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Discovery and optimization of imidazo[1,2-*a*]pyridine inhibitors of insulin-like growth factor-1 receptor (IGF-1R)

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ABSTRACT

The optimization of imidazo[1,2-*a*]pyridine inhibitors as potent and selective inhibitors of IGF-1R is presented. Further optimization of oral exposure in mice is also discussed. Detailed selectivity, in vitro activity, and in vivo PK profiles of an optimized compound is also highlighted.

Insulin-like growth factor-1 receptor (IGF-1R) is a member of the insulin receptor family of tyrosine kinases. IGF-1R is a tetrameric transmembrane-spanning protein with an intracellular kinase domain. It plays an important role in several critical signaling cascades. By activating both the Ras/Raf/MAPK and PI3K/Akt pathways, IGF-1R is a key component in the proliferation, survival, transformation, and metastasis of cancer cells.[1] Monoclonal antibodies specifically targeting IGF-1R are currently being evaluated in clinical trials.[2] In addition, several small molecule approaches to IGF-1R inhibition have also recently been disclosed.[3] There are multiple examples demonstrating growth inhibition of human tumor xenografts grown on mice through daily treatment with small molecule IGF-1R inhibitors such as NVP-AEW541,[4] NVP-ADW742,[5] BMS-554417,[6] BMS-539264,[7] PQIP,[8] and OSI-906.[9] Because of the high homology between IGF-1R and the closely related insulin receptor (IR), small molecule IGF-1R inhibitors typically have activity against IR as well. Since IGF-1R is an attractive target for cancer therapy, a program focused on the discovery of novel small molecule inhibitors of IGF-1R was initiated.

An ongoing effort directed toward multi-targeted kinase inhibitors identified an imidazo[1,2-*a*]pyridine lead series as exemplified by compound **1**, which had modest IGF-1R activity in both the IGF-1R enzyme[10] and the cellular mechanistic assay[11] (Table 1). An important early discovery in this effort was that reversal of the amide connectivity resulted in a significant increase in IGF-1R potency to the template. For example, **2** showed an approximately 20-fold improvement in both the enzyme and cellular mechanistic assays compared to **1**. Encouraged by this potency, further efforts to optimize this series were made in the context of this amide orientation.

Synthesis of these imidazo[1,2-*a*]pyridine inhibitors began with commercially available isophthalic monoester **3** (Scheme 1). Treatment with oxalyl chloride and DMF yielded the corresponding acid chloride, which upon exposure to 2,6-difluoroaniline afforded the aryl amide **4**. Subsequent treatment with the anion derived from pyrimidine **5** produced ketone **6** in high yield. Generation of the reactive α-bromoketone followed by immediate treatment with 2-aminopyridine resulted in cyclization to afford the imidazo[1,2-*a*]pyridine intermediate **7**. Acid-catalyzed reaction of **7** with a variety of anilines **8** at elevated temperatures afforded the target inhibitors **9**. This transformation was carried out in either

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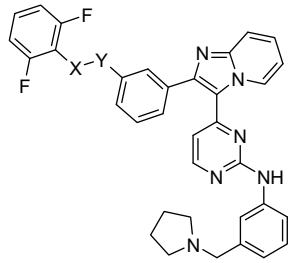
Table 1
Early imidazo[1,2-*a*]pyridine leads



Compound	X	Y	IGF-1R enzyme IC_{50}[a] (nM)	IGF-1R cellular IC_{50}[a] (nM)
1	C=O	NH	180	5930
2	NH	C=O	10	247

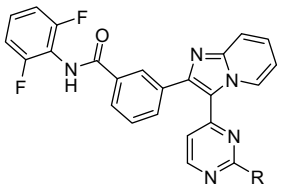
[a] Data are $n = 1$.

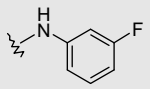
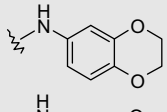
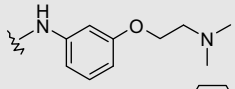
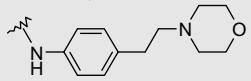
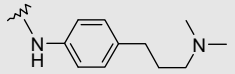
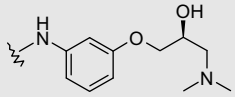
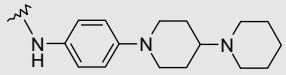
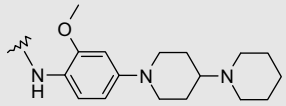
trifluoroethanol or isopropanol with either mild heating in a sealed vessel or utilizing microwave irradiation.

Initial investigations into the structure–activity relationships of the imidazo[1,2-*a*]pyrimidines focused on modification of the aniline attached to the pyrimidine ring, which binds the hinge of the kinase (Table 2). Due to the potentially undesirable high molecular weight of the inhibitors being generated, removal of the 2-aniline was investigated. Disappointingly, this modification resulted in a significant loss of potency (**10** vs **2**). Simple anilines (**9a** and **9b**) restored potency to moderate levels. Incorporation of anilines with basic amine functionality further enhanced potency (**9c**–**9f**). For example, the analog containing a 4-(1,4′-bipiperidin-1-yl)aniline group (**9g**) possessed an IC_{50} of less than 100 nM in the cellular assay, a feature considered highly desirable. Cellular potency could be further enhanced with the incorporation of a methoxy substituent at the 2-position of the aniline (**9h**).

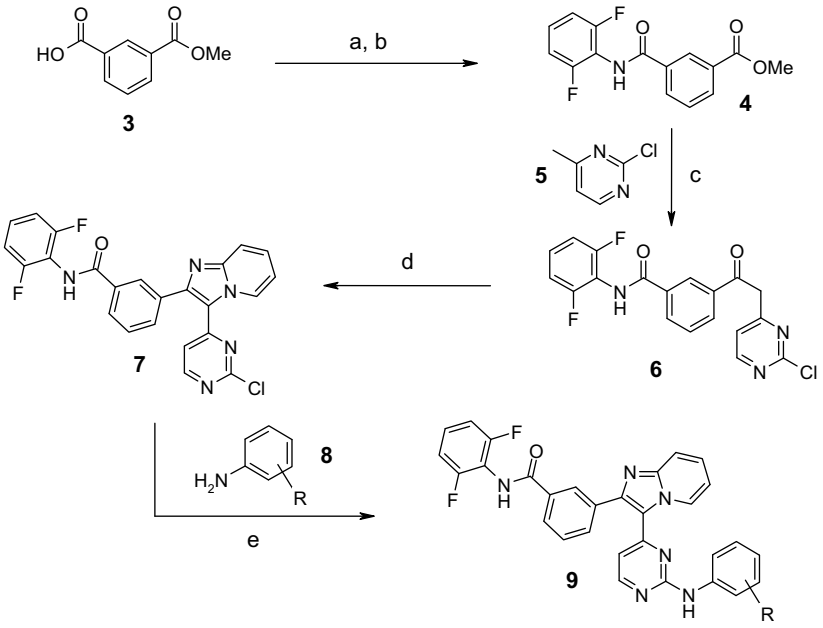
Early analogs in this series were found to also inhibit Aurora B kinase. Small molecule inhibitors of Aurora B have been shown to inhibit cell division and compromise cell proliferation.[12] Avoiding the potential for masking or confusing the phenotype observed from inhibiting IGF-1R was desirable. The 2-methoxy substituent

Table 2
Aniline modifications

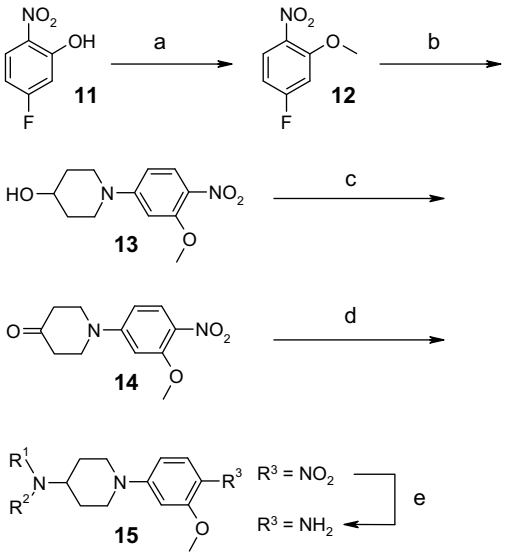


Compound	R	IGF-1R enzyme IC_{50}[a] (nM)	IGF-1R cellular IC_{50}[a] (nM)
10	$-NH_2$	440	>30,000
9a		28	509
9b		7	431*
9c		1	177
9d		4	174
9e		2	344
9f		1	244*
9g		2	87
9h		2	38

[a] Data are the average for $n \geqslant 2$, except for those with (*), which are $n = 1$.



Scheme 1. Reagents and conditions: (a) $(ClCO)_2$, DMF, CH_2Cl_2; (b) 2,6-difluoroaniline, pyridine, CH_2Cl_2 (84%, 2 steps); (c) $LiN(SiMe_3)_2$, THF (83%); (d) NBS, CH_2Cl_2, then 2-aminopyridine, dioxane, 60 °C (77%); (e) HCl or p-TSA·H_2O, trifluoroethanol or isopropanol, 80–100 °C or 140–180 °C (μw) (50–90%).



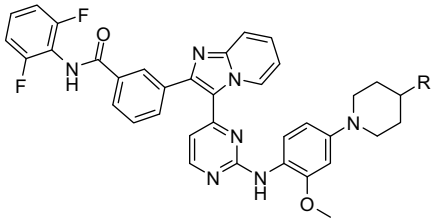
Scheme 2. Reagents and conditions: (a) K_2CO_3, MeI, DMF (92%); (b) 4-piperidinol, K_2CO_3, DMSO (99%); (c) $(ClCO)_2$, DMSO, NEt_3, CH_2Cl_2, −78 °C to rt (97%); (d) NEt_3, AcOH, $NaBH(OAc)_3$, $HN(R^1)R^2$, DCE or PhMe (60–80%); (e) hydrogenation or $NiCl_2{\cdot}6H_2O$, $NaBH_4$, MeOH (50–90%).

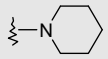
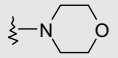
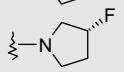
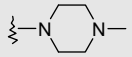
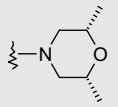
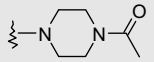
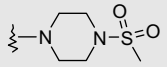
found in **9h** imparted a level of selectivity over Aurora B not previously observed (Aurora B enzyme IC_{50} = 9 nM and 130 nM for **9g** and **9h**, respectively). The anilino-pyrimidine moiety of the molecule contacts the hinge of the kinase ATP binding site, and the methoxy substituent is believed to sterically conflict with Tyr_{156} of Aurora B. The corresponding residue in IGF-1R is Leu_{1078}, which is smaller and able to accommodate the methoxy substituent.

Oral dosing of compound **9h** (7.3 mg/kg) in mice[13] resulted in lower than desirable plasma levels (DNAUC = 239 ng h/mL/(mg/kg)). In an effort to further improve the oral exposure of these molecules, a strategy focused on variation of the terminal piperidine ring was initiated. The preferred synthetic route for the requisite aniline intermediates began with methylation of commercially available phenol **11** (Scheme 2). Addition of 4-piperidinol to **12** afforded the nitrobenzene intermediate **13**. Swern oxidation[14] of alcohol **13** afforded ketone **14** which was subsequently reacted with the appropriate secondary amine in the presence of sodium triacetoxyborohydride to afford 4-aminopiperidines **15**. The final reduction of the nitro group to the corresponding anilines **15** was accomplished using either a catalytic hydrogenation or a combination of nickel chloride and sodium borohydride.[15] Alternatively, commercially available or custom made 4-aminopiperidines could be coupled with intermediate **12**. The highlighted route was considered superior as chemical diversity was introduced later in the synthetic sequence.

Variation of the terminal amine yielded some encouraging results (Table 3). In some cases the exposure was similar or inferior to that observed with **9h** (**16**, **17**, **19**, and **21**); however, there were clear examples of improved oral exposure (**18**, **20**, and **22**). In each case the molecules with improved exposure were among those with less basic terminal amine groups. Certainly, reducing the basicity of the amine did not guarantee improved oral exposure (**16**, **19**, and **21**), but it did appear to increase its probability. It is possible that in the more neutral environment of the gut, a higher proportion of these amines are in their uncharged state leading to better permeability and thus better absorption.[16] It is unlikely that the dramatic difference in oral exposure between **9h** and **22** is due to reduced metabolism as clearance in mice was low for both molecules (Cl = 13.4 and 13.6 mL/min/kg for **9h** and **22**, respectively).

Table 3
Terminal amine SAR

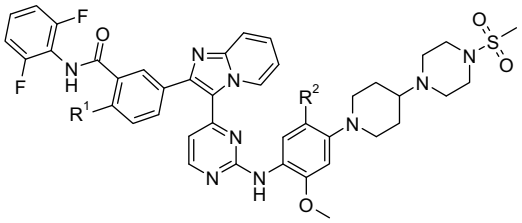


Compound	R	IGF-1R enzyme IC_{50}[a] (nM)	IGF-1R cellular IC_{50}[a] (nM)	Mouse PO DNAUC[b]
9h		2	38	239
16		2	41	276
17		2	38	121
18		2	40	494
19		2	28	203
20		4	68	549
21		3	65	216
22		5	36	1060

[a] Data are the average for $n \geqslant 2$.
[b] DNAUC units are ng h/mL/(mg/kg) determined from 0 to 6 h.

As expected, sulfonamide **22** was a potent dual inhibitor of both IGF-1R and IR; however, the non-familial kinase activity also remained fairly broad in nature (Table 4).[17] Further optimization of the selectivity profile was accomplished through additional modifi-

Table 4
Final optimization of selectivity



Kinase	IC_{50}[a] (nM)		
	22 R^1 = H R^2 = H	**23** R^1 = H R^2 = Et	**24** R^1 = OMe R^2 = Et
IGF-1R	5	7	27
IR	5	7	25
EGFR	57	22	>10,000
ErbB4	74	140*	>25,000
ErbB2	72	54	>10,000
B-Raf V600E	80	320	> 2000
Aurora B	200	1300	4500
p-38α	440	1800*	11,000
Aurora A	1600	>9500	>1500
LCK	1200	710*	>3000

[a] Data are the average for $n \geqslant 2$, except for those with (*), which are $n = 1$.

cations of the template. For example, incorporation of an ethyl group at the 5-position of the aniline (**23**) resulted in a compound with similar potency at the desired target (IGF-1R cellular IC_{50} = 66 nM). However, this small change improved selectivity against multiple kinases although potency against the ErbB family kinases and LCK remain similar to **22**. Addition of a methoxy substituent at the 4-position of the inner phenyl ring further enhances selectivity (**24**).

Although the enzyme potency of **24** against IGF-1R (IC_{50} = 27 nM) is lower than earlier compounds such as compound **22** (IC_{50} = 5 nM), cellular potency was actually improved (IGF-1R cellular IC_{50} = 22 nM). Compound **24** was confirmed to be an ATP-competitive inhibitor; however, kinetic studies showed that both the association and disassociation rates for this compound with IGF-1R and IR were quite slow ($t_{½}$ > 180 min). As the standard enzyme assay does not include a pre-incubation of enzyme and inhibitor and is only one hour in length, the potency of **24** in this assay was therefore underestimated. Assays which included pre-incubation of enzyme and compound were developed to more accurately reflect inhibitor potency.[18] The apparent K_i values for **24** in these assays were 1.6 ± 0.1 nM and 1.3 ± 0.1 nM against IGF-1R and IR, respectively. Apparent K_i values for **22** were much closer to the enzyme IC_{50} values, 5.2 ± 0.8 nM and 2.2 ± 0.3 nM against IGF-1R and IR, respectively.

A crystal structure of **24** in an IR double mutant (C981S, D1132N) was solved to 2.2 Å (Fig. 1).[19] As expected, the anilinopyrimidine contacts the β-strand, with the piperazino-piperidine oriented in the direction of the solvent exposed area. The 2,6-difluoroamide head group is oriented deep into the back pocket of the kinase, indicative of an 'inactive-like' conformation of the protein. It is possible that this binding mode contributes to the aforementioned slow kinetics seen with this inhibitor. This type of binding is a characteristic of type II kinase inhibitors and similar to those observed with marketed drugs such as imatinib, sorafenib, and lapatinib.[20]

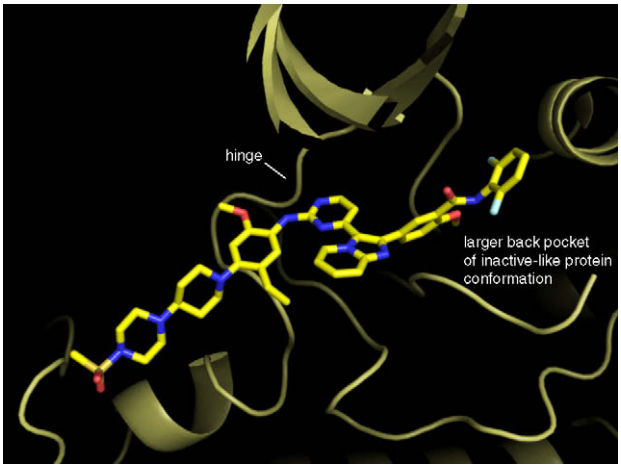


Figure 1. X-ray crystal of **24** bound to IR double mutant.

Table 5
Profile of **24** in cellular assays

Target		IC_{50}[a] (nM)
Mechanistic assays (receptor autophosphorylation)		
IGF-1R		22
IR		19
Cell line	Tumor type	IC_{50}[a] (nM)
Proliferation assays		
NCI-H929	Multiple myeloma	81
LP-1	Multiple myeloma	104
TC-71	Ewing's sarcoma	35
SK-ES	Ewing's sarcoma	61
COLO205	Colon	124
A549	Lung	>20,000
HFF	Normal foreskin fibroblast	>20,000

[a] Data are the average for $n \geqslant 2$.

Table 6
PK profile of **24** in various species

	PO dose mg/kg	Cl mL/min/kg	V_{SS} L/kg	PO DNAUC[a]	*F* (%)
Rat	3.0	5.8	7.5	1220	63
Dog	7.2	8.7	4.7	870	47
Monkey	2.1	13.9	9.8	1070	97

[a] DNAUC units are ng h/mL/(mg/kg).

Compound **24** was further profiled in additional cellular assays (Table 5). Not surprisingly, it was an equipotent inhibitor of both IGF-1R and IR phosphorylation in the mechanistic assays. A broader panel of tumor cell lines representing various tumor types were treated with **24** in order to measure the compound's ability to inhibit cellular proliferation.[21] The potency was excellent in a number of cell lines representing multiple tumor types, such as myeloma, sarcoma and colon. Certain cell lines such as A549 lung showed no response to treatment with **24**. Interestingly, **24** showed good selectivity towards normal HFF cell line when compared to most tumor cell lines.

Oral exposure of **24** in mice was similar to **22** (DNAUC = 1150 ng h/mL/(mg/kg)). Additional pharmacokinetic properties of **24** were evaluated in multiple species (Table 6).[22] The molecule proved to be a low clearance compound with good to excellent oral bioavailability. It is worth noting that the encouraging oral exposure observed with **24**, given its rather large molecular weight (M_W = 851), certainly places it outside the range that is considered desirable (⩽500).[23] Given the structural requirements necessary to achieve sufficient potency and selectivity within this chemical series, significant optimization was necessary in order to identify **24**, which demonstrated a considerably better pharmacological profile than would be predicted based on its large molecular weight.

In conclusion, a highly selective, potent inhibitor of IGF-1R and IR was discovered in an imidazo[1,2-*a*]pyrimidine chemical series. Although inhibition of IR has the potential for metabolic alteration, recent evidence points to a role for IR signaling in cancer, suggesting a potential therapeutic advantage of inhibiting both IGF-1R and IR.[24] Should an IGF-1R inhibitor with substantial selectivity over IR ultimately prove necessary, strategies directed toward the design of inhibitors that bind outside of the ATP binding site may be required. Optimization of mouse oral exposure was used as a tool in the imidazo[1,2-*a*]pyrimidine chemical series for the ultimate identification of **24**, a compound with good oral exposure in multiple pre-clinical species. Further communications regarding the biological profile of **24** both in vitro and in vivo will be forthcoming in the near future.

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10. Enzyme assays: Baculovirus-expressed GST-tagged proteins encoding the intracellular domain of IGF-1R (amino acids 957–1367) and IR (amino acids 979–1382) were used for determination of IC_{50}s. Kinases were activated by pre-incubating the enzyme (2.7 μM final concentration) in 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 0.1 mg/mL BSA, 2 mM ATP (all chemical reagents from Sigma–Aldrich, St. Louis, MO, unless otherwise noted). Compounds were diluted in dimethyl sulfoxide (DMSO) and dispensed into the assay plates (100 nL/well). Kinase reactions contained (in 10 μL volume) 50 mM Hepes, pH 7.5, 3 mM DTT, 0.1 mg/mL BSA, 1 mM CHAPS, 10 mM $MgCl_2$, 10 μM ATP, 500 nM substrate peptide (biotin-aminohexyl-AEEEEY̲MMMMAKKKK-NH_2; QPC, Hopkinton, MA) and 0.5 nM activated enzyme. Reactions were stopped after 1 h at room temperature with 33 μM EDTA. Peptide phosphorylation was measured by time resolved fluorescence with 7 nM streptavidin-Surelight™ allophycocyanin (Perkin-Elmer, Waltham, MA) and 1 nM Europium-conjugated phospho-tyrosine antibodies (Perkin-Elmer). Plates were read in a Victor X5 or Viewlux 1430 ultra HTS microplate imager (Perkin-Elmer).
11. Cellular mechanistic assays: NIH-3T3-hIGF-1R and NIH-3T3-hIR cell lines were seeded in culture medium in collagen-coated 96-well tissue culture plates (BD Biosciences, Franklin Lakes, NJ). After 24 h, cells were treated with DMSO or various concentrations of inhibitors and stimulated 2 h later with 30 ng/ml human IGF-1 or 3 μg/mL bovine insulin (Sigma–Aldrich) for 15 min. Cells were lysed in RIPA buffer (Roche Diagnostics, Indianapolis, IN) and lysates were transferred to wells of 96-well assay plates (MaxiSorp™, NalgeNunc, Rochester, NY) coated with either anti-IGF-1R (R&D Systems, Minneapolis, MN) or anti-IRβ (Santa Cruz Biotechnology, Santa Cruz, CA). Plates were incubated at 4 °C, overnight, washed and incubated with Eu-labeled phospho-tyrosine antibody PT66 (Perkin-Elmer, Waltham, MA) for the detection of phosphorylated IGF-1R and IR. Total IGF-1R and IR were detected with anti-IGF-1Rβ (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-IR (Lab Vision/Thermo Fisher Scientific, Fremont, CA), respectively. Secondary antibodies used were Eu-labeled goat anti-rabbit IgG (for IGF-1R) and Eu-labeled goat anti-mouse IgG (for IR) (Perkin-Elmer). Eu-fluorescence was quantified using a Victor Multilabel Counter (Perkin-Elmer; excitation 340 nm/emission 615 nm). The ratio of phosphorylated kinase to total kinase was determined, and concentration–response curves were plotted. IC_{50} values were determined from inhibition curves using XLfit4 software (Guildford, UK).
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13. Low dose mouse PK studies were conducted with a 1% DMSO, 10% solution in water formulation. Composite sampling was used with three mice per time point. AUC was determined from 0 to 6 h.
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17. Other kinases were screened using conditions and assays optimized for each.
18. A filter binding assay was used for apparent K_i determinations. IGF-1R and IR kinase domains were activated by pre-incubating at 500 nM in assay buffer A [50 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 0.2 mg/ml BSA, and 1× phosphatase inhibitor cocktail II (Sigma–Aldrich)] with 1 mM ATP for 30 min. Reactions typically contained (in 40 μL) 0.1 nM activated enzyme, 40 μM ATP (Teknova, Hollister, CA), and 7.5 μM peptide substrate (21st Century Biochemicals, Marlboro, MA; 1× Km and 1.5× Km concentration, respectively, for both IGF1R and IR) and [γ-^{33}P]ATP (100–500 cpm/pmol, 10 mCi/mL in 10 mM Tricine, Perkin-Elmer). The peptide substrate had the same sequence as that used for the time-resolved fluorescence assay, but without the biotin moiety. Inhibitor dilutions were prepared in DMSO and dispensed (1 μL/well) into the wells of a 96-well, half-area nonbinding surface (NBS) plates (Corning, Corning, NY). Reaction mix containing ATP and peptide was added to the plate and reactions were initiated by addition of enzyme mix. Reactions were quenched after 240 min by addition of 1% H_3PO_4. For compound **24**, reaction conditions were altered slightly due to slow inhibition kinetics. Final enzyme concentration was increased to 0.5 nM. Reaction mix containing enzyme and ATP was added and incubated with inhibitor for 6 h to allow the inhibitor–enzyme binding to reach equilibrium. Reactions were initiated by the addition of the reaction mix containing ATP and peptide substrate. The reactions were quenched after 120 min. After quenching, all reactions were filtered through MAPHN0B50 filter plates (Millipore), washed, and the product was quantified by liquid scintillation counting using MicroScint-20 (Perkin-Elmer) in a MicroBeta-1450 plate-reader (Perkin-Elmer). IC_{50} values were determined using a two-parameter fit (Hill coefficient and IC_{50}) using GraFit software (Erithacus, Surrey, UK). The apparent K_i^* values were calculated from the IC_{50} values using the Cheng–Prusoff relationship for ATP competitive inhibition.
19. IR protein was expressed and purified as previously described (Li, S.; Covino, N. D.; Stein, E. G.; Till, J. H.; Hubbard, S. R. *J. Biol. Chem.* **2003**, *278*, 26007). Protein at 10 mg/ml was complexed with a 3-fold molar excess of inhibitor for 1 h prior to crystallization. Crystals were grown by hanging drop vapor diffusion at 22° from 0.1 M MOPS, pH 7.0, 1.0 M trisodium citrate. Crystals were flash frozen in PFO prior to data collection. The structure was solved by molecular replacement using PDB:1P14 as a starting model.
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21. Cell proliferation assays: Cells were seeded in 96-well dishes, incubated overnight at 37 °C, and treated with various concentrations of inhibitors for 72 h. Cell proliferation was quantified using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI), following the manufacturer's recommendations. IC_{50} values were determined from using a 4-parameter curve fit software package (XLfit4).
22. PK studies were conducted as a solution in 1.0% DMSO, 20% Captisol® in saline (IV) or water (PO). The PO study in dogs was run as a partial suspension. IV doses ranged from 0.6 to 1.3 mg/kg, and PO doses ranged from 2.1 to 7.2 mg/kg depending on species. Rats were male Sprague–Dawleys. Dogs were male beagles. Monkeys were male cynomolgus. Reported data are for 0–24 h.
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