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Novel fragment-like inhibitors of EphA2 obtained by experimental screening and modelling

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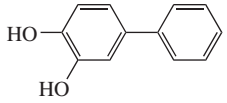
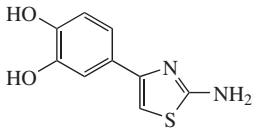
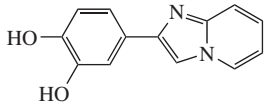
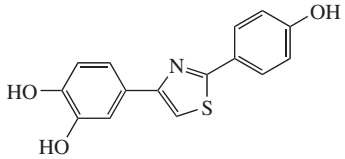
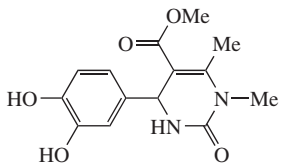
A set of novel fragment-like catechol derivatives were identified as EphA2 inhibitors and were further profiled against a panel of 19 tyrosine kinases. In addition to EphA2, the recovered hits were active against EGFR, FGFR1, FGFR2, Abl and PDGFR-a, and according to molecular modelling studies catechol moiety was capable of forming two or more correlated hydrogen bonds with the kinase hinge region, suggesting prospects of its further optimization as an EphA2 inhibitor.

Receptor protein tyrosine kinases (RPTKs) are important in a broad spectrum of cellular functions including proliferation, differentiation and migration. EphA2 protein tyrosine kinase belongs to the largest RPTK subfamily, which is Eph/Eck.[1] In contrast to the rest of the subfamily members, EphA2 has a broad tissue distribution and is expressed in brain, lung, kidney and other tissues that contain high amounts of epithelial cells.[2,3]

Table 1 Novel inhibitors of EphA2.

Compound		IC_{50}[a]/μM	Ki_{calc}[b]/μM
1		2.2±0.3	8.4
2		0.6±0.2	5.1
3		1.2±0.2	1.9
4		1.0±0.3	0.3
5		1.0±0.2	1.4

[a]IC_{50} values (mean values±SD) were calculated graphically as the concentrations causing 50% inhibition of the kinase. [b]Inhibition constants were estimated from the dG-score calculated by the Lead Finder software.

The recruitment of PI3-kinase by phosphorylated EphA2 is critical for RAC1 GTPase activation and vascular endothelial cell migration and assembly.[4,5] Elevated expression of EphA2 combined with downregulation of its native ligand Ephrin-A1 in multiple malignant tumor types such as carcinomas and gliomas[6] make the former a promising anticancer target. Despite that, no small molecule drugs are available that primarily target EphA2.

In order to discover novel fragment-like inhibitors of EphA2, we have applied a high-throughput screening technique† combined with molecular modelling.‡ The inhibitors found were additionally profiled against a panel of 19 PTKs and showed noticeable inhibition in many cases.

Primary screening determined five compounds with detectable inhibition of EphA2 activity. The activity of these compounds was further validated by fourfold inhibition measurement at the inhibitor concentration of 10 μM (Table 1). The inhibition curves generated for EphA2 in the presence of increasing concentrations of compounds **1**–**5** showed dose-dependent reductions of kinase activities with the IC_{50} values presented in Table 1.

Database searches performed with Chemical Abstracts Service and PubChem BioAssay§ showed that the identified compounds have not yet been described as kinase inhibitors. Given the fragment-like nature of the recovered hits, one can interpret them as novel scaffolds for the development of original series of potent kinase inhibitors.

Interestingly, the discovered compounds lack typical kinase inhibitor pharmacophore composed of an aromatic amino group (a hydrogen bond donor) with an adjacent aromatic nitrogen or carbonyl oxygen atom (a hydrogen bond acceptor). This moiety is known to form two correlated hydrogen bonds with the kinase hinge region. Instead, these compounds possess a catechol moiety which was hypothesized to be capable of forming the system of hydrogen bonds mentioned above. A docking study was performed in order to predict the binding mode of the compounds upon interaction with the ATP binding site of EphA2. It was shown that the catechol moiety can form at least two hydrogen bonds with backbone atoms of hinge region residues E693 and M695 (residue numbering corresponds to PDB struc-

ture 1mqb) in the manner characteristic of kinase inhibitors (Figure 1). In order to obtain the further proof of the predicted binding mode, we have performed a search for similar patterns of kinase inhibition in the Protein Data Bank[¶] using the catechol moiety as a ligand substructure query. The subsequent visual analysis of the matching protein-ligand complexes allowed us to find a single structure representing a complex of PI3-kinase with flavonoid myricitin (PDB ID 1E90) in which a 2,5-dihydroxyphenol moiety of the flavonoid was similar to the predicted binding mode of catechol moiety (Figure 1). However, the same substructure search retrived a different binding mode, which was present in the majority of flavonoid/kinase complexes and involved hydrogen bonding between the hinge region and the ketone group with the adjacent OH group of a flavonoid. This observation allows one to distinguish between catechol and flavonoid scaffolds based on the differences of the hinge-interacting moiety. Thus, our preliminary results prove that catechol may constitute a novel pharmacophore moiety of EphA2 inhibitors presumably interacting with the kinase hinge region.

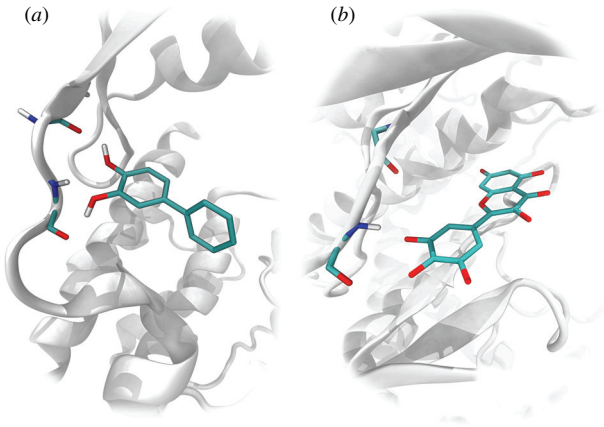


Figure 1 (*a*) Modeled binding mode of compound **1** in the active site of EphA2. The catechol moiety forms hydrogen bonds with the hinge region residues E693 and M695. (*b*) Binding mode of myricitin in the active site of PI3-kinase (PDB ID 1e90, hydrogen atoms not shown).

To further characterize the discovered compounds, they were screened against the panel of 19 PTKs. As can be seen in Table 2, notable inhibition of other kinases and a moderate selectivity was determined for all of the compounds screened. Novel inhibitors were especially active against EGFR, FGFR1, FGFR2, Abl and PDGFR-a. Molecular docking results indicate that the binding mode of the compounds upon interaction with several protein kinases, namely Csk, EGFR, FGFR1, FGFR2 and KDR remains the same with that observed for EphA2. Thus, the 2-hydroxyphenol group represents a general pharmacophore for tyrosine kinase inhibitors, which is important for targeting several kinases simultaneously, for example, EphA2 and EGFR.

However, there are multiple issues to be addressed regarding the use of catechol moiety in drug design. Despite being present in marketed drugs, the 2-hydroxyphenol group is known to be readily metabolized.[9] It is also implicated in activating Nrf2 *via* oxidative modification of KEAP1[10] and in inhibiting lipoxygenase activity.[11] Both of these actions enhance cellular defense and should be carefully considered for they might increase drug

Table 2 Inhibition of tyrosine kinases by compounds **1**–**5** at 10 μM concentration.[a]

Compound	**1**	**2**	**3**	**4**	**5**
Syk	6±4	9±4	13±5	8±1	3±1
CSK	48±12	90±16	81±13	39±5	14±14
IGF1R	4±3	4±4	64±11	26±3	2±5
EGFR	60±11	57±13	44±12	48±7	27±13
InsR	35±21	48±12	35±9	4±1	7±10
Lyn	68±10	80±16	65±29	55±5	39±19
FGFR1	80±20	91±15	58±30	62±12	54±14
FGFR2	76±20	85±17	64±24	67±18	54±11
Alk	28±14	28±15	36±11	22±6	7±9
Abl	77±12	10±9	64±24	68±10	64±15
ACK	13±6	0	9±13	32±18	12±4
BLK	60±19	0	58±28	74±13	0
CSFR	30±19	0	34±14	34±16	14±14
CTK	31±7	39±15	24±12	63±33	30±20
PDGFR-a	84±28	18±7	103±13	104±3	77±36
PYK	0	0	0	63±27	24±25
KIT	0	0	0	0	0
KDR	12±4	13±4	29±17	93±14	0
YES	0	10±2	14±6	46±4	0

[a]Percentage of inhibition [average±SD, n = 2 or data from two experiments (first – in duplicates, second – in quadruplicates) were combined].

[†] The screening was performed against chemical compounds from the kinase set of ChemBridge Corp. historical library, http://chembridge.com/datasheets/KINASet.pdf. All compounds were dissolved in DMSO to give 1 mM stock solutions that were further stored at –20 °C.

Twenty human PTKs in the form of full length proteins or fragments containing kinase domains, namely, Abl, ACK1, ALK, Blk, CSF1R, Csk, Ctk, EGFR, Eph A2, FGFR1, FGFR2, IGFR1, Insr, KDR, Kit, Lyn, PDGFR-a, Pyk2, Syk and Yes with N-terminal 6xHis-tags were obtained from SF9 insect cells by baculoviral expression followed by metal-chelating chromatography on Ni^{2+} Sepharose. The concentrations of isolated proteins were quantified by a Bradford assay; the molecular weights and homogeneity of PTKs were controlled by Coomassie staining of SDS-PAAG, and specific activities of the enzymes were determined as the amount of phosphate transferred to the poly(Glu-4 Tyr) substrate per minute per mg of protein by a radioactive phosphorylation assay.[7]

In order to evaluate the inhibitors kinase-Glo Plus Luminescent ATP consuming kinase assay (Promega) was used to detect PTKs activity *via* the decrease of ATP. Purified enzymes in the amount enough to consume 60–80% ATP in a kinase reaction (usually, 30–100 ng) were incubated for 30 min with an inhibitor (10 μM) or DMSO (1%) in a 1x kinase buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM $MnCl_2$, 5 mM $MgCl_2$, 0.01% Tween-20, 0.5 mM Na_3VO_4 and 2 mM DTT. Upon incubation, kinase reactions were started by the addition of an ATP/substrate mixture and allowed to proceed for 90 min at 30 °C. Assays were performed manually or with MultiPROBE II (Packard) in the total volume of 30 μl containing 10 μM ATP and 50 μg of poly(Glu-4 Tyr) substrate in 384-well opaque OptiPlates and were developed with 15 μl of ATP consumption luminescent reagent using a Fusion Universal Microplate Analyzer (Perkin–Elmer). All the experiments were performed twice either in duplicate (primary screening) or in quadruplicate (validation). Each assay plate was carrying kinase(+) and kinase(–) control wells with ATP and pan protein kinase inhibitor staurosporine.

For the inhibition curves generation, series of twofold dilutions of inhibitors in a 1x kinase buffer were tested as described above. Experiments were done in triplicates and repeated two times. IC_{50} values (mean values±SD) were calculated graphically as the concentrations causing 50% inhibition of kinase. The amount of undigested ATP in kinase reaction was kept at 50±10% from starting ATP level during the IC_{50} determination experiments.

[‡] Ligand docking was performed with the Lead Finder software[8] v. 1.1.11 using its default configuration parameters. Full-atom protein kinase models were prepared from the corresponding raw PDB structures by adding hydrogen atoms and assigning ionization states of the amino acids using the Model Builder module of the Lead Finder software package. The formation of hydrogen bonds with the hinge region of a kinase for each top-scoring ligand pose obtained by docking was monitored with the Structure Filter module of Lead Finder. If at least one hydrogen bond with the hinge region was formed, the ligand was recognized as a potential kinase inhibitor. Ligand binding energies and inhibition constants were estimated from the dG-score calculated by Lead Finder.

[§] Chemical Abstracts Service: http://www.cas.org/expertise/cascontent/registry/index.html

PubChem BioAssay database: http://www.ncbi.nlm.nih.gov/sites/entrez?db=pcassay

[¶] http://www.rcsb.org

resistance in cancer cells. A probable way to overcome these obstacles could involve the methylation of one hydroxyl group.

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