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Discovery of the First Potent and Selective Inhibitor of Centromere-Associated Protein E: GSK923295

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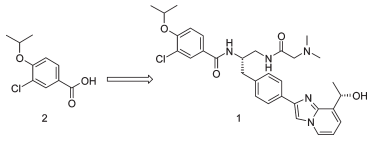
ABSTRACT Inhibition of mitotic kinesins represents a novel approach for the discovery of a new generation of anti-mitotic cancer chemotherapeutics. We report here the discovery of the first potent and selective inhibitor of centromere-associated protein E (CENP-E) 3-chloro-*N*-{(1*S*)-2-[(*N*,*N*-dimethylglycyl)amino]-1-[(4-{8-[(1*S*)-1-hydroxyethyl]imidazo[1,2-*a*]pyridin-2-yl}phenyl)methyl]ethyl}-4-[(1-methylethyl)oxy]benzamide (GSK923295; **1**), starting from a high-throughput screening hit, 3-chloro-4-isopropoxybenzoic acid **2**. Compound **1** has demonstrated broad antitumor activity *in vivo* and is currently in human clinical trials.

KEYWORDS CENP-E, inhibitor, GSK923295, mitotic kinesin



Kinesins are a superfamily of motor proteins that utilize the energy from ATP hydrolysis to transport cellular cargoes along microtubules.[1−3] Mitotic kinesins are a functional class of kinesins essential for mitotic spindle assembly and function during cell division.[4,5] Perturbation of mitotic kinesin activity results in cell cycle arrest in mitosis and subsequent cell death.[6] The past decade has seen an increased interest in the development of small-molecule inhibitors of mitotic kinesins as a new generation of anti-mitotic agents.[7−10] Since these agents specifically target dividing cells, mitotic kinesin inhibitors have the potential of capturing the therapeutic benefits of antimicrotubule (MT) agents, such as the taxanes, while minimizing toxicities on nondividing cells, thereby mitigating side effects such as peripheral neuropathies.[6−10] The most advanced mitotic kinesin inhibitors in clinical development target kinesin spindle protein (KSP or *Hs*Eg5), a mitotic kinesin required for spindle pole separation during prometaphase.[7−13] Centromere-associated protein E (CENP-E) is a mitotic kinesin directly involved in coupling the mechanics of mitosis with the mitotic checkpoint signaling machinery, regulating the cell-cycle transition from metaphase to anaphase.[14−17] During mitosis, CENP-E is localized to the region of mitotic chromosomes responsible for interaction with spindle microtubules and it is essential for prometaphase chromosome movements that contribute to metaphase chromosome alignment. Disruption of CENP-E function using a variety of methods, including antibody microinjection and ablation of gene expression with siRNA, induces mitotic arrest and a cellular phenotype characterized by misaligned chromosomes arrayed on bipolar spindles, and leads to subsequent cell death.[18−23]

From a high-throughput screen of a 700K-member small molecule compound library looking for inhibitors of the microtubule-stimulated ATPase activity of CENP-E, we identified a low-molecular-weight fragment (benzoic acid **2**, Figure 1) with a biochemical IC_{50} of 6.7 μM and no detectable cellular effect at 40 μM. Although we were unable to pursue a typical fragment based optimization approach utilizing X-ray crystallography or NMR,[24] the good ligand binding efficiency[25,26] (ΔG/number of non-hydrogen atoms, LE = 0.50), selectivity vs other kinesins, and structural features amenable to rapid creation of analogues made **2**

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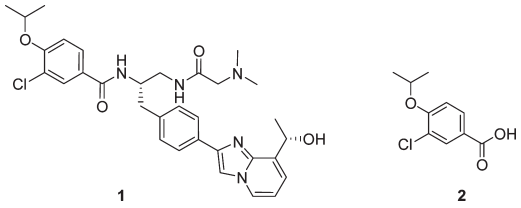
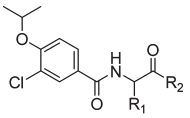


Figure 1. Structures of **1** and **2**.

Table 1. Biochemical Activity of Benzamide Analogues

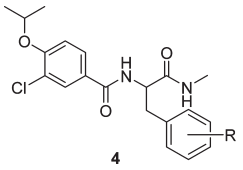


compd	R_1	R_2	CENP-E IC_{50} (μM)
3a	H	NHMe	> 100
3b	iPr	NHMe	> 100
3c	CH_2OH	NHMe	> 100
3d	CH_2CONH_2	NHMe	> 100
3e	Ph	NHMe	> 100
3f	CH_2CH_2Ph	NHMe	> 100
3g	CH_2Ph	NHMe	6.5
3 h	CH_2Ph	NH_2	5.4
3i	CH_2Ph	$N(Me)_2$	> 100
3j	CH_2Ph	OMe	> 100
3k	CH_2Ph	OH	61

an attractive starting point for further optimization. Reasoning that the lack of cellular activity might be due to the poor permeability related to the presence of the carboxylate, a small library of amide analogues was prepared by coupling **2** with a set of amino acid derivatives bearing a variety of side chains and different C-terminal capping groups.[27] Representative examples are shown in Table 1. Compounds **3g** and **3h**, which contain a benzyl group side chain and a primary or methyl amide C-terminus, were found to have IC_{50} values similar to screening hit **2**. The simple glycine amide analogue (**3a**) was inactive, as were analogues with side chains containing a simple alkyl chain (**3b**), H-bond donors or acceptors (**3c** and **3d**), or benzyl group homologues (**3e** and **3f**). A tertiary amide (**3i**) and methyl ester (**3j**) at the C-terminus also rendered compounds inactive. Carboxylic acid analogue **3k** retained some potency but was 10-fold less active than **3g**.

With the identification of the phenylalaninamide as a new active scaffold, we explored the substitution on the side chain phenyl group to introduce further structural diversity. A systematic Topliss scan[28] revealed that substitution was tolerated roughly equally at all positions with either electron-withdrawing or electron-donating groups (**4a**−**4f**) as shown in Table 2. Substitution with a larger phenyl group was dramatically more sensitive. A phenyl ring appended at the 4-position (**4g**) improved the biochemical potency by 10-fold whereas the same substitution at the 2- and 3-positions (**4h** and **4i**) dramatically attenuated potency. Encouragingly,

Table 2. Biological Activity of Phenylalanine Amide Analogues



compd	R	CENP-E IC_{50} (μM)	SKOV-3 IC_{50} (μM)
3g	H	6.5	> 25
4a	4-OH	3.3	> 25
4b	3-OH	6.2	> 25
4c	2-OH	8.8	> 25
4d	4-F	9.7	> 25
4e	4-Cl	7.2	> 25
4f	4-Me	5.4	> 25
4g	4-Ph	0.36	6.2
4h	3-Ph	36	> 25
4i	2-Ph	> 100	> 25
4j	4-(2-Me)-imidazol-4-yl	1.0	> 25
4k	4-(2-^{t}Bu)-imidazol-4-yl	0.13	1.2
4l	4-(4-^{t}Bu)-imidazol-2-yl	0.19	4.4
4m	4-benzimidazol-2-yl	0.066	3.1

4g also showed the first sign of antiproliferative effect in the SKOV-3 human ovarian carcinoma cell line with an IC_{50} of 6.2 μM. In light of these results, we investigated heterocyclic substitution at the 4-position of the phenyl ring as a means to optimize physicochemical properties while further improving biochemical and cellular activity. An imidazolyl group linked via the 2- or 4-position was found to be the most active among a variety of five- and six-membered heterocycles explored. In going from a modestly potent methyl substituent (**4j**) to a bulky *tert*-butyl group (**4k** and **4l**) or fusion with a phenyl ring (i.e., benzimidazolyl, **4m**), biochemical and cellular potencies were further improved.

Having a potent compound (**4m**) in hand, we examined whether there was a stereochemical preference for binding to CENP-E. Both (*S*)- and (*R*)-enantiomers of **4m** were synthesized using enantiomerically pure starting materials, and final chiral purity was > 98% ee by chiral HPLC for each antipode. As shown in Table 3, the (*S*)-enantiomer (**5a**) was > 400 times more potent than the (*R*)-enantiomer (**5b**), demonstrating a pronounced stereochemical bias at the binding pocket for the (*S*)-antipode. This stereoselectivity translated well into cellular activity as cells treated with **5a** displayed the characteristic mitotic arrest phenotype (bipolar spindles with misaligned chromosomes), whereas **5b** showed no effect, providing strong support for an on-target mechanism for inhibition of cell proliferation based on the inhibition of CENP-E motor protein.

Although the biochemical IC_{50} was greatly improved upon incorporation of a biaryl group in the side chain, the cellular activity of these compounds was still 10−100-fold below their biochemical potency. Our efforts shifted to modification of the C-terminus in an attempt to narrow this

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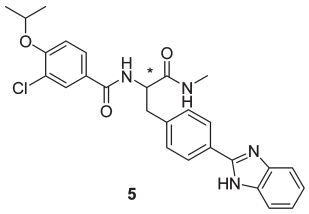
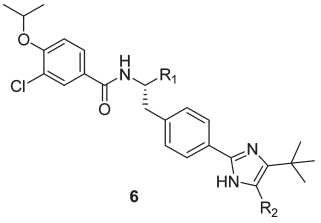
Table 3. Stereoselectivity of Phenylalanine Amide Analogues



5

compd	stereo	CENP-E IC_{50} (μM)	SKOV-3 IC_{50} (μM)
5a	*S*	0.032	2.0
5b	*R*	13.8	> 20

Table 4. Effect of C-Terminal Modification on Biological Activity



6

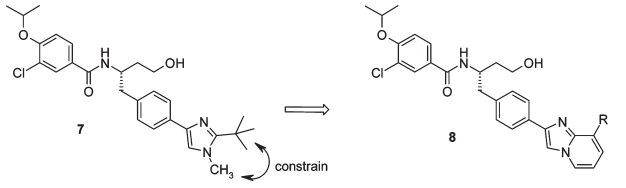
compd	R_1	R_2	CENP-E IC_{50} (μM)	SKOV-3 IC_{50} (μM)
4l	$CONHCH_3$	H	0.19	4.4
6a	CH_2OH	H	0.056	2.7
6b	CH_2OH	Me	0.043	1.1
6c	CH_2NH_2	Me	0.050	1.4
6d	CH_2CH_2OH	Me	0.022	0.094
6e	$CH_2CH_2NH_2$	Me	0.10	7.2

differential. Reduced C-terminal groups such as hydroxymethyl (**6a** and **6b**), aminomethyl (**6c**) and aminoethyl (**6e**) gave modest improvements to both the biochemical and cellular potency (Table 4). Remarkably, however, a one-carbon extension to a hydroxyethyl group (**6d**) significantly improved cellular potency (SKOV-3 IC_{50} = 94 nM), with only a modest improvement in biochemical activity. Detailed mechanistic studies of **6d** and **4g** revealed that there was a significant difference in their modes of inhibition. Steady-state kinetic studies showed that **6d** was uncompetitive with ATP, while **4g** was ATP-competitive. Since the ATP concentration in cells is much higher than that used in the biochemical assay (500 μM), this could help explain the disparity between the biochemical and cellular activities for ATP-competitive inhibitors such as **4g**.

We postulate that both **4g** and **6d** interact with the enzyme in an allosteric binding cleft adjacent to the ATP binding site and that the relatively minor structural modification perturbs the ATP pocket such that a change in inhibitor modality is observed.[29] Most importantly, the improved cellular activity proved to be general with the incorporation of the hydroxyethyl into the inhibitor template and fueled further exploration.

Maintaining the hydroxyethyl moiety, we further explored substitution of the side chain phenyl group. Transposition of

Table 5. Biological Activity of Selected Imidazopyridine Analogues



7 **8**

compd	R	CENP-E IC_{50} (nM)	SKOV-3 IC_{50} (nM)	sol[30] (μM)
7		56	41	23
8a	Me	3	15	1
8b	(*S*)-$CH(CH_3)OH$	7	14	21

one of the imidazole nitrogens to give isomeric imidazole **7** showed no advantage. However, constraining the methyl and *tert*-butyl groups to form an imidazopyridine ring (**8a**) resulted in a significant increase in biochemical and cellular activity (Table 5). Unfortunately, **8a** was much less soluble,[30] and since our goal was to identify a drug that could be administered intravenously, maximizing aqueous solubility was crucial. Encouragingly, the addition of a hydroxyl group (**8b**) to the imidazopyridine side chain restored the solubility with no attenuation of biochemical and cellular potency.[31]

Although significant gains in enzyme and cellular potency had been realized, the rat pharmacokinetic (PK) profiles of these analogues were characterized by high clearances and short half-lives. In an attempt to rationalize the underlying poor PK, the *in vitro* metabolism of selected analogues in rat and human hepatocytes and microsomes was investigated. The results indicated that hydroxylation of the biaryl side chain and oxidation of the primary hydroxyl group were the most prevalent metabolic pathways, and derivatives that incorporated modifications likely to reduce or block these putative metabolic sites were targeted.

Modification of the side chain, including the incorporation of fluorine atoms at various positions on the side chain aryl groups, had little effect on PK.[32] We next examined alternatives to the C-terminus hydroxyethyl group, keeping in mind that maintaining the ATP-uncompetitive mechanism of inhibition would be necessary for retention of good cellular activity. Although various cyclic and acyclic groups maintained high levels of enzyme and cellular potency, substituted glycinamides also provided a moderate boost in exposure coupled with significantly improved solubility. These efforts ultimately led to the identification of GSK923295 (**1**), a potent inhibitor of human CENP-E.[27,33,34]

Under steady-state kinetic conditions, **1** behaves as an uncompetitive inhibitor of both ATP and MT (Table 6). To ascertain its kinesin selectivity, **1** was evaluated against a panel of mitotic human kinesins and showed only minimal inhibitory activity (< 25%) at 50 μM.

Compound **1** exhibits inhibition of cell proliferation in a broad panel of human solid tumor and hematological cell lines and induces mitotic arrest leading to apoptosis and cell death.[33,34] Consistent with CENP-E inhibition, cells treated

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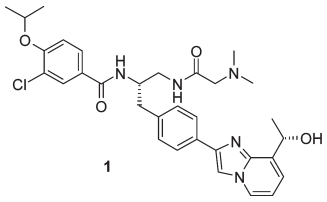
Table 6. Biological Activity Profile of **1**



1

hCENP-E K_i		3 nM
mode of inhibition	ATP	uncompetitive
	MT	uncompetitive
kinesin selectivity[a]		< 25% inhibition @ 50 μM
cell IC_{50}	SKOV-3	22 nM
	Colo205	22 nM

[a] Panel: KSP, Kif1A, MKLP1, RabK6, HSET, MCAK, Kif4.

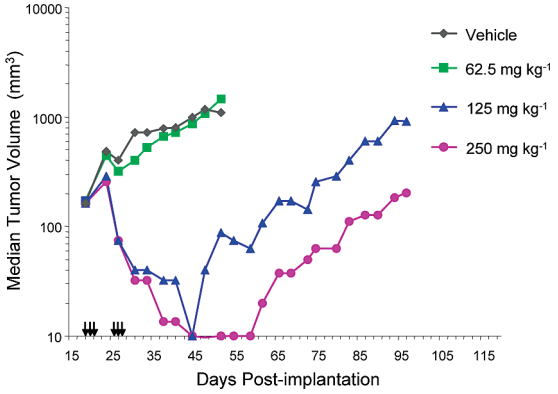


Figure 2. Efficacy of **1** in Colo205 human tumor xenograft study in mice. Arrows denote dosing days.

with the drug display a phenotype characterized by bipolar mitotic spindles with misaligned chromosomes.

Compound **1** also showed dose-dependent activity in a Colo205 human tumor xenograft efficacy model.[35−37] Mice were administered **1** as a single dose three consecutive days per week for two weeks (6 total doses) resulting in significant effects with tumor regression observed for the top doses (125 and 250 mg kg^{-1}) (Figure 2). In other studies, **1** showed broad spectrum activity against a range of human tumor xenografts in mice.[36]

Compound **1** showed an overall profile suitable for clinical development, including a solubility of 5 mg mL^{-1} in 0.1 M acetate buffer at pH 5.6. Compound **1** exhibited half-lives in the rat and dog of 1.3 and 2.1 h, respectively. This has translated to dose-proportional PK profile in humans with a mean terminal elimination half-life of ∼12 h.[38]

In summary, starting from a fragment based screening hit, benzoic acid **2**, SAR studies culminated in the discovery of **1**, a highly potent and selective inhibitor of CENP-E. Inhibition of CENP-E with **1** induces mitotic arrest in human tumor cells and tumor regressions *in vivo*. CENP-E inhibition is expected to have a beneficial effect on cancer therapy, and **1** is being evaluated in human clinical trials for the treatment of cancer.[39]

SUPPORTING INFORMATION AVAILABLE

Experimental procedures and analytical data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

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ABBREVIATIONS

CENP-E, centromere-associated protein E; KSP, kinesin spindle protein; LE, ligand efficiency; MT, microtubules.

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(29) Evidence for the proposed binding site was consistent with site directed mutagenesis and photolabeling experiments. Details of these studies will be the focus of a future publication. For another example of allosteric ATP-competitive kinesin inhibitors, see: Parrish, C. A.; Adams, N. D.; Auger, K. R.; Burgess, J. L.; Carson, J. D.; Chaudhari, A. M.; Copeland, R. A.; Diamond, M. A.; Donatelli, C. A.; Duffy, K. J.; Faucette, L. F.; Finer, J. T.; Huffman, W. F.; Hugger, E. D.; Jackson, J. R.; Knight, S. D.; Luo, L.; Moore, M. L.; Newlander, K. A.; Ridgers, L. H.; Sakowicz, R.; Shaw, A. N.; Sung, C. M.; Sutton, D.; Wood, K. W.; Zhang, S.; Zimmerman, M. N.; Dhanak, D. Novel ATP-Competitive Kinesin Spindle Protein Inhibitors. *J. Med. Chem.* **2007,** *50*, 4939–4952.
(30) Solubility was measured routinely using a high throughput HPLC based method. Compounds were dissolved in DMSO and diluted with pH 7.4 phosphate buffer, resulting in a final mixture that contained 2% DMSO and a theoretical maximum compound concentration of 200 μM.
(31) The corresponding alcohol diastereomer, R = (*R*)-CH(CH_3)OH, was ~10-fold less active (data not shown).
(32) Only minor modifications to the 3-chloro-4-isopropoxybenzamide portion of the template were tolerated. Further details of these additional SAR studies will be the subject of a future publication.
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