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In silico identification of novel EGFR inhibitors with antiproliferative activity against cancer cells

Claudio N. Cavasotto,[a,*] María A. Ortiz,[b] Ruben A. Abagyan[c] and F. Javier Piedrafita[b,*]

[a]*Molsoft LLC, 3366 N. Torrey Pines Ct. Ste. 300, La Jolla, CA 92037, USA*
[b]*Sidney Kimmel Cancer Center, 10835 Road to the Cure, San Diego, CA 92121, USA*
[c]*The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, USA*

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Abstract—Inhibition of the epidermal growth factor receptor (EGFR) tyrosine kinase activity by small molecules has proved effective for the treatment of cancer. To the best of our knowledge, the crystal structure of EGFR has been used for the first time to identify novel inhibitor chemotypes by docking-based in silico screening of a large virtual chemical library followed up by experimental validation. We identified several compounds with antiproliferative effects on cancer cells. Amongst them, a C(4)-N(1)-substituted pyrazolo[3,4-*d*]pyrimidine MSK-039 (**39**) was discovered as a low-micromolar inhibitor of EGFR tyrosine kinase activity. The predicted binding mode of **39** opens a new avenue toward the optimization of novel chemical entities to develop potent and selective inhibitors of EGFR signaling.

The design of protein kinase ATP-competitive inhibitors was initially considered a challenging task due to the similarities in the binding pockets across the kinase family. However, many potent inhibitors have been reported exhibiting an acceptable degree of selectivity;[1] recently highly selective inhibitors of the ribosomal protein S6 kinase were designed using a structural bioinformatics approach.[2] Interestingly, based on the information available, it was asserted that small changes in kinase inhibitors may have a strong influence on the binding mode and the binding kinetics.[3] This evidence opens up an opportunity to improve existing leads and, moreover, provides motivation to further develop novel kinase inhibitors, since therapeutic response might not be correlated with the smallness of the variation in the chemical structure.

The epidermal growth factor (EGF) is the prototype of a large family of peptide ligands that bind to cell membrane receptors and activate a myriad of intracellular signaling pathways to control tumor cell growth, proliferation, survival, metastasis, and angiogenesis.[4] The EGF receptor (EGFR, ErbB1, or HER1) is one of a four-membered family of transmembrane receptors that, similar to HER2, is frequently overexpressed in cancer cells, correlating with poor prognosis.[5] EGFR therefore represents a rational target for the development of novel anticancer therapies. Besides monoclonal antibody-based therapies, a number of small molecule inhibitors of EGFR tyrosine kinase are under clinical development.[6,7] The anilinoquinazoline derivatives ZD1839 (gefitinib, Iressa) and OSI-774 (erlotinib) are ATP-competitive selective EGFR inhibitors that have recently been approved for the treatment of patients with advanced non-small cell lung carcinoma and are being clinically evaluated in patients with various types of cancers. Also under clinical evaluation are the EGFR/HER-2 dual inhibitors, quinazoline GW572016 and pyrrolopyrimidine PKI-166, and the irreversible inhibitors CI-1033 (PD183805) and EKB-569.[8] ZD1839 has shown excellent anticancer activity in vitro and in pre-clinical studies against a variety of tumor cells when used alone or in combination with other chemotherapeutic agents.[9–11] Despite these promising results and rapid FDA approval, the clinical response to Iressa is very variable with 27% of Japanese patients versus only 10% of US/European patients responding.[12] Additional variability was observed in male versus female and in patients that had or had not previously smoke. Furthermore, disap-

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* Corresponding authors. Tel.: +1 858 625 2000x112; fax: +1 858 625 2888 (C.N.C); tel.: +1 858 410 4188; fax: +1 858 450 3251 (F.J.P); e-mail claudio@molsoft.com; jpiedrafita@skcc.org

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pointing results were found in subsequent clinical trials in which Iressa was given in addition to traditional chemotherapy. The reasons for this unpredictable clinical response were unclear until the recent discovery of somatic mutations in the *EGFR* gene that occur in patients with non-small cell lung cancer that responded to treatment.[13–15] Most of the mutations included missense substitutions and deletions located within the kinase domain rendering proteins that were more sensitive to Iressa-mediated inhibition.[13,14] These mutant EGFR selectively activate anti-apoptotic pathways, including Akt and STAT.[16] Therefore, genetic screening prior to treatment would identify patients that could benefit from Iressa. The fact that 80–90% of lung cancer patients do not have mutant EGFR and do not respond to the currently available EGFR inhibitors encourages additional research to develop novel small molecule inhibitors that can effectively inhibit both wild type as well as mutant EGFR proteins.

Two crystal structures of EGFR bound to inhibitors are available,[17,18] and virtual screening (VS) based on docking and scoring of chemical libraries to a protein target has not been reported to date, to the best of our knowledge, to discover new inhibitors of EGFR. VS is an in silico alternative to high-throughput screening that imposes a structure-based filter on a chemical database, thus generating a prioritized 'targeted' or 'focused' library. This reduces the number of compounds to be tested experimentally to those that fit better within the binding pocket.

Our goal was to explore through VS of a diverse chemical library the possibility of discovering novel hits that may be developed into drug leads. The ICM VS program[19,20] was used, which has been benchmarked on protein kinases[21–23] and recently successfully used to discover novel antagonists of the thyroid hormone receptor[24] and inhibitors of the peptide deformylase.[25] The ICM-based VS procedure is based on fast docking of a flexible ligand to a grid representation of the receptor[26] followed by scoring. The ICM-score takes into account the ligand–receptor interaction energy, conformational strain energy of the ligand, conformational entropy loss, and desolvation effects.[27] The 3D structure of EGFR bound to OSI-744 (PDB code 1M17) was screened against a collection of 315,102 compounds of the ChemBridge Express Library (the second crystal structure of EGFR bound to GW572016, PDB code 1XKK, had not been yet released at the time the VS was performed). Due to strong contacts with the receptor, the water molecule within the binding site was included in the VS (remarkably, the crystal structure 1XKK, which exhibits significant structural differences in the binding site, also has this conserved water molecule). The presence of this water molecule was needed to redock the EGFR inhibitor AG1478, used as a control.

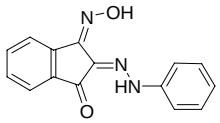
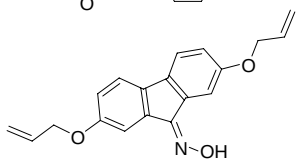
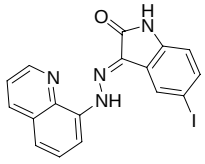
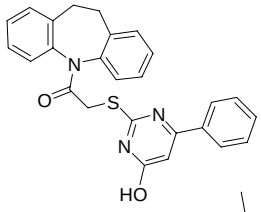
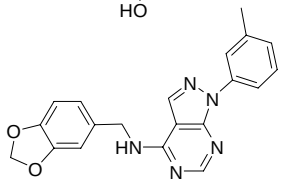
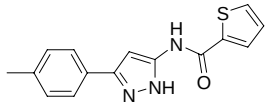
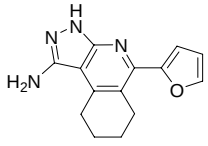
Self-docking of co-crystallized ligand OSI-744 showed a score of −35. Thus, a permissive cutoff score was chosen as −28, to take into account limitations stemming from the rigid representation of the receptor and inaccuracies in the scoring function. There is also strong evidence that in the protein kinase family, inhibitors make hydrogen bonds to hinge-region backbone atoms (corresponding in EGFR to carboxylic O in Q767, and carboxylic O and HN in M769). For this reason, 1246 compounds (from a total of 315,102) scoring better than −28 and making at least two hydrogen bonds with the hinge-region backbone were clustered according to chemical similarity and visual inspection, resulting in a final list of 212 compounds. The 50 best-scoring molecules were evaluated for their effect on EGFR tyrosine kinase activity and their antiproliferative activity in cancer cells.

A preliminary screening was performed with EGFR purified from A431 cells that was pre-incubated with 100 μM of the 50 compounds. The concentration of ATP in the reaction mixture was kept at 2.5 μM, four times lower than the K_m calculated for this enzyme preparation. Table 1 shows compounds that exhibited more than 40% inhibition of EGFR tyrosine kinase activity (the inhibition activity of all 50 compounds is shown in Supplementary data). Some of the molecules evaluated -including compound MSK-039 (**39**)-precipitated in the reaction mixture and were tested at lower concentrations at which no precipitates were observed. Except for **39** (see below), no significant activity was observed with reduced concentrations of those compounds (not shown). Next, titration experiments were performed with those molecules that exhibited significant EGFR tyrosine kinase activity. EGFR kinase activity was inhibited by as much as 40% with 10 μM compound **39**, which exhibited an estimated IC_{50} of 15 μM (Fig. 1A). We noticed increasing amounts of precipitate when compound **39** was used at concentrations higher than 30 μM, which could explain in part why maximum inhibition of EGFR activity was seen at this concentration of the inhibitor. All other compounds exhibited dramatically less inhibition of EGFR when used at 30 μM and were not active when the concentration was decreased to 10 μM (Fig. 1A and data not shown).

The inhibition of EGFR kinase activity by **39** was barely affected by the amount of enzyme (not shown). In contrast, increasing the concentration of ATP in the reaction mixture up to two times the K_m (20 μM) completely prevented the inhibition of EGFR by **39**, while reducing amounts of ATP had the opposite effect (Fig. 1B). These data support that compound **39** behaves as a true ATP competitor.

Compounds with significant inhibition of EGFR kinase activity were further evaluated for their antiproliferative activity against cancer cells. We chose A431 epidermoid carcinoma cells as a cellular model that expresses very high levels of EGFR. All EGFR inhibitors significantly prevented cell growth when tested at 50 μM, with the exception of compound MSK-041 (**41**) (Fig. 2A). Similar results were also observed when the antiproliferative activity of these compounds was investigated in breast (BT-474, MDA-MB-435, and MDA-MB-468) and ovarian (SkOv3) carcinoma cells. In contrast, these molecules elicited poor antiproliferative activity against A549 and H441 lung carcinoma cells (not shown). Compound MSK-032 (**32**) elicited the strongest inhibition at

Table 1. Effect of small molecules on EGFR kinase activity in vitro

Compound	Structure	Kinase activity (% of control)[a]	Chemical name
2		50.3 ± 6.1 (n = 13)	1*H*-Indene-1,2,3-trione-2-(phenylhydrazone) 1-oxime
16		55.3 ± 6.7 (n = 8)	2,7-Bis(allyloxy)-9*H*-fluoren-9-one oxime
30		50 ± 5.1 (n = 6)	5-Iodo-1*H*-indole-2,3-dione 3-(8-quinolinylhydrazone)
32		41.1 ± 4.7 (n = 7)	2-{[2-(10,11-Dihydro-5*H*-dibenzo[*b*,*f*]azepin-5-yl)-2-oxoethyl]thio}-6-phenyl-4-pyrimidinol
39		48.4 ± 6.7 (n = 6)	*N*-(1,3-Benzodioxol-5-ylmethyl)-1-(3-methylphenyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine
41		44.1 ± 7.9 (n = 11)	*N*-[3-(4-Methylphenyl)-1*H*-pyrazol-5-yl]-2-thiophenecarboxamide
47		44.5 ± 7.9 (n = 9)	5-(2-Furyl)-6,7,8,9-tetrahydro-3*H*-pyrazolo[3,4-*c*]isoquinolin-1-amine

[a] Purified EGFR was pre-incubated with 100 μM of the different molecules for 30 min, when kinase activity was measured as described in Experimental information (see Supplementary data). As control, EGFR was incubated in the presence of 2% DMSO. The data show averages ± standard deviation obtained in 2–5 experiments performed in triplicate.

50 μM and completely inhibited cell growth of the three breast cancer cell lines analyzed (data not shown). The reason why compound **41** elicited no antiproliferative activity despite inhibiting EGFR kinase activity is not completely understood. It is possible that **41** is not cell permeable or it is metabolized into an inactive compound.

Titration experiments were next performed with active molecules. With the exception of compounds MSK-002 (**2**) and MSK-030 (**30**), the inhibition of cell growth by other EGFR inhibitors (including **39**, which significantly inhibited EGFR in vitro at low concentrations) was notably reduced when the concentration was lowered to 25 μM or below. Figure 2B shows the dose–response curves for **2** and **30**. The antiproliferative activity of compound **30** did not increase at concentrations above 2 μM ($IC_{50} \sim 1$ μM), while almost maximum antiproliferative activity was reached at 8 μM with compound **2** ($IC_{50} \sim 6$ μM). The reason why compounds **2** and **30** are such strong antiproliferative molecules at lower concentrations at which they exhibit no inhibition of EGFR kinase activity in vitro (see Fig. 1A) is not known. They could probably be acting through additional signaling pathways besides EGFR or they could be metabolized into more active compounds inside the cell. Both **2** and **30** induced morphological changes reminiscent of apoptosis. Whether or

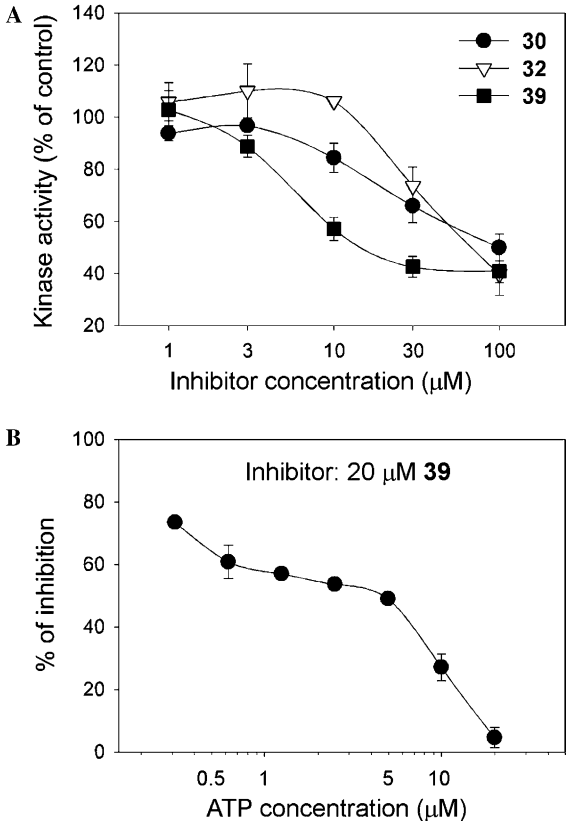


Figure 1. Titration of novel EGFR inhibitors. (A) Purified EGFR was incubated with the indicated concentrations of **30**, **32**, or **39** for 30 min at room temperature, after which the kinase reaction was initiated by addition of ATP/$MgCl_2$ and continued for 30 min at 30 °C. The percentage of activity with respect to control reactions performed in the absence of EGFR inhibitor is shown. The data represent averages ± standard deviation from 1 to 3 experiments performed in triplicate. (B) Inhibition of EGFR by **39** is competed by ATP. Purified EGFR was pre-incubated with 20 μM **39** for 30 min when kinase reaction was initiated by addition of increasing concentrations of ATP. Control reactions contained equal amounts of ATP and EGFR, but not inhibitor. The experiment was performed twice with identical results and shown here are the results of one experiment performed in triplicate.

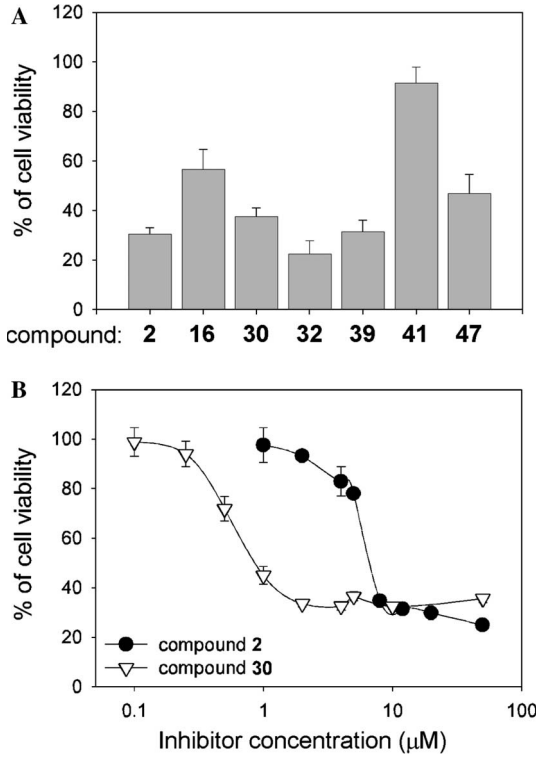


Figure 2. Antiproliferative activity of novel EGFR inhibitors. (A) A431 cells were incubated with 50 μM of the indicated EGFR inhibitors for 2 days, when cell viability was measured by MTT. Control cells were grown in the presence of 0.5% DMSO and the averages ± standard deviation percentage obtained in three experiments performed in quadruplicate is shown. (B) Titration of **2** and **30**. A431 cells were grown in the absence (control) or in the presence of increasing concentrations of **2** (closed circle) or **30** (inverted triangle). Cell proliferation was measured by MTT 48 h after treatment. The results of two experiments performed in triplicate are shown.

not these compounds cause cell death in A431 and other cancer cell lines is currently under investigation.

The structure of the EGFR-**39** complex was refined using a flexible-ligand–flexible-receptor methodology,[28] unfixing also the six degrees of freedom of the water molecule. The predicted binding pose of **39** is shown in Figure 3. The amino H and the N(5) of the pyridimine ring make hydrogen bonds with backbone atoms of M769. The interaction of the ligand with T766 is mediated by a water molecule, and the 3-methylphenyl moiety substituted at N(1) is buried within the binding pocket. This shows that *N*-(1,3-benzodioxol-5-ylmethyl)-1-(3-methylphenyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (**39**) binds in a different way compared to the predicted mode of C(3)–C(4) substituted pyrazolo[3,4-*d*]pyrimidines, where N(1) and N(7) make a donor–acceptor system hydrogen bonded to the backbone.[29] In that work, the authors applied a pharmacophore approach using an EGFR model based on the cyclic-AMP-dependent protein kinase to optimize micromolar hits. Thus, the difference in binding modes arises from the difference in substitution points between these two classes of compounds. Figure 4 shows the superposition of **39** with OSI-744 (PDB 1M17). Both compounds superimpose well, however it is evident that the amine nitrogen plays a completely different role in each of the compounds, since in fact the amine N of OSI-744 corresponds to the pyrazole N(1) in **39**. The binding mode of the pyrazolo[3,4-*d*]pyrimidine ring resembles more the binding of the ATP purine ring (not shown).

In summary, to the best of our knowledge, the crystal structure of EGFR has been used for the first time to perform a virtual screening of a large chemical library and select only those compounds that best fit into the binding site for subsequent biological evaluation. The discovery of *N*-(1,3-benzodioxol-5-ylmethyl)-1-(3-methylphenyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (**39**) as a low-micromolar EGFR inhibitor with a unique binding mode opens a new avenue toward the development of novel drug scaffolds as potent and selective inhibitors of EGFR tyrosine kinase activity. Among the different EGFR inhibitors described here, **39** is not the one with highest growth inhibitory activity against cancer cells. Other molecules (**2** and **30**) elicited stronger antiprolifer-

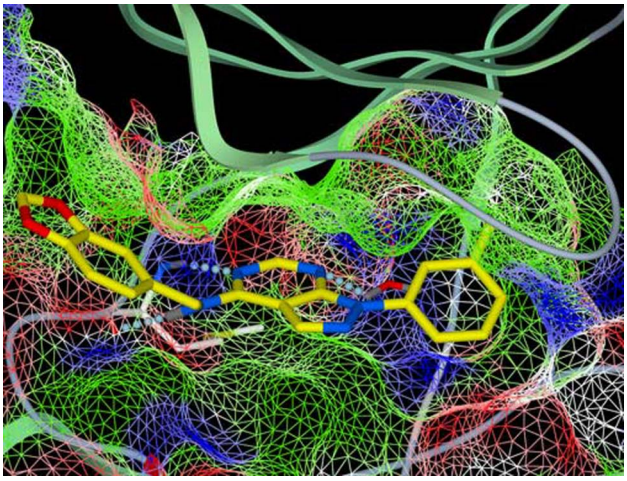


Figure 3. Predicted binding pose of **39** within the binding site of EGFR. The ligand is anchored by hydrogen bonds from O and HN of M769 with amino H and the N(5) of the pyridimine ring, respectively. The water molecule makes hydrogen bonds with the N(7) of the pyridimine ring and the T766. The 3-methylphenyl moiety is buried into the hydrophobic cavity. The receptor is shown in gray ribbon representation and the surface of the binding site is displayed as a mesh colored according to binding properties: hydrophobic, green; blue, hydrogen bond donor; red, hydrogen bond acceptor. Color codes for atoms: carbon, yellow; oxygen, red; nitrogen, blue; polar hydrogens, dark gray. Hydrogen bonds are depicted as small balls. (Image generated with ICM.)

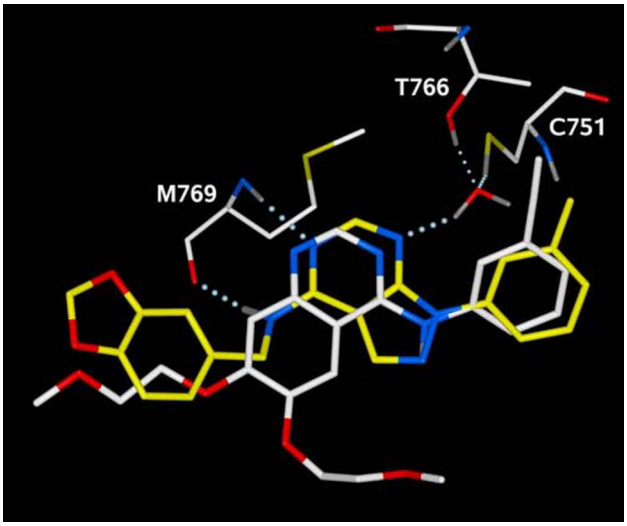


Figure 4. Comparison of the predicted binding mode of **39** and OSI-744 (PDB code 1M17). Relevant side chains of the complex EGFR-**39** are displayed. Color codes: oxygen, red; nitrogen, blue; polar hydrogens, dark gray; sulfur, green. Carbon atoms are shown in yellow (**39**) and white (protein and OSI-744). Hydrogen bonds are shown as small balls. (Image generated with ICM.)

ative activity even at concentrations at which no inhibition of EGFR kinase activity was observed in vitro. Taken together, our data suggest that compounds **2** and **30** may be targeting additional cellular pathways besides EGFR, whereas **39** could be more selective toward EGFR. This warrants further structure–activity relationship studies with compound **39** as a lead for EGFR-based drug discovery. Of particular interest for clinical applications will be to analyze the effect of optimized inhibitors on wild type and mutant EGFR proteins.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.12.067.

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