key structural feature that these compounds share with HMD is the highly polar glycohydrazide ring, which may limit the compounds' cell permeability. Other compounds tested that were inactive included hydrazones 28c, 28e, 28l, 28n, and 28v. These compounds either had low CDK inhibitory activity (28c and 28l) or sulfonamide groups that seemed to inhibit cellular activity (28e, 28n, and 28v).

The compounds with the highest inhibition of cell growth were indole-hydrazone analogs. Compounds 28p, 28w, and 28z (Figure 3, rows 3 and 4) arrested cells in G2/M phase at concentrations as low as 3.8 μ M (Supplemental Figure S3C and Table S2 at <http://www.chembiol.com/cgi/content/full/11/2/247/DC1>). Compounds 28t, 28g, and 28ai did not alter the percentage of cells in G2/M phase but significantly inhibited cell proliferation. These compounds may have a more complex kinase inhibitory profile (inhibiting targets that result in G1 arrest in addition to CDK1/cyclin A) or act by other mechanisms that cause cell death.

Significance

Several protein kinase inhibitors have advanced to clinical trials based on the indolocarbazole nucleus of Staurosporine (PKC412, Novartis; CEP-701, Cephalon; LY-333531, Eli Lilly). The pyrrole[2,3-c]azipine scaffold of HMD also exhibits great potential for development as a therapeutically relevant kinase inhibitor scaffold. The present work facilitates this goal by demonstrating that analogs of HMD can be prepared by a variety of synthetic routes and that these compounds possess altered potencies and selectivities toward a variety of therapeutically relevant kinases.

Experimental Procedures

Preparation and Use of Affinity Reagents

Affi-Gel 10 Gel (Bio-Rad Laboratory) (1 ml bed volume) was rinsed with anhydrous DMSO (4 ml \times 3) and transferred into a vial containing a solution of linker tethered compound 4 or 7 in DMSO (3 μ M, 1 ml). The mixture was agitated at room temperature for 1 day in the presence of triethylamine (the progress of the coupling reaction was monitored by LC-MS). Upon the completion of the coupling, excess amount of ethanolamine (8 μ l) was added to block any unreacted groups that remain on the resin. The resulting beads were washed with DMSO (4 ml \times 3) and PBS (4 ml \times 3) and stored in PBS (1 ml) at 4°C until use.

Extraction of Proteins

Mouse brains were homogenized in lysis buffer (25 mM MOPS, 15 mM EGTA, 15 mM MgCl₂, 2 mM DTT, 1 mM sodium orthovanadate,

1 mM NaF, 1 mM phenyl phosphate, 15 mM nitrophenyl phosphate, 60 mM β -glycerophosphate, 100 mM benzamidine, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin [pH 7.2]) using tissue grinders (Kontes). The resulting homogenate was centrifuged for 30 min at 20,000 \times g and 4°C. The supernatant was recovered and stored at -80°C until used.

Affinity Chromatography

Affinity chromatography was performed as described in [54]. Affinity matrices (30 μ l) were washed with bead buffer (50 mM Tris HCl, 5 mM EDTA, 5 mM EGTA, 5 mM NaF, 250 mM NaCl, 0.1% Nonidet P-40, 100 mM benzamidine, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin [pH 7.4]) twice before use and resuspended in 400 μ l of bead buffer. Proteins (200 μ g) in 400 μ l of lysis buffer were added and incubated with rotation for 1 hr at 4°C. The resins were then washed 4 times with bead buffer and bound proteins were eluted by heating at 95°C for 3 min in the presence of 2 \times Lamli sample buffer. The resulting supernatants were loaded onto SDS-PAGE gels, and the separated proteins were visualized by silver staining or Western blot analysis. To identify proteins by peptide mass fingerprinting, proteins (1 mg) were loaded onto the same amount of resins and eluted proteins were stained by Coomassie brilliant blue G (Bio-Rad Laboratories).

Silver Staining

Silver staining was performed as described in [70]. Gels were fixed in 50% methanol/5% acetic acid for 20 min then washed with water for 1 hr. They were sensitized by incubating in 0.02% (w/v) Na₂S₂O₃ for 1 min and then washed twice with water for 1 min before being incubated in 0.1% (w/v) AgNO₃ for 20 min. After being washed again twice with water for 1 min, gels were developed in a solution containing 0.04% (v/v) formaldehyde and 2% (w/v) Na₂CO₃. The reaction was terminated by discarding the reagent followed by addition of 5% acetic acid in water.

Protein In-Gel Digestion

In-gel digestion was performed according to the published protocol [72] with minor modifications. Protein bands in Coomassie-stained gel were excised and rinsed with water for 15 min. They were then incubated in 100 mM NH₄HCO₃ (100 μ l) for 15 min. The same volume of CH₃CN was added and incubated for 15 min. After the supernatant was removed, protein bands were shrunk by dehydration in CH₃CN. CH₃CN was removed by vacuum centrifuge and gel pieces were swollen in a digestion solution containing 50 mM NH₄HCO₃ and 12.5 ng/ μ l of modified porcine trypsin (Promega) in an ice-cold bath. The supernatant was removed 45 min later and replaced with 50 mM NH₄HCO₃ (15 μ l) to keep gel pieces wet during the overnight enzymatic digestion at 37°C.

Sample Preparation for MS Analysis and Peptide Mass Fingerprinting Using MALDI-TOF MS

Tryptic digests were introduced to microscale purification [73], which allowed efficient concentration of peptides and cleanup of analytes. A micro purification column was prepared using a Gel-oader tip (Eppendorf, Germany), in which Poros 50 R2 (PerSeptive Biosystems) was packed and equilibrated. Samples were loaded onto the column and briefly washed with formic acid (5%, 15 μ l). Peptides bound to the resin were eluted directly onto a MALDI target with 1 μ l of saturated α -cyano-4-hydroxy-*trans*-cinnamic acid in acetonitrile-formic acid-water (50:5:45) solution.

The MALDI-TOF mass spectrometer (BIFLEX; Bruker Daltonics, Germany) was operated in the reflector mode. Ion signals produced by trypsin autodigestion peptides were used as internal mass calibrants. Proteins were identified by searching against NCBI human nonredundant (nr) and SwissProt databases with the list of mono-isotopic tryptic peptide masses using 200 ppm mass tolerance. Database searching was performed using a web-based search program, PROFOUND (http://prowl.rockefeller.edu/profound_bin/webProFound.exe) developed by Rockefeller University and Proteomatrix [74].

Radioenzymatic RSK1 Kinase Assay

The RSK1 (p90S6K) kinase inhibitory activity of HMD and HMD-linker was evaluated following manufacturer's (Upstate) recommendations using the peptide KKLNRTLVA as a substrate.

In Vitro Kinase Assay Using SPA Assay

SPA assay [75] was performed using SPA assay kit (Amersham Pharmacia Biotech, UK). Inhibitors dissolved in DMSO were transferred to 5 μ l of enzyme in assay buffer (50 mM MOPS [pH 7.2] and 5 mM MgCl₂). Enzymatic reactions were initiated by adding 10 μ l of solution containing 1.5 μ M ATP, 1.5 μ M biotinylated substrate, 0.01 μ Ci [γ -³³P]ATP (NEN Life Science) and incubated at room temperature for 1 hr in 384-well ProxiPlates (Perkin Elmer). Reactions were stopped by the addition of 10 μ l PBS solution containing 50 μ g of streptavidin-tagged polyvinyltoluene bead, 50 mM ATP, 5 mM EDTA, and 0.1% Triton X-100. Before counting the signals with TopCount (Packard Bioscience), the plates were spun for 3 min at 2000 rpm. For GSK3 β , sequence of the peptide substrate was biotin-YRR AAVPPSPSLSRHSSPHQ(pS)EDEEE (Upstate). In CDK5/p25 assay, the peptide was biotin-aminohexanoic acid-AGAKKAVKTPKAKKP derived from Histone H1.

Cell Cycle Studies

786-O renal adenocarcinoma cells were cultured in DMEM with 10% fetal bovine serum supplemented with glutamine and antibiotics. Cells were plated in black clear bottom 384-well microtiter plates (Greiner) and cultured for 8 hr. Compounds of interest were added to cells (<1% DMSO final concentration) and incubated for 30 hr at 37°C, with four replicate wells for each concentration. After washing with PBS, cells were fixed with 3.5% paraformaldehyde in PBS for 20 min. Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (200 ng/ml) in 0.1% TritonX-100/PBS for 1 hr. After washing with PBS, cells were imaged on an EIDAQ100 (Q3DM) automated inverted fluorescence microscope (Nikon TE300 with a Cohu video camera) with a 10 \times /0.5 objective (Nikon). Sixteen images were collected per well. Image analysis was performed with CytoShop software (Q3DM). After images were shade corrected and background subtracted, objects were extracted and single cells defined using geometric and total fluorescence parameters. Histograms plotting number of events versus total nuclear intensity for representative wells were used to define nuclear fluorescence boundaries for G1, S, and G2/M cells. Percentages of cells in each category were determined for all wells and replicate wells were averaged for each condition.

Supplemental Data

General synthetic methods and spectroscopic data for all compounds included in the main text, as well as cell proliferation and DNA content redistributions in the population of 786-O renal adenocarcinoma cells after treatment with selected HMD analogs, are included in the Supplemental Data at <http://www.neuron.org/cgi/content/full/11/2/247/DC1>.

Acknowledgments

We are grateful to Dr. Priscilla Yang and Dr. Tetsuo Uno for revising this article and providing valuable suggestions.

Received: September 19, 2003

Revised: November 18, 2003

Accepted: November 24, 2003

Published: February 20, 2004

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