ACS Medicinal Chemistry Letters

LETTER

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Host-Directed Inhibitors of Myxoviruses: Synthesis and in Vitro Biochemical Evaluation

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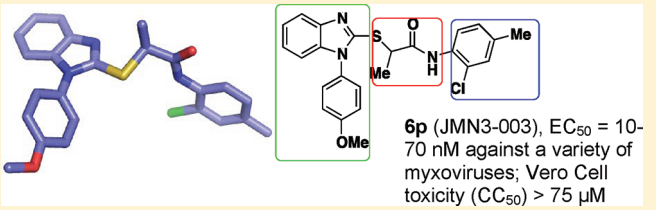
Supporting Information

ABSTRACT: Drugs targeted to viral proteins are highly vulnerable to the development of viral resistance. One little explored approach to the treatment of viral diseases is the development of agents that target host factors required for virus replication. Myxoviruses are predominantly associated with acute disease and, thus, ideally suited for this approach since the necessary treatment time is anticipated to be limited. High-throughput screening previously identified benzimidazole 22407448 with broad antiviral activity against different influenza virus and paramyxovirus strains. Hit to lead chemistry has generated **6p** (JMN3-003) with potent antiviral activity against a panel of myxovirus family members exhibiting EC_{50} values in the low nanomolar range.



KEYWORDS: Host-directed, non-nucleoside, small molecule inhibitor, influenza virus, myxovirus, benzimidazole

Myxoviruses are responsible for the majority of human morbidity and mortality cases due to viral respiratory illness globally.[1] Influenza virus is the leading cause of these events in North America, although vaccine prophylaxis is widely available. Despite extensive research, no vaccines currently exist for several major pathogens of the paramyxovirus family such as respiratory syncytial virus (RSV) or different human parainfluenza viruses (HPIVs). Ribavirin (RBV) is a synthetic nucleoside analogue with broad-spectrum antiviral activity. Although RBV is approved for the treatment of hepatitis C virus, RSV, and Lassa fever virus infections, its efficacy is limited, and the drug is compromised by several side effects.[2−5] Previously, we utilized high-throughput screening (HTS) to identify small molecule inhibitors against measles virus (MeV) RNA-dependent RNA polymerase (RdRp) activity.[6−8] However, viral adaptation has demonstrated that robust resistance to inhibition by these compounds can originate from single point mutations in the targeted viral protein.[9,10] In an attempt to counteract viral escape from inhibition, we have explored targeting host factors required for viral replication rather than viral proteins directly. Anticipated advantages of this strategy include a decreased frequency of viral escape from inhibition and a broadened pathogen target spectrum. HTS in combination with counterscreening for a broadened viral target spectrum that extends to other pathogens of the myxovirus families has identified several antiviral hits that likely target host cells.[11] This approach yielded several benzimidazoles of the same class [22407448 (BM-1),[12] 22404943, and 22407466] as well-behaved inhibitors of MeV. Potencies (EC_{50}) against MeV are 0.2, 0.7 and 2.1 μM, respectively (Figure 1). Hit BM-1 in particular is unusual since it shows broad-spectrum antiviral action against various paramyxoviruses in the low micromolar to nanomolar range.[11]

To confirm the activity, BM-1 was resynthesized and reassayed. For hit confirmation, dose−response curves were generated. Synthesized BM-1 revealed behavior identical to the original library member against CDV, HPIV3, and MeV. In parallel, MTT assays were employed to determine compound-induced cytotoxicity in the absence of viral infection. Synthesis was initiated by coupling of 1-fluoro-2-nitrobenzene **1** with *p*-anisidine **2** in the presence of potassium carbonate to provide *N*-(4-methoxyphenyl)-2-nitroaniline **3**. Reduction of **3** gave diamine **4**, which was treated with 1,1-thiocarbonyldiimidazole in dichloromethane to afford 1-(4-methoxyphenyl)-1*H*-benzimidazole-2-thiol **5**. The 2-thio-imidazole **5** was transformed to its potassium salt and coupled with 2-bromo-*N*-(3,5-dichloropyridin-2-yl)propanamide to give BM-1. The approach has been

Received: May 24, 2011
Accepted: August 23, 2011
Published: August 23, 2011

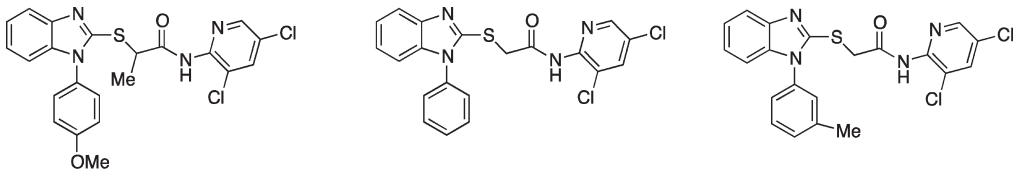
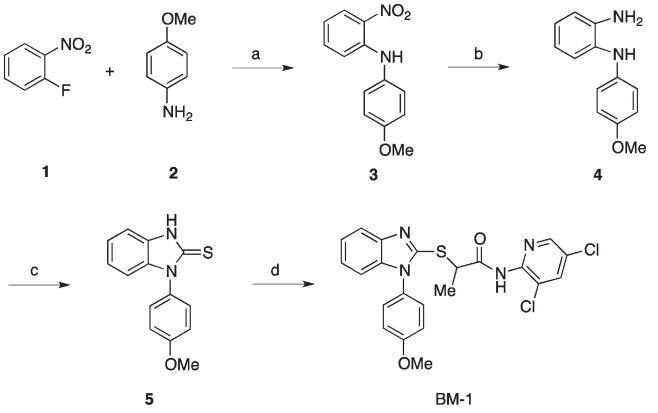


Figure 1. Anti-MeV hits identified by high-throughput screening.

Scheme 1. Synthesis of Screening Hit BM-1[a]



[a] Reagents: (a) K_2CO_3, 160 °C, 5 h. (b) Pd/C (10%), H_2 (50 psi), MeOH, 2 h. (c) 1,1-Thiocarbonyldiimidazole, CH_2Cl_2, room temperature, 4 h. (d) KOH, EtOH, reflux, 2 h, and then 2-bromo-*N*-(3,5-dichloropyridin-2-yl)propanamide **7**.

used for the synthesis of a set of BM-1 derivatives, which are described below (Scheme 1).

A structure−activity profile has begun to emerge by examination of the three molecular fragments circumscribed in Figure 2, namely, the benzimidazole (A), the α-thio-amide linker (B) and the substituted pyridine ring (C).

The first stage of hit optimization focused on introducing a variety of aromatic rings as pyridine replacements, since modification of the C sector is achieved in a straightforward fashion by employing the same synthetic methodology utilized for the preparation of BM-1 (Scheme 1). A small and compact library of 30−50 analogues was obtained by utilizing different α-halide amides for the final coupling step. Substituted pyridines, pyrazoles, triazines, thiazoles and other functionalities were used instead of **7**. A second group of analogues was prepared by employing alternative anilines in sector A. The *para*-methoxy group was replaced by hydrogen, ethoxy, fluoro and hydroxyl, among others. Sector C analogues with chloro, methyl and trifluoromethyl substituents show activities very similar to BM-1 (**6a** and **6d**, Table 1). The corresponding ethoxy analog delivers slightly better activity (entry **6c**), while pyrazole and isoxazole replacements furnish 2-fold reduced potency (entries **6j** and **6n**). The fluoro-analogue is virtually equipotent to BM-1 (entry **6b**). The thiazole functionality reduces activity by 10-fold (entry **6m**), while triazines are significantly weaker still (entries **6i** and **6k**). However, most of the active compounds listed in Table 1 are toxic in the Trypan blue exclusion assay with CC_{50} values of 1−10 μM.

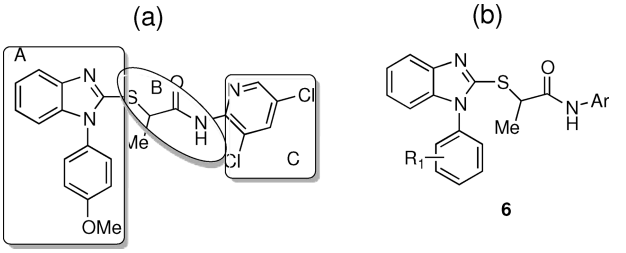


Figure 2. (a) Structure−activity modification strategy for hit compound BM-1; (b) modification of A and C sectors of BM-1.

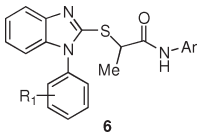
The *p*-ethoxy analogue is the exception with reduced toxicity at 20 μM (entry **6c**). Encouragingly, **6p** (JMN3-003) with a substituted phenyl in sector C demonstrated strong antiviral activity (EC_{50} = 170 nM) against MeV and cell cytotoxicity over 75 μM (entry **6p**, Table 1). As outlined in the context of the recently reported detailed molecular characterization of JMN3-003, antiviral activities were evaluated using actively dividing cells, while CC_{50} values were determined for confluent cell populations, thus reflecting acute toxicity. Independent assessment of cell proliferation revealed a cytostatic effect for JMN3-003. This was found, however, not to be the basis for the antiviral effect.[13] To understand the relationship between chirality and potency, R- and S-isomers of **6p** were isolated by chiral HPLC. Both isomers were subjected to the MeV inhibition assay. Potency of the S-isomer was essentially identical to the racemic mixture **6p**, while the antiviral capacity of the R-isomer was slightly reduced (Table 1). A newly developed methodology for the asymmetric synthesis of both isomers will be reported elsewhere.

For part A of hit modification, we initially tried to replace the fused benzene of the benzoimidazole with pyridine and functionalize the benzene moiety by substitution with Me, Br or COOEt. Unfortunately, most of the analogues experienced either significant reduction or complete loss of activity. Thus, we did not pursue this series further.

For replacement of the 2-thioacetamide linker, five different variations of the central tether with an equivalent number of chain atoms were prepared. Compound designations **11**−**15** (Schemes 2−4) and the corresponding activities are recorded in Table 2, illustrating a significant reduction or loss of activity relative to BM-1. Systematic structural modification revealed that substitution of benzyl for phenyl at the benzimidazole *N*1 position delivers analogues with similar anti-MeV viral activity (Figure 2, sector A). Benzyl derivatives **20** and **21** deliver fairly good potency with EC_{50} at 0.5 and 0.4 μM, respectively (Table 2). Thus, both derivatives **11** and **13** bear a benzyl group instead of the *p*-methoxy phenyl group as shown

dx.doi.org/10.1021/ml200125r |*ACS Med. Chem. Lett.* 2011, 2, 798–803

Table 1. Antiviral Activity and Cytotoxicity of Various Substituted Anilides 6



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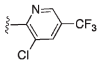
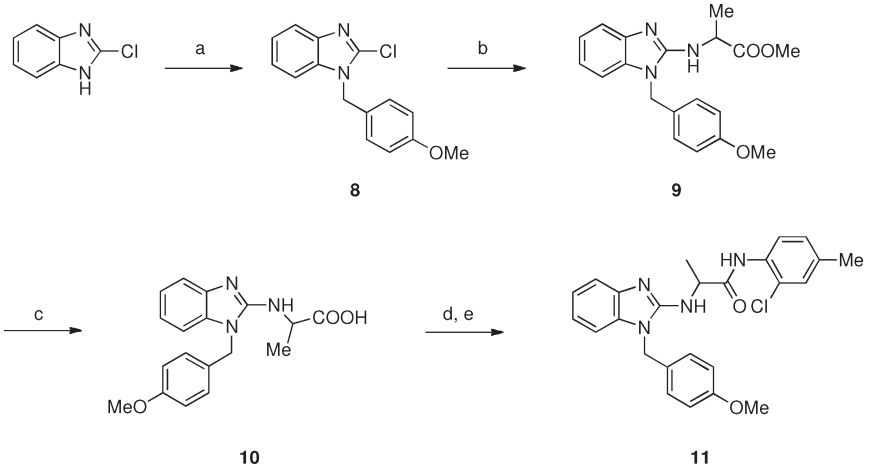
Entry	Comp.	R_1	Ar	EC_{50} ± SEM (μM)[a] (MeV-Alaska) CPE inhibition	CC_{50} (μM)[b] (Vero cells)
hit	BM-1	*p*-OMe		0.35±0.03	>75
6a	AS92	*p*-OMe		0.4±0.05	9.2-9.8
6b	AS93	*p*-F		0.1±0.00	5.9-6.2
6c	AS94	*p*-OEt		0.1±0.01	18.5-22.2
6d	AS102	*p*-OMe		0.2±0.01	7.25-7.8
6e	AS80b	*p*-OMe		>75	ND[c]
6f	AS103b	*p*-OMe		0.5±0.05	9.7-11.9
6g	AS103a	*p*-OMe		0.05±0.00	0.88-0.92
6h	AS109	*p*-OEt		1.2±0.22	ND[c]
6i	AS112	*p*-F		17.4±6.11	ND[c]
6j	AS114	*p*-OMe		0.5±0.09	75
6k	AS115a	*p*-OMe		9.3±2.65	ND[c]
6l	AS120	*p*-OMe		>75	ND[c]
6m	JMN2-173	*p*-OMe		2.8±0.17	ND[c]
6n	JMN2-183	*p*-OMe		0.4±0.01	>75
6o	AS86	*p*-OMe		1.4±0.11	ND[c]
6p	JMN3-003	*p*-OMe		0.2±0.00	>75
(S)-6p	(s)-JMN3-003	*p*-OMe	—	0.3±0.07	>75
(R)-6p	(r)-JMN3-003	*p*-OMe	—	0.2±0.03	>75

[a] 50% inhibitory concentrations were calculated using the variable slope (four parameters) nonlinear regression-fitting algorithm embedded in the Prism 5 software package (GraphPad Software). Values represent averages of four experiments ± SEMs (standard errors of the mean); highest concentration assessed, 75 μM. [b] CC_{50} values represent range of two experiments; highest concentration assessed, 75 μM. [c] CC_{50} not determined (ND) when EC_{50} > 1.0 μM.

in Table 2. Compound **11**, incorporating NH in the central linker, introduces additional hydrogen bond capacity and a new pK_a center, while **12**, **13**, **14** and **15** (CH_2, O, SO and SO_2, respectively) sustain somewhat different linker geometries by comparison with the sulfur-containing tether of BM-1. The importance of sulfur versus other atoms was also observed during discovery of the HIV reverse transcriptase inhibitor RDEA-806,[14,15] which shares the central 2-thioacetamide linker with BM-1 and has achieved success in clinical trials (Figure 3). Obviously, a sulfur atom in the linker is essential. However, its precise role at the binding site remains to be fully defined. Comparative conformational

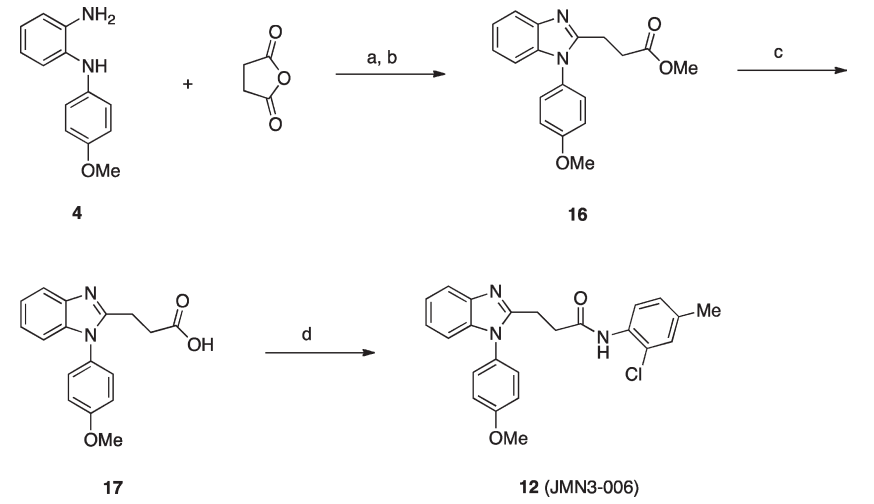
dx.doi.org/10.1021/ml200125r |ACS Med. Chem. Lett. 2011, 2, 798–803

Scheme 2. Synthesis of 2-Aminobenzimidazole[a]



[a] Reagents and conditions: (a) *p*-Methoxybenzyl bromide, K_2CO_3, CH_3CN, reflux. (b) DL-Alanine methyl ester, K_2CO_3, CH_3CN, 165 °C, microwave, 1 h. (c) HCl (conc.)/H_2O, reflux, 2 h. (d) Oxalyl chloride (2.0 equiv of 2.0 M in CH_2Cl_2), CH_2Cl_2, DMF (cat.). (e) 2-Chloro-4-methylaniline, 4-(dimethylamino)pyridine, (DMAP) (cat.), pyridine.

Scheme 3. Synthesis of the Carbon Analogue of 6p[a]



[a] Reagents and conditions: (a) CH_2Cl_2, 40 °C. (b) HCl (4 N), MeOH, 100 °C. (c) LiOH, THF/H_2O. (d) ^{i}PrOCOCl, 4-methylmorpholine, 2-chloro-4-methylaniline, CH_2Cl_2/DMF.

searches for otherwise identical analogues with O, NH and CH_2 replacements for S suggest that sulfur is possibly unique in providing an energetically accessible bioactive conformation.[16]

Analogue **11** was prepared as outlined in Scheme 2. 2-Chloro-benzimidazole was treated with *p*-methoxybenzyl bromide in the presence of potassium carbonate to provide chloro-benzimidazole **8**. The latter was coupled with racemic alanine methyl ester in the microwave at 165 °C for 1 h to afford 2-aminoimidazole **9**, which was hydrolyzed to the corresponding acid **10**. The latter was treated with oxalyl chloride followed by coupling with 2-chloro-4-methylaniline to furnish the final product **11** (Scheme 2).

The synthesis of carbon analogue **12** proceeded from reaction of diamine **4** with succinic anhydride to give the benzimidazole **16**. Hydrolysis of **16** under basic conditions with lithium hydroxide (LiOH) in a mixture of THF and water delivered acid **17**, which was further coupled with 2-chloro-4-methylaniline in the presence of isopropyl chloroformate and 4-methylmorpholine to afford **12** (Scheme 3).

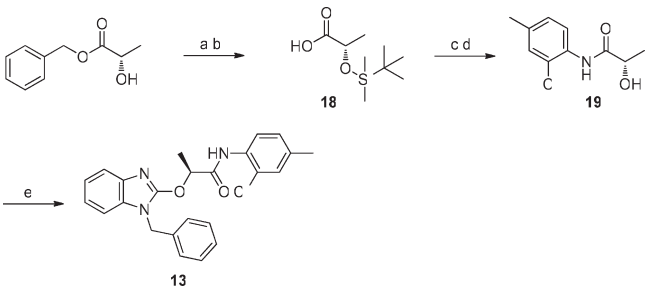
The synthesis of the oxygen analogue **13** commenced with the protection of benzyl-L-lactate as a silyl ether, which on hydrogenolysis furnished acid **18**. BOP-mediated coupling of **18** with 2-chloro-4-methylaniline followed by cleavage of the silyl group furnished alcohol **19**. Treatment of **19** with NaH facilitated coupling with 1-benzyl-2-chloro-1*H*-benzimidazole to afford **13** (Scheme 4).

dx.doi.org/10.1021/ml200125r |*ACS Med. Chem. Lett.* 2011, 2, 798–803

Sulfoxide **14** and sulfone **15** can be easily obtained by oxidation of **6p** with 1.0 and 2.0 equiv of 3-chloroperoxybenzoic acid (MCPBA), respectively. Benzene analogue **6p** has surfaced as the most promising candidate of this compound series with superb antiviral potency and low cytotoxicity. The compound shows potent activity against MeV and a selection of clinically significant members of the para- and orthomyxovirus families. We also compared the antimyxovirus activity of **6p** with the previously reported MeV RdRp inhibitor AS-136a. The latter shows high selectivity against MeV, while **6p** exhibits a broad range of antimyxovirus activities with EC_{50} values ranging from 10 to 70 nM in virus yield reduction assays depending on the target virus. Detailed biological evaluation and target examination have been reported elsewhere.[13]

In summary, HTS has identified several hits in the benzimidazole class with potent anti-MeV activities. Follow-up counterscreening assays uncovered BM-1 as a well-behaved inhibitor with the ability to block replication of a broad range of myxovirus family members. By optimization of BM-1, we have developed preliminary SAR within the three pharmacophoric sectors highlighted in Figure 2. A variety of structural modifications essentially abolish antiviral activity or result in high cytotoxicity. Particularly influential for the SAR of these agents are the structural constitutions of the amide substituents and the sulfur atom of the central tether. The most potent analogue **6p** was generated by replacing the pyridine ring in BM-1 with a substituted phenyl ring. The compound shows activity against MeV at 170 (viral CPE-reduction assay) and 30 nM (virus yield reduction assay) and does not display any detectable acute cytotoxicity. Compound **6p** was also evaluated for its antiviral activity against a selection of clinical-relevant paramyxovirus (RSV, MuV, and HPIV3) and orthomyxovirus (influenza) family members. The compound exhibits superb inhibitory activity against all of the viruses tested with EC_{50} values ranging from 10 to 70 nM in virus yield reduction assays.[13] These results demonstrate that **6p** has great potential as a lead for development of host-directed antiviral drugs. Pharmacokinetics evaluation, in vivo efficacy, and expansion of **6p** library are currently in progress.

Scheme 4. Synthesis of the Oxygen Analogue of 6p[a]



[a] Reagents and conditions: (a) *tert*-Butyldimethylsilyl chloride, imidazole, DMF. (b) H_2, Pd/C, MeOH. (c) Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), *i*-Pr_2NEt, 2-chloro-4-methylaniline, CH_2Cl_2. (d) Tetrabutylammonium fluoride (TBAF), THF. (e) NaH, 1-benzyl-2-chloro-1H-benzimidazole, DMF, 100 °C.

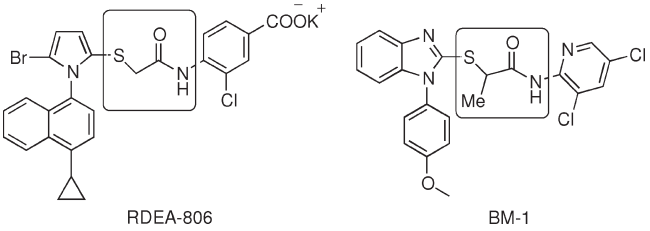
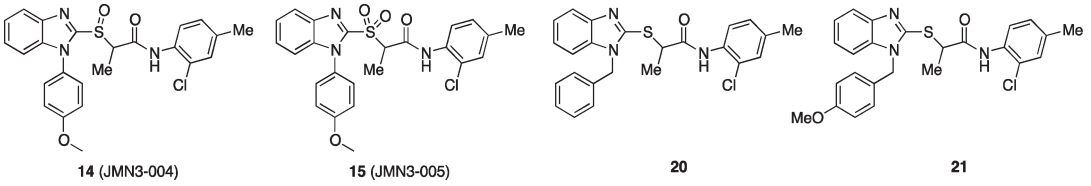


Figure 3. Structural comparison of RDEA-806 and BM-1.

Table 2. Anti-MeV EC_{50} Values for S-Atom Replacements



ID	compd	EC_{50} (μM)[a] (MeV-Alaska)	CC_{50} (μM)[b] (Vero cells)
11	AS-228	>150	ND[c]
12	JMN3-006	>150	ND
13	JMN8-096	>150	ND
14	JMN3-004	1.3 ± 0.06	>75
15	JMN3-005	>150	ND
20	JMN5-010	0.4 ± 0.06	>75
21	JMN4-023	0.7 ± 0.04	>75

[a] 50% inhibitory concentrations were calculated using the variable slope (four parameters) nonlinear regression-fitting algorithm embedded in the Prism 5 software package (GraphPad Software). Values represent averages of four experiments ± SEMs; highest concentration assessed, 75 μM. [b] Values represent averages of two experiments; highest concentration assessed, 75 μM. [c] CC_{50} not determined (ND) when EC_{50} > 150 μM.

dx.doi.org/10.1021/ml200125r |*ACS Med. Chem. Lett.* 2011, 2, 798–803

■ ASSOCIATED CONTENT

Supporting Information. Experimental details for the synthesis and characterization of **3**−**11**, **12**, **13**, **15**, **20** and **21** and experimental details for biology assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding Sources

We are grateful to Dr. Dennis Liotta for generous financial support and scientific discussions. This work was supported, in part, by Public Health Service Grants AI071002 and AI085328 (to R.K.P.) from the NIH/NIAID and by Public Health Service Grant HG003918-02 (to J.P.S.) from the NIH.

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(12) 22407448 is called BM-1 through the following parts of the paper for simplicity.

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(16) Conformational searches of the S, O, NH and CH_2 analogues were performed with the OPLS-2005 force field, and a putative binding model was compared with the global minimum in each case.