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5-Benzylidene-hydantoins as new EGFR inhibitors with antiproliferative activity

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Abstract—A series of 1,5-disubstituted hydantoins, whose structure was designed to interact at the ATP binding site of EGFR, was synthesized and evaluated for inhibition of EGFR kinase activity and antiproliferative action. Some of these compounds, characterized by a 1-phenethyl and a 5-(*E*)-benzylidene substituent, inhibited EGFR autophosphorylation and polyGAT phosphorylation, and also inhibited the growth and proliferation of human A431 cells, which overexpress EGFR. These compounds can therefore be regarded as examples of a new scaffold for tyrosine kinase inhibitors.

Receptor protein kinases play a central role in signal transduction pathways, regulating cell division and differentiation. Among them, the epidermal growth factor receptor (EGFR) is involved in the regulation of several key processes such as cell proliferation, survival, adhesion, migration, and differentiation.[1] Overexpression of EGFR tyrosine kinase is reported in a variety of human tumors and is associated with poor prognosis.[2] Therefore, inhibition of EGFR kinase activity has emerged as a promising new approach to cancer therapy and several small molecule tyrosine kinase inhibitors are currently in clinical use or development. The 4-anilinoquinazolines gefitinib (Iressa™) and erlotinib (Tarceva™), which have both been approved for the treatment of non-small-cell lung cancer, are potent, competitive inhibitors at the tyrosine kinase ATP-binding site (Fig. 1).

Extensive SAR studies of 4-anilinoquinazolines[3] and co-crystallization of erlotinib within the catalytic domain of the EGFR[4] provided detailed information on the interactions at molecular level between this class of inhibitors and the adenine-binding portion of the ATP-binding site. The EGFR-erlotinib co-crystal structure showed that N1 of the quinazoline nucleus is involved in a hydrogen bond with the backbone NH of Met769, the CH in 2 is very close to the backbone C=O of Gln767, and the N3 interacts with Thr766 through a water molecule.[4]

Employing the EGFR coordinates from this complex, and following the hypothesis that hydantoin could work as a bioisoster of the quinazoline nucleus, we designed and docked 1,5-substituted derivatives into the erlotinib-binding site. The imide fragment of hydantoin ring has the pharmacophoric elements described above, only needing appropriate lipophilic groups in 1 and 5 to mimic the aniline portion and the second benzene ring in anilinoquinazolines. In fact, 1-phenethyl-5-benzylidene-hydantoin (**2a** in Table 1) can be easily superimposed on the active conformation of erlotinib, and docked into the ATP-binding site of EGFR, as shown in Figure 2. In this docking model, the hydantoin ring undertakes hydrogen bonds in the hinge region resembling those of the quinazoline nucleus, while the side chains in positions 1 and 5 can occupy hydrophobic pockets in the ATP-binding site of EGFR.

To validate our hypothesis, and to get a starting exploration of structural requirements, a series of hydantoins

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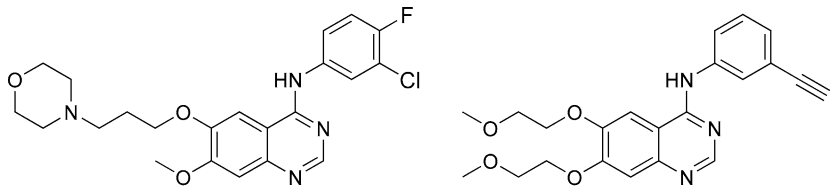
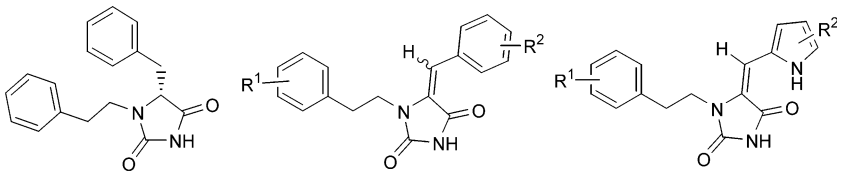


gefitinib (Iressa™) erlotinib (Tarceva™)

Figure 1. EGFR TK inhibitors recently approved for the clinical use.

Table 1. Inhibition of EGFR kinase and antiproliferative activity for compounds **1** and **2a–h**



1 **2a-f** **2g-h**

Compound	R^1	R^2	Isomer	Kinase assay inhibition (%)[a,c]	Cell proliferation assay inhibition (%)[b,c]
1				0.0	0.0
2a	H	H	*E*	45.1	25.6
2b	H	H	*Z*	39.7	14.9
2c	H	3-Cl	*E*	43.4	16.2
2d	H	3-OH	*E*	52.8	30.3
2e	H	4-OH	*E*	60.9	39.6
2f	3-Cl	H	*E*	46.8	9.5
2g	H	H	*E*	23.9	12.9
2h	H	3,5-CH_3	*E*	42.1	30.4

[a] Percentage of inhibition at 10 μM; mean values of three independent experiments are reported.
[b] Percentage of inhibition at 20 μM; mean values of three independent experiments are reported.
[c] Gefitinib[10] showed 65.4% inhibition of the polyGAT phosphorylation when tested at 0.001 μM concentration in the kinase assay; it showed 36.6%

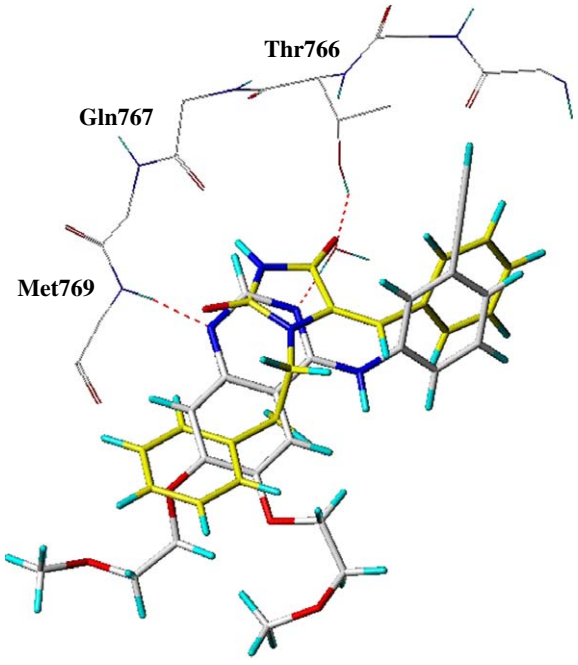
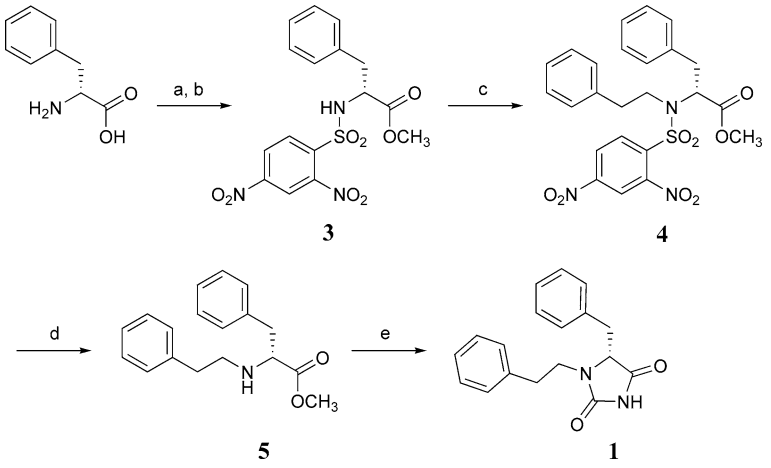
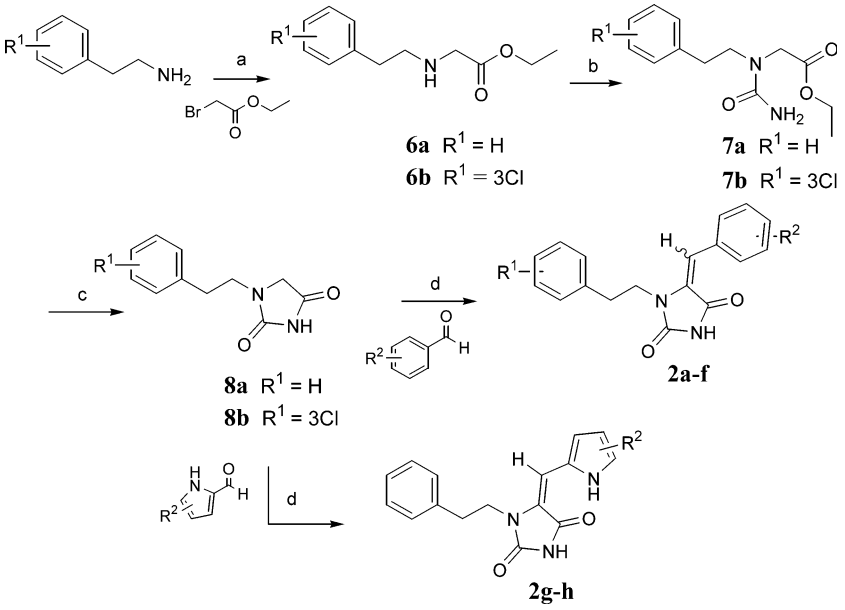


Figure 2. Compound **2a** (yellow carbons) docked within the catalytic domain of EGFR and compared to the co-crystallized erlotinib (gray carbons).

(Table 1) was prepared and tested on EGFR activity and on cell proliferation assays. In particular, (*R*)-5-benzyl-1-phenethylhydantoin **1** was synthesized starting from D-phenylalanine (Scheme 1): the 2,4-dinitrobenzensulfonamide **3**, readily prepared from 2,4-dinitrobenzensulfonyl chloride and D-phenylalanine methyl ester, was alkylated under Mitsunobu conditions according to a previously reported procedure.[5] Easy deprotection of **4** was achieved by treatment with thioglycolic acid and triethylamine in CH_2Cl_2. Finally, cyclization of **5** with potassium cyanate in acetic acid afforded the hydantoin **1** in good yield. Compounds **2a–f** and **2g–h**, carrying at the 5-position of the hydantoin ring a benzylidene or a (pyrrol-2-yl)methylidene substituent, respectively, were obtained by a four-step synthetic route (Scheme 2). 1-Phenethylhydantoin **8a** was prepared starting from phenethylamine and ethyl bromoacetate. The ethyl ester of *N*-phenethylglycine was converted to the urea **7a** with potassium cyanate, and then submitted to acid catalyzed cyclization to hydantoin.[6] This synthetic pathway was also applied to the preparation of the chloro-derivative **8b**, starting from 3-chlorophenethylamine. The products **2a–f**, obtained by the reaction between hydantoins **8a–b** and benzaldehyde or its derivatives in piperidine, were isolated as



Scheme 1. Reagents and conditions: (a) anhydrous CH_3OH, HCl gas, reflux, 10 min, 88%; (b) 2,4-dinitrobenzensulfonyl chloride (1 equiv), pyridine (3 equiv), CH_2Cl_2, rt, 16 h, 78%; (c) $PhCH_2CH_2OH$ (2 equiv), DEAD (2 equiv), PPh_3 (2 equiv), benzene, rt, 20 min, 94%; (d) $HSCH_2CO_2H$ (1.3 equiv), Et_3N (2 equiv), CH_2Cl_2, 23 °C, 1 h, 97%; (e) KOCN (2 equiv), CH_3CO_2H, rt, 4 h, 75%.



Scheme 2. Reagents and conditions: (a) anhydrous $CHCl_3$, rt, 2 h, 94–97%; (b) HCl (1.5 equiv), KCNO (1.5 equiv), water, rt, 20 h, 84–89%; (c) HCl, 25%, reflux, 4 h, 99%; (d) dry piperidine, 130 °C, 1 h, 25–69%.

mixtures of *E* and *Z* isomers in about 50% proportions. Only the *E* isomer was obtained for compounds **2g** and **2h**. The geometry of the products was assigned from NMR spectral data, after isolation of the stereoisomers by silica gel chromatography. Purity of all compounds, fully characterized by NMR and MS, was assessed by elemental analysis. This four-step synthetic route was flexible enough to give good yields for these and other different products (data not shown), being thus suitable for a combinatorial expansion of the series.

The hydantoin derivatives **1** and **2a**–**h** were evaluated in EGFR tyrosine kinase assays (Table 1). The ability of these compounds to inhibit the phosphorylation of the peptide substrate polyGAT by EGFR was measured.[7] Inhibitory activities are given as percentage of inhibition at 10 μM. In addition, the ability of compound **2e** to inhibit EGFR autophosphorylation was also assessed.[8] The antiproliferative activity of these inhibitors was assayed using the human epidermoid carcinoma cell line A431 which overexpresses EGFR.[9] Activities are given as percentage of inhibition of cell growth at 20 μM.

Compound **1** showed no effect on the enzymatic and cellular assays at concentrations up to 50 μM. As one of its minimum-energy, extended conformations could be easily overlaid onto that of the anilinoquinazoline (data not shown), we attributed this lack of activity to the entropic effect due to the free rotation around the C(5)–CH_2Ph bond. In fact, ^{1}H NMR suggested that the conformational equilibrium at this bond was shifted toward an eclipsed conformation, with the benzyl group above the hydantoin ring.[11] This behavior had also been previously observed in similar cases.[12] In our docking model,

the folded conformation of compound **1** was too sterically hindered to efficiently occupy the ATP-binding pocket of EGFR.

Therefore, it was supposed that an extended conformation should be necessary for the interaction at the ATP-binding site. To maintain the substituent at the 5-position on the same plane of the hydantoin ring, the benzyl group was replaced with a benzylidene moiety in compounds **2a** and **2b**. The *exo*-cyclic double bond led to derivatives whose phenyl group strictly overlaid the position of the anilino moiety of erlotinib in the complex with EGFR (Fig. 2). As expected, compound **2a** partially inhibited substrate phosphorylation, thus showing its ability to interact with EGFR, even if with a potency much lower than that of the anilinoquinazoline template. **2a** (*E*), represented in Figure 2, showed slightly higher inhibition on the enzymatic test than the *Z* isomer **2b**, which could be superimposed on the erlotinib structure only inverting the orientation of the 5-benzylidene and 1-phenethyl groups. While rapid conversion of the *Z* isomer to the *E* one was observed for the most potent compound (**2e**), no data on the conformational preference for the other, less active, compounds are available at the moment.

The planar compounds **2c**–**h** showed percentage of inhibition ranging from 45.1% to 60.9% when tested at 10 μM concentration on enzyme assay. Inhibitory activity increased when the benzylidene group was substituted by a hydroxyl group at both *meta* and *para* positions. A *meta*-chloro substituent on the *N*-1 phenethyl group led to **2f**, with only a small activity enhancement with respect to **2a**, while the chloro substitution at the *meta* position on the benzylidene group (**2c**) did not affect EGFR kinase activity. Replacement of the benzylidene group by a (pyrrol-2-yl)methylidene one[13] (**2g**) significantly reduced the inhibition of kinase activity. However, compound **2h**, having two methyl substituents in positions 3 and 5, showed a percentage of inhibition close to the parent compound **2a**.

Before proceeding to SAR analysis based on enzymatic inhibition, which was hampered by the low potency of these compounds and by the noise-to-signal ratio of the test at these concentrations, we looked for further indications that this class can be considered interesting for TK inhibition.

Compound **2e**, giving the highest enzyme inhibition on the substrate-based assay and showing an IC_{50} value of 0.71 μM (Fig. 3), also evidenced inhibitory activity toward EGF-stimulated EGFR autophosphorylation in A431 cells (Fig. 4). To assess whether the compound was able to inhibit the phosphorylation of EGFR at the level of Tyr1173, a phosphospecific anti-EGFR (Tyr1173) antibody was used. The percentage of inhibition of EGFR autophosphorylation at 20 μM concentration reached 50% after 4 h of incubation.

Compounds **2a**–**h** also inhibited the growth of A431 cell line overexpressing EGFR. A qualitative correlation was observed between enzymatic and cellular

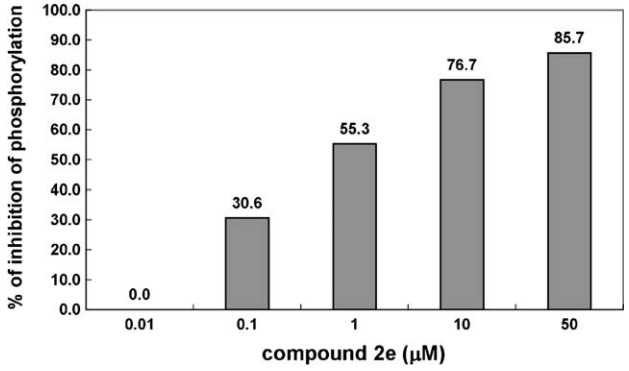


Figure 3. Percentage of inhibition of polyGAT phosphorylation by EGFR for **2e** (0.01–50 μM); mean values of two experiments are reported.

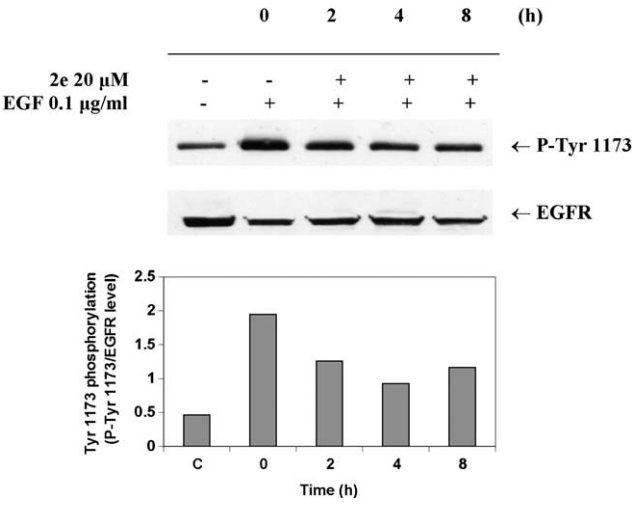


Figure 4. Western blot for phospho-Tyr1173 in A431 exposed for different times to **2e** at 20 μM and stimulated for 5 min with 0.1 μg/ml EGF. The levels of p-Tyr1173 at each point were quantified by densitometric analysis and normalized to the levels of EGFR.

results for these derivatives (Table 1). Compounds **2d** and **2e** showed the highest antiproliferative activity on A431 cell line, and the result parallels the potency observed in the enzyme assay. Moreover, **2e** showed an IC_{50} value of 27 μM in the cellular assay, which is consistent with the percentage of inhibition observed at 20 μM.

In conclusion, hydantoin derivatives showed inhibition of the EGFR kinase activity and antiproliferative effects toward A431 cells. The *exo*-cyclic double bond at the 5-position is essential for both enzyme and cell growth inhibition, suggesting that a rigid planar system is necessary to interact with the molecular target. The best results were obtained for compound **2e**, carrying a *para*-hydroxyl group on the benzylidene at the C-5 position. This compound was also active in the EGFR autophosphorylation assay. Even if further structure–activity investigation is needed for potency optimization within this class, particularly in the enzymatic test, the ability to control cell proliferation

indicates that access to the biophase is not a main drawback for these compounds.

Finally, the easy and flexible synthesis and workup for the 5-*exo*-methylene-substituted derivatives, which can be exploited to introduce different substituents at positions 1 and 5, allowing easy exploration of physicochemical space, highlight the potential of this scaffold to generate new kinase inhibitors.

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7. EGFR kinase assay was performed in 100 μL reaction mixtures containing kinase buffer (10 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM Na_3VO_4 and 0.1 mg/mL BSA), 4 U EGFR (Sigma Chemicals, MO, USA), 10 μL of 66 nM EGF (Sigma Chemicals, MO, USA), 10 μL of 5 mg/ml polyGAT peptide (Sigma Chemicals, MO, USA). Compounds (1 μL) dissolved in DMSO and conveniently diluted were added and the reactions initiated by adding 1 μL of 100 μM ATP, 2 μCi of [γ-^{32}P]ATP. After 60 min at 30 °C, the reactions were stopped by adding 900 μL TCA 11% and stored at 4 °C for 3 h. Afterwards, each reaction solution was spotted onto a phosphocellulose disc (∅2.5 cm, Whatman, P81), previously equilibrated in 1 mM ATP. Then, each disc was washed three times with TCA 5% at rt, plunged in TCA 5% for 40 min, dried, and incorporated ^{32}P was counted in a scintillation counter. Gefitinib 1 nM was used as an internal standard in each experiment. Percent inhibition of compounds was calculated by comparison with DMSO-treated controls.
8. Procedures for protein extraction, solubilization, and analysis by 1-D PAGE were described in Petronini, P. G.; Alfieri, R.; De Angelis, E.; Campanini, C.; Borghetti, A. F.; Wheeler, K. P. *Br. J. Cancer* **1993**, *67*, 493, A mouse anti-phospho-EGF receptor (Tyr1173) monoclonal antibody at 1:2500 dilution (Upstate, Cell Signaling Solution, Lake Placid, NY, USA) and a horseradish peroxidase (HRP)-secondary antibody at 1:20,000 dilution (Amersham Pharmacia Biotech, Buckinghamshire, UK) were used.
9. The cell line A431, purchased from the American Type Culture Collection (Rockville, MD, USA), was maintained in DMEM 4.5 g/L containing 10% FCS, at 37 °C in a water saturated atmosphere of 5% CO_2 in air. Proliferation rate was evaluated as described in Cavazzoni, A.; Petronini, P. G.; Galetti, M.; Roz, L.; Adriani, F.; Carbognani, P.; Rusca, M.; Fumarola, C.; Alfieri, R.; Sozzi, G. *Oncogene* **2004**, *23*, 8439.
10. Gefitinib was synthesized as described in Gilday, J. P.; David, M. WO Patent WO2004024703, **2004**.;
11. The vicinal H, H coupling constants, H–C(5)–CH_aH_bPh $^3J_{H5Ha} = {}^3J_{H5Hb} = 4.7$ Hz, were measured by ^{1}H NMR at Bruker 300 MHz Avance spectrometer. DMSO-d_6 was taken as solvent.
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