Bioorganic & Medicinal Chemistry Letters 21 (2011) 2224–2228

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Discovery of the first non-ATP competitive IGF-1R kinase inhibitors: Advantages in comparison with competitive inhibitors

Dominique Lesuisse [b,*], Jacques Mauger [a], Conception Nemecek [b], Sébastien Maignan [e], Janine Boiziau [d], Greg Harlow [a], Augustin Hittinger [b], Swen Ruf [c], Hartmut Strobel [c], Anil Nair [a], Kurt Ritter [c], Jean-Luc Malleron [b], Anne Dagallier [e], Youssef El-Ahmad [b], Jean-Pierre Guilloteau [e], Houlfa Guizani [b], Hervé Bouchard [b], Corinne Venot [d]

[a] Highthroughput Medicinal Chemistry, Sanofi-aventis, 2090 East Innovation Park Drive, Oro Valle, AZ 85755-1965, USA
[b] Medicinal Chemistry, Sanofi-aventis, 13 Quai Jules Guesde, 94300 Vitry-sur-Seine, France
[c] Medicinal Chemistry, Sanofi-aventis, Industriepark Hoechst, Frankfurt am Main 65926, Germany
[d] Oncology Therapeutic Domain, Sanofi-aventis, 13 Quai Jules Guesde, 94300 Vitry-sur-Seine, France
[e] Structural Biology and Molecular Modelling, Sanofi-aventis, 13 Quai Jules Guesde, 94300 Vitry-sur-Seine, France

ARTICLE INFO

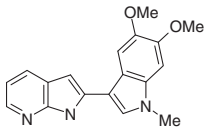
Article history:
Received 3 January 2011
Revised 28 February 2011
Accepted 1 March 2011
Available online 4 March 2011

Keywords:
IGF-1R
Kinase inhibitors
ATP
Noncompetitive
Slow binding inhibitor

ABSTRACT

A new series of IGF-1R inhibitors related to hydantoins were identified from a lead originating from HTS. Their noncompetitive property as well as their slow binding characteristics provided a series of compounds with unique selectivity and excellent cellular activities.

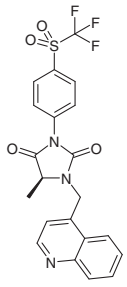
The IGF-1R (Insulin like Growth Factor 1 Receptor) tyrosine kinase is the main receptor involved in the transduction of growth metabolic signals[1] of IGF-1 (Insulin Growth Factor 1). Several lines of evidence link this growth factor and receptor to the emergence and proliferation of various tumors.[2] During the course of a programme aimed at finding inhibitors of IGF-1R[3] we initiated a high throughput screening (HTS) for compounds with affinity for this receptor. The initial lead identified was an azaindole **1** that was optimized and previously described.[4]



1
IC_{50} = 55 nM

The present communication describes a chemically unrelated and new series that originated from HTS and was expeditiously optimized by our combinatorial chemistry group.

IGF-1R is widely expressed in cells that have undergone oncogenic transformation. IGF-1, the canonical growth factor of IGF-1R, induces receptor autophosphorylation on Tyr1131, Tyr1135 and Tyr1136 in the activation loop of the cytoplasmic domain.[5] IGF-1R inhibitors were identified in an autophosphorylation-HTRF



2
IC_{50} = 0.5 μM (Table 1)

* Corresponding author. Present Address: Sanofi-Aventis, 1 av Pierre Brossolette 91385 Chilly Mazarin, France. Tel.: +33 1 60496857; fax: +33 1 60497492.
E-mail address: dominique.lesuisse@sanofi-aventis.com (D. Lesuisse).

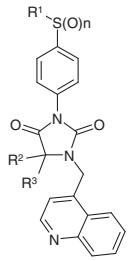
0960-894X/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2011.03.003

assay[6] with a nonphosphorylated recombinant GST-IGF-1R (aa882-1367) protein. This led to the identification of a hit **2** belonging to a hydantoin chemical series.

As **2** was generated in a full combinatorial library, hundreds of analogues were available to establish the initial SAR. The key structural features are as follows: The oxidation state of the sulfur was not essential to the activity as the sulfone **2**, displayed the same activity as the thioether **4**, both only about 5-fold more active than the corresponding sulfoxide **3**. On the other hand, the activity was totally lost when replacing the trifluoromethyl group by a methyl group as in **5**. The methyl on the hydantoin was important for the activity as shown by the loss of activity of unsubstituted **6** but both enantiomers **2** and **7** displayed about the same level of activity, as the analog incorporating two methyl groups **8** (Table 1). Larger alkyl or polar groups in this position all resulted in loss of activity (data not shown).

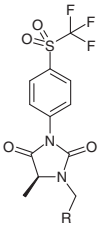
On the other hand, there was very little flexibility on the 4-quinolyl group in the southern part of the molecule (Table 2). It could be replaced by a 4-pyridyl group with only a 3-fold loss in activity. All other replacements like 3- and 2-pyridyls (compounds **10** and **11**), 3-quinolyl (**12**), 4-imidazolyl (**13**), 4-piperidyl (**14**), phenyl (**15**) were much less active as well as the oxides of the 4-pyridyl and 4-quinolyl derivatives (**16** and **17**).

Table 1
SAR around **2**: variations of R^1, R^2, R^3 and *n*



Compd	R^1	R^2	R^3	Chirality	*n*	HTRF (μM)
2	CF_3	Me	H	*S*	2	0,5
3	CF_3	Me	H	*S*	1	2,52
4	CF_3	Me	H	*S*	0	0,41
5	CH_3	Me	H	*S*	2	>100
6	CF_3	H	H	—	2	5,4
7	CF_3	Me	H	*R*	2	0,3
8	CF_3	Me	Me	—	2	0,46

Table 2
SAR around **2**: replacement of the 4-quinolyl group



				
2 0.5 μM	**9** 1.6 μM	**10** 79.7 μM	**11** >100 μM	**12** >100 μM
				
13 >100 μM	**14** >100 μM	**15** >100 μM	**16** >100 μM	**17** >100 μM

Table 3
Evaluation of **1** in IGF-1-related assays

	IGF-1R (HTRF) (μM)	IGF-1R (ELISA) (μM)	IGF-1-induced proliferation (μM)
1	0.055	0.145	1.6
2	0.5	0.6	0.3

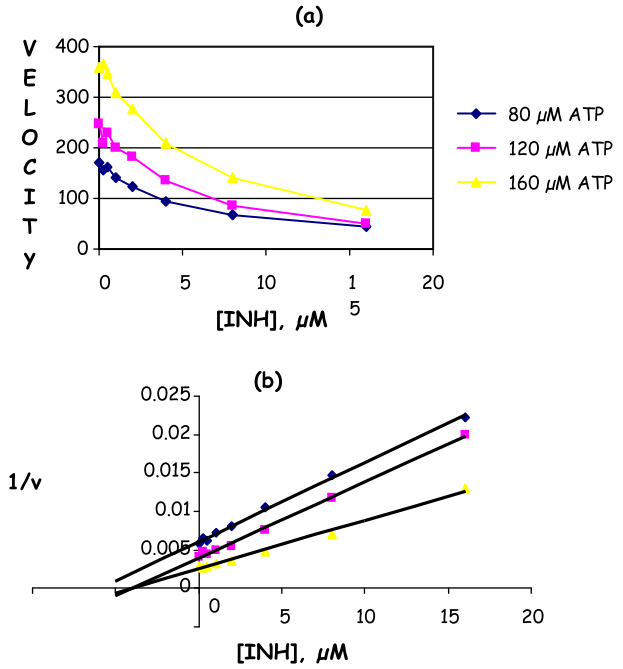


Figure 1. (a) Inhibition of IGF-1R by **2** as a function of ATP concentration; (b) Dixon-plot analysis of the above curves.

Compound **2** was evaluated against a panel of 23 kinases (a combination of Serine/Threonine and Tyrosine proteins)[7] and found inactive on all of them except the highly homologous IRK (Insulin Receptor Kinase) with an IC_{50} of 0.3 μM.

As **2** showed high permeability in a model of intestinal absorption (TC7 cells)[8] along with good metabolic stability in hepatic mouse and human microsomes (83% and 82% compound remaining after 15 min, respectively), it was engaged in a PK experiment. The absolute bioavailability following oral administration to the female Balb/c mouse at a dose level of 10 mg/kg was found to be in excess of 100% (i.e., dose 1 mg/kg). Systemic exposure to **2** was good after oral dosage at 10 mg/kg ($AUC_{0-\infty}$ = 13234 h ng/ml and Cp_{max} = 1491 ng/ml) with evident levels up to 24 h. The compound was not an inhibitor of CYP3A4, 1A2, 2D6 and 2C19 (IC_{50}'s >15 μM) and a slight inhibitor of CYP2C9 (IC_{50} = 0.7 μM). The solubility of **2** was rather low (9.9–13 μg/ml). These properties prompted us to engage in an optimization programme with the objectives of improving the solubility and optimizing the potency on the target to low nanomolar cellular potency.

Compound **2** was evaluated in a cellular IGF-1 phosphorylation assay (ELISA) and in an IGF-1-dependant cell proliferation assay (MEF).[9] The antiproliferative activity of **2** was in fact quite striking compared to that of our initial azaindole lead **1**.[4] While **1** displayed a 30- and 80-fold lower potency than enzymatic activity, **2** was more potent in the antiproliferative assay than in the enzymatic assay (0.3 vs 0.5 μM) (Table 3).

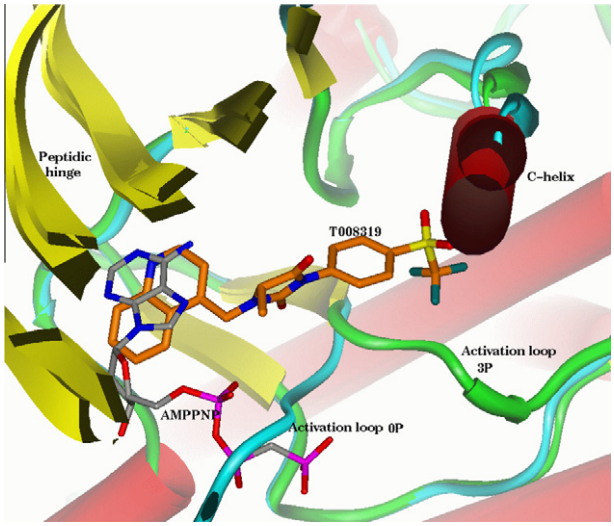


Figure 2. Overlay of IGF-1R kinase domain 3P (green) and 0P (cyan), respectively, in complex with AMPPNP (nonhydrolysable form of ATP) (grey) and 2 (orange). The activation loop conformations are different. It is DFG-out for the complex with **2**.

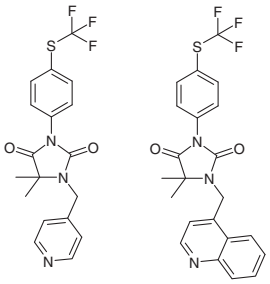
This result, along with the high selectivity of this compound motivated us to elucidate the mechanism of action of **2** with IGF-1R versus ATP. The reactions were done using the same HTRF protocol as before and using three concentrations of ATP (80, 120, and 160 μM). Compound **2** showed mixed uncompetitive/noncompetitive type kinetics with respect to ATP (Fig. 1a and b).

The noncompetitive kinetics might explain the good selectivity of this compound. These observations also suggested a novel binding mode with the kinase. We had hypothesized from the importance of nitrogen position on the activity (see Table 2) that the quinolyl nitrogen was involved in the hinge binding with the kinase, but we had no structural information. Several compounds from our azaindole series had been co-crystallized in the 2P (bisphosphorylated form) of IGF-1R. However, this form as well as the 3P phosphorylated active form of IGF-1R exposed to **2** (co-crystallization and soaking) did not allow us to obtain measurable crystals. The success came from the nonphosphorylated (inactive form) of IGF-1R. (Fig. 2).

This structure confirmed that the quinolyl nitrogen was interacting with the hinge binding region of the kinase.[10] In addition, it also pointed to a DFG-out binding with the kinase.[11] This conformation of the protein is characterized by a large change of position and orientation of the DFG motif found at the beginning of the activation loop in most kinases. Whereas the phenylalanine side chain in the DFG-in conformation occupies a volume outside of the active site, in the DFG-out conformation it lies close to the location of the ribose pocket. In the process it allows access to a secondary pocket at the back of the active site. Part of **2** binds in that secondary pocket.

One rational way to enhance affinity of **2** for the kinase was to reinforce the hinge interaction by adding an H-bond donor in the close vicinity of the H-bond acceptor as to be able to obtain the classical two-pronged interaction. One of our objectives being also the optimization of the solubility, it appeared to us that this properties could also be favourably impacted by the addition of polar groups on this aromatic ring.

From the previous SAR studies, it was decided to start from compound **18** incorporating the simplest features required for the activity, namely a trifluoromethyl thioether, a nonchiral dimethylhydantoin backbone and a pyridine as hinge interaction motif (this was also in turn more potent than the corresponding quinoline **19** on this particular scaffold).



18 **19**

As hypothesized the compounds possessing an NH in 2-position of the hinge binding nitrogen, like the amino pyridine **43** and its acetyl derivative **42** indeed displayed an improved activity, whereas bulkier groups in this position tended to impede the hinge contact (compounds **20**–**23**).

The small amino substituents of **40**, **42**, **43** also led to marked increase in solubility (Table 4). An additional amine in 6-position of the pyridine to further enhance the hinge binding did not lead to improved activity (data not shown).

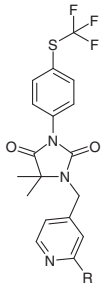
The best compounds **18**, **40**–**43** were evaluated in the cellular IGF-1 phosphorylation assay and IGF-1-dependant cell proliferation assay.[7] The results are outlined in Table 5. **42** turned out to be extremely potent in the proliferation assay, once again much more potent than anticipated from its inhibitory potency on IGF-1R.

The X-ray structure of **2** bound to IGF-1R along with its noncompetitive behavior strongly suggested that this series of inhibitors was stabilizing an inactive form of the kinase, therefore it was anticipated that they should behave as slow binding inhibitors.[12] An experiment was set up to verify this assumption. The apparent inhibitory potency of the hydantoin **42**, the azaindole **1** and staurosporine (a pan-kinase inhibitor) was monitored as a function of preincubation time between rec-IGF-1R and compound (max. of 2 h for enzyme stability). Only for the hydantoin **42** did we observe that the apparent IC_{50} value was clearly dependent on the preincubation time with an almost 300-fold decrease over an hour of time. This behavior was later shown to be common to all compounds belonging to this series albeit the extent of IC_{50} lowering differed from one compound to the other (data not shown). This time-dependent kinetic was clearly correlated to a better cellular activity (Table 6).

The compounds from combinatorial libraries (compounds **2**–**17**) were synthesized on solid phase as shown in Scheme 1.[13] Fmoc protected Rink resin **A** was deprotected in a mixture of 20% piperidine/DMF and capped by a Fmoc protected amino acid in the presence of diisopropylaminecarbodiimide and hydroxybenzotriazole. The immobilized Fmoc amino acid **B** was then deprotected and reacted with aldehydes in a mixture of THF/triethyl orthoformate to afford the corresponding Schiff base that upon reduction, using sodium cyanoborohydride, provided the amines **C**. Isocyanates (commercially available or prepared from the corresponding amines by reaction with phosgene) were condensed with the resulting amine to give the respective ureas **D**. Final cleavage from the solid support (95% TFA/water) directly provided the hydantoins **E** as their salts.

The compounds from the optimization program (**18**–**43**) were obtained as depicted in Schemes 2–5.[14] The synthesis of a first set of hydantoins was performed analogously as on solid phase: the unsubstituted hydantoin **44** was obtained starting from 4-trifluoromethylthiophenyl isocyanate and methyl α-aminoisobutyrate. From there, condensation with various 2-substituted 4-bromomethylpyridines[15] afforded compounds **18**, **24**, **29**, **30**, **35** and **38** (Scheme 2).

Table 4
SAR from pyridine substitution



Compd	R	Sol (μg/ml)[a]	IGF-1R HTRF (nM)
18	H	<0.02	264
20	1-Piperidyl	<0.024	>10000
21	4-Morpholinyl	0.037	>10000
22	1-(4-Me-Piperaziny	0.159	>10000
23	1-Piperazinyl	<0.024	>10000
24	CN	<0.021	>10000
25	COOH	0.142	>10000
26	$NHSO_2Me$	<0.025	4706
27	NMe_2	<0.022	4501
28	$CONH_2$	ND	2950
29	F	0.032	2897
30	OEt	ND	1851
31	NH-cyclohexyl	<0.025	1241
32	$NHCH_2PhOMe$	<0.027	1089
33	NHPh	<0.025	1061
34	NHBn	<0.026	1052
35	Cl	<0.022	995
36	NHCOO*t*Bu	ND	784
37	CONHMe	<0.023	703
38	Et	<0.022	574
39	NH*i*Pr	<0.023	453
40	NHMe	0.075	202
41	NHEt	<0.022	154
42	NHAc	0.035	98
43	NH_2	0.113	92

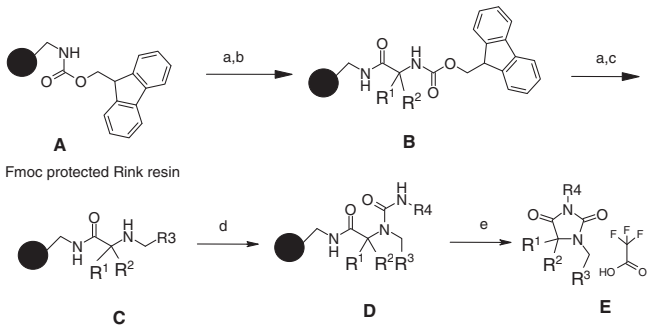
[a] Nephelometric solubility.

Table 5
Evaluation of **18** and **40**–**43** in IGF-1-related assays

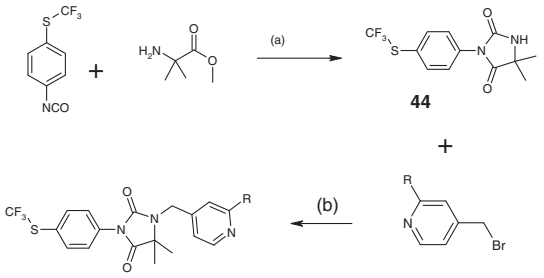
Compd	IGF-1R (HTRF) 10 min (nM)	IGF-1R (ELISA) (nM)	IGF-1-induced proliferation (nM)
18	264	117	282
40	202	43	312
41	154	47	104
42	98	7	8
43	92	34	97

Table 6
Activity as a function of preincubation time

	Preincubation time (min)	IC_{50}'s (nM)		
		Staurosporine	**1**	**42**
IGF-1R in vitro (HTRF, nM)	0	1132	39	1975
	5	1325	34	175
	10	1701	34	87
	30	1533	33	28
	60	1646	35	7
IGF-1R in cell (ELISA, nM)	60	—	180	7
IGF-1R-dep proliferation (nM)	—	—	439	8

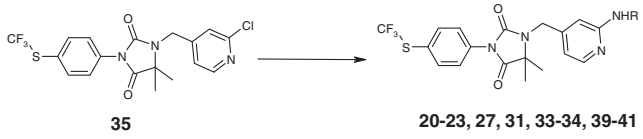


Scheme 1. Reagents and conditions: (a) 20% piperidine, DMF; (b) FmocAA(OH) (AA = S-Ala (compounds **2**–**5** and **9**–**17**), R-Ala (compd **7**), Gly (compd **6**) or Me-Ala (compd **8**), Diisopropylcarbodiimide (DIC), HOBt, DMF; (c) R^3CHO, $HC(OEt)_3$, THF; $NaBH_3CN$; (d) R4-N=C=O, DCM; (e) TFA, H_2O.

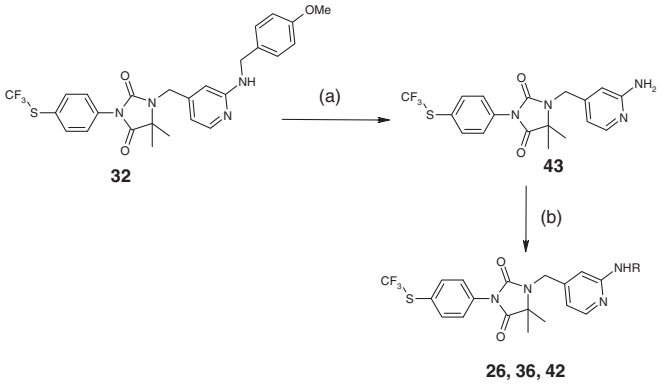


18 R = H; **24** R = CN; **29** R = F; **30** R = OEt; **35** R = Cl; **38** R = Et

Scheme 2. Reagents and conditions: (a) toluene/TEA; (b) NaH/DMF.



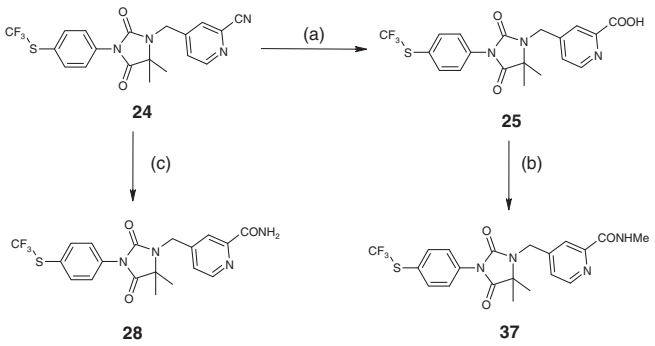
Scheme 3. Reagents and conditions: RNH_2/microwave, NMP or DMF.



Scheme 4. Reagents and conditions. (a) TFA/DCM; (b) **26** $MeSO_2Cl$/Py; **36** $(BOC)_2O$/*t*BuOH/rt; **42** Ac_2O/80 °C.

From the 2-chloropyridine hydantoin **35** a series of amines were prepared by direct displacement (Scheme 3).

Trifloracetic acid deprotection of the 2-(*p*-methoxybenzylamino-pyridine hydantoin **32** afforded the free amine which could then



Scheme 5. Reagents and conditions: (a) HCl 5 N, reflux; (b) $MeNH_2$, EDCI, HOBT, DCM; (c) H_2SO_4, 40 °C.

be acylated or sulfonylated to afford compounds **26**, **36** and **42** (Scheme 4).

Finally a series of hydantoins could be obtained from the 2-cyanopyridine hydantoin derivative **24** after hydrolysis to the corresponding carboxylic acid **25** followed by amide formation with methylamine to afford **37**. Alternatively it could be directly hydrolyzed to the primary amide **28** (Scheme 5).

A lead from HTS was optimized and characterized as a noncompetitive slow binding inhibitor of IGF-1R. To our knowledge, this is the first reported IGF-1R DFG-out kinase inhibitor. The potential advantages of these types of inhibitors both in terms of selectivity[16] and sustained effects[17] (in vitro and in vivo) could be quite considerable. In addition slow kinetics often leads to extended target occupancy and improved therapeutic responses. The optimization of this series bears promises for the treatment of IGF-related malignancies.

Acknowledgements

We are grateful to Bill Metz and Carlos Garcia-Echeverria for their help on reviewing the manuscript and to the analytical departments for compound analyses.

References and notes

1. Kim, J. J.; Accili, D.; Naomi, B. *Growth Horm. IGF. Res.* **2002**, *12*, 84; Li, R.; Pourpak, A.; Morris, S. W. *J. Med. Chem.* **2009**, *52*, 4981.
2. Furstenberger, G.; Senn, H.-J. *Lancet Oncology* **2002**, *3*, 298; Gross, J. M.; Yee, D. *Cancer Metast. Rev.* **2003**, *22*, 327; Yu, H.; Rohan, T. *J. Natl. Cancer Inst.* **2000**, *92*, 1472; Bohula, E. A.; Playford, M. P.; Macaulay, V. M. *Anti-Cancer Drugs* **2003**, *14*, 669.
3. For a general review on IGF1-R inhibitors see: Gualberto, A.; Pollak, M. *Oncogene* **2009**, *28*, 3009.
4. Nemecek, C.; Wentzler, S.; Venot, C.; Dagallier, A.; Maignan, S.; Guilloteau, J.-P.; Bernard, F.; Henry, A.; Grapinet, S.; Lesuisse, D. *Chem. Biol. Drug Des.* **2010**, *76*, 100.
5. Favelyukis, S.; Till, J. H.; Hubbard, S. R.; Miller, W. T. *Nat. Struct. Biol.* **2001**, *8*, 1058.
6. *Briefly, the activities were determined as follows:* IGF-1R–HTRF: Inhibition of autophosphorylation activity was determined using a time-resolved fluorescent assay. The cytoplasmic domain of human IGF-1R has been cloned as glutathione *S*-transferase (GST) fusion into the pFastBac–GST tagged baculovirus expression vector. The protein has been expressed in SF21 cells and purified to about 80% homogeneity. Kinase activity was determined in 50 mM Hepes pH 7.5 containing 5 mM $MnCl_2$, 50 mM NaCl, 3% glycerol, 0.025% Tween 20, 120 μM adenosine triphosphate. Enzyme reactions were terminated by the addition of 100 mM Hepes buffer pH 7.0, containing 0.4 M KF, 133 mM EDTA, BSA 0.1% containing an anti-GST antibody labelled with XL665 and an anti-phosphotyrosine antibody conjugated to a europium cryptate (Eu-K). Features of the two fluorophores, XL-665 and Eu-K are given in Mathis et al., *Anticancer Res.* **1997**, *17*, 3011. The specific long time signal of XL-665, produced only when the IGF1R enzyme is autophosphorylated, was measured on a Victor analyser (Perkin–Elmer). Inhibition of IGF1R kinase activity with compounds of the invention was expressed as percentage inhibition of control activity exhibited in the absence of test compounds.
7. Internal panel of kinases: Akt, Fak, p38, Tie2, Jnk3, Plk1, Pak3, Aurora2, GSK3b, Cdk4; CEREP panel of kinases: Abl, CAMKII, CKII, Cdk1, Cdk2, EGF, IRK, MAP, MEK1, PKCa, Src, ZAP70.
8. Turco, L.; Catone, T.; Caloni, F.; i Consiglio, E. D.; Testai, E.; Stammati, A. *Toxicol. in Vitro* **2011**, *25*, 13.
9. *Briefly, the activities were determined as follows:* IGF-1R–ELISA: Inhibition of autophosphorylation in MCF7 cell line after IGF-1 stimulation was evaluated by ELISA technique. MCF-7 cells were seeded at 6,00,000 cells per well in 6-multiwell plates, left over night in 10% serum and then serum-starved for 24 h. Compounds are added to medium 1 h before IGF-1 stimulation. After 10 min of IGF1 stimulation, cells are lysed with Hepes 50 mM pH 7.6, Triton X100 1%, orthovanadate 2 mM, protease inhibitors. Cell lysates are incubated on 96-multiwell plates pre-coated with anti-IGF-1R antibody, followed by incubation with an anti-phosphotyrosine antibody coupled to peroxydase enzyme. Peroxidase activity level (measured by OD with a luminescent substrate) reflects receptor phosphorylation status.
IGF-1-Induced MEF cell proliferation: MEF (Mouse Embryo Fibroblast 3T3) Tet off cells (BD Bioscience) were stably transfected with IGF-1R tetracycline-regulatable expression plasmid. MEF-IGF-1R cells were seeded in a 96-well Cytostar microplates (Amersham) at 5000 cells/well under 0.2 ml of EMEM culture medium and incubated at 37 °C for 18 h. After this period of time, cells were washed twice with serum-free EMEM and left in this culture medium for 24 h. Compounds were then added in the presence of rhIGF1 (100 ng/ml; RD Systems) and 0.1 μCi of Thymidine [methyl-^{14}C]. After an incubation time of 72 h in the presence of the compounds, at 37 °C under 5% CO_2, the ^{14}C-thymidine incorporation was measured on a Micro-beta counter (Perkin–Elmer).
10. The crystal structure of **2** with IGF-1R has been deposited in the PDB with accession code 3O23.
11. For precedents of kinase DFG-out inhibitors see for instance: Liu, Y.; Grey, N. S. *Nat. Chem. Biol.* **2006**, *2*, 358; Liao, J. J.-L. *J. Med. Chem.* **2007**, *50*, 409.
12. For general reviews on slow binding inhibitors see for instance: Morrison, J. F.; Walsh, C. T. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1988**, *61*, 201; Schloss, J. V. *Acc. Chem. Res.* **1988**, *21*, 348.
13. Patek, M.; Nair, A.; Hittinger, A.; Nemecek, C.; Bond, D.; Harlow, G.; Bouchard, H.; Mauger, J.; Malleron, J.-L.; Palermo, M.; Al_Obeidi, F.; Strobel, H.; Faitg, T.; Strobel, H.; Ruf, S.; Ritter, K.; El-Ahmad, Y.; Lesuisse, D.; Benard, D. PCT Patent Publication N WO2004/070050 A2, August 19th 2004.
14. Patek, M.; Nahir, A.; Hittinger,A.; Nemecek, C.; Bond, D.; Harlow, G.; Bouchard, H.; Mauger, J.; Malleron, J.-L.; Palermo, M.; Al-Obeidi, F.; Faitg, T.; Strobel, H.; Ruf, S.; Ritter, K.; El-Ahmad, Y.; Lesuisse, D. US2004/248884 A1.
15. The starting bromomethylpyridine precursors of compounds **18** and **24** were commercially available; the precursors of compounds **30**, **35** and **38** were prepared from the corresponding methyl ester after sodium borohydride reduction and dibromotriphenylphosphine bromination; finally the 2-fluoro-4-bromomethyl-pyridine was prepared from the 4-methyl analog after benzoylperoxide-catalyzed NBS bromination.
16. See for instance: Liu, H.; Kuhn, C.; Feru, F.; Jacques, S. L.; Deshmukh, G. D.; Ye, P.; Rennie, G. R.; Johnson, T.; Kazmirski, S.; Low, S.; Coli, R.; Ding, Y.-H.; Cheng, A. C.; Tecle, H.; English, J. M.; Stanton, R.; Wu, J. C. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4885.
17. Copeland, R. A.; Pompliano, D. L.; Meek, T. D. *Nat. Rev. Drug Disc.* **2006**, *5*, 730; Tummino, P. J.; Copeland, R. A. *Biochemistry* **2008**, *47*, 5481.