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Long-lasting inhibition of EGFR autophosphorylation in A549 tumor cells by intracellular accumulation of non-covalent inhibitors

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ABSTRACT

In the present study, a small set of reversible or irreversible 4-anilinoquinazoline EGFR inhibitors was tested in A549 cells at early (1 h) and late (8 h) time points after inhibitor removal from culture medium. A combination of assays was employed to explain the observed long-lasting inhibition of EGFR autophosphorylation. We found that EGFR inhibition at 8 h can be due, besides to the covalent interaction of the inhibitor with Cys797, as for PD168393 (**2**) and its prodrug **4**, to the intracellular accumulation of non-covalent inhibitors by means of an active cell uptake, as for **5** and **6**. Compounds **5–6** showed similar potency and duration of inhibition of EGFR autophosphorylation as the covalent inhibitor **2**, while being devoid of reactive groups forming covalent bonds with protein thiols.

The epidermal growth factor receptor (EGFR), belonging to the ErbB family of receptor tyrosine kinases, is a validated target in anti-cancer therapy. The first-generation reversible EGFR tyrosine-kinase inhibitors (TKIs) gefitinib[1] (**1**, Iressa, Fig. 1) and erlotinib[2] (Tarceva, Fig. 1) are approved for the treatment of non-small cell lung cancer (NSCLC) in patients carrying activating mutations in the EGFR-TK domain.[3] The emergence of secondary resistance mutations to TKIs, such as the gatekeeper T790M mutation in EGFR,[4] gave rise to the development of a second generation of inhibitors, endowed with a covalent mechanism of action. Several irreversible inhibitors, such as afatinib (BIBW2992, Fig. 1) and dacomitinib (PF299804, Fig. 1), are currently in clinical trials.[5] The ability of irreversible inhibitors to covalently interact with Cys797, within the ATP-binding site of the kinase domain, leads to long-lasting inhibition and improved efficacy towards gatekeeper-mutant and wild-type EGFR.

Long-lasting inhibition of EGFR autophosphorylation in cell cultures measured 8 h after compound removal from the culture medium, has been traditionally considered as an indirect clue of irreversible inhibition of the target.[6–8] Accumulating evidence suggested that persistent inhibition of EGFR autophosphorylation could be ascribed not only to direct covalent interaction between the inhibitor and the target,[6] but also to in situ bioconversion of a prodrug into the corresponding reactive species[9] as well as accumulation of a reversible inhibitor within the cell.[10] In particular, the role of intracellular accumulation of EGFR inhibitors on the outcome of cell-based assays of reversibility has not been addressed so far in a systematic way, despite trans-membrane transport and intracellular metabolism had been shown to play a role for gefitinib-sensitive and resistant cell lines.[11] In the present

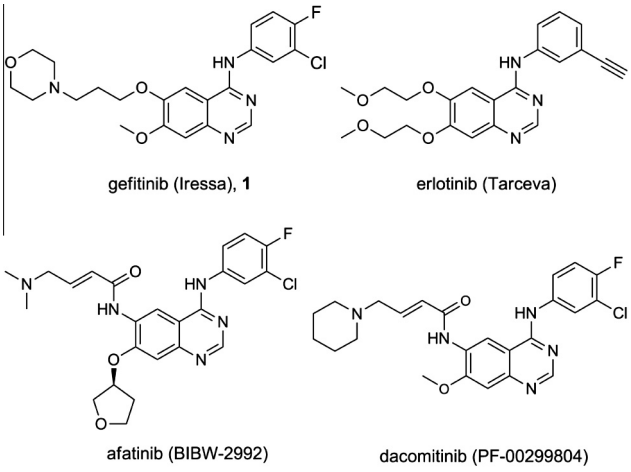

Figure 1. Chemical structures of reversible and irreversible EGFR-TK inhibitors.

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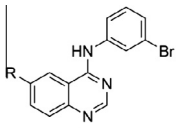
study, we present new data showing that persistent (8 h) inhibition of EGFR autophosphorylation by different drugs can be ascribed either to covalent binding or to intracellular accumulation in tumor cells. Furthermore, we provide a set of suitable and straightforward in vitro assays which can be usefully employed to interpret the cell-based data and to get insight into the mechanism of action of newly synthesized EGFR inhibitors.

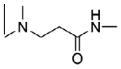
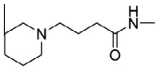
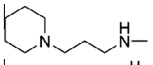
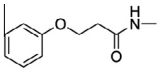
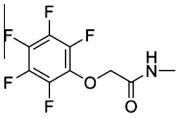
We selected a set of compounds from different chemical classes, endowed with different potential mechanisms of interaction with EGFR-TK (Table 1). The set included: (i) a covalent irreversible inhibitor (**2**, PD168393),[6] bearing a highly reactive acrylamide warhead; (ii) a 3-dimethylaminopropanamide prodrug (**4**), non-reactive per se, but able to generate the acrylamide **2** via β-elimination in the intracellular environment;[9] (iii) the non-covalent reversible EGFR inhibitors (**3**)[8] and gefitinib (**1**); (iv) the putative reversible inhibitors (**5**) and (**6**); and (v) the potentially irreversible inhibitors (**7**) and (**8**).[8] The 4-aminobutanamide **5** and the 3-(piperidinyl)propylamine **6** are expected to behave as reversible inhibitors as they do not present any reactive group that could directly or indirectly form a covalent bond with Cys797. Differently, the 3-phenoxypropanamide **7** could potentially undergo a β-elimination with subsequent release of phenol and conversion into a reactive acrylamide, following the mechanism already reported for **4** and in the literature.[12] The perfluorophenoxyacetamide **8** is potentially able to undergo a nucleophilic substitution by Cys797 of EGFR on the α-methylene group, with formation of a covalent conjugate with the enzyme and in situ release of a phenol leaving group. In fact, slow irreversible inactivation of caspase-1 catalytic cysteine had been reported for fluorophenoxymethylketones inhibitors.[13]

The 4-anilinoquinazoline compounds **4**–**7** were synthesized as described in Scheme 1. 6-Amino-4-(3-bromoanilino)quinazoline **3**[14] was reacted with 3-chloropropionylchloride or 4-chlorobutanoylchloride to obtain the amide intermediates **9** and **10**, respectively. As previously described,[9] substitution of the chlorine atom in **9** with dimethylamine or piperidine gave the 3-aminopropanamide compounds **4** and **11**, while substitution of **10** with piperidine under the same conditions led to the 4-aminobutanamide **5**. The amide group of **11** was reduced to the corresponding amine **6** with sodium bis(2-methoxyethoxy)aluminium hydride (Red-Al). Finally, the 3-phenoxypropanamide **7** was synthesized from **3** by coupling with 3-phenoxypropanoic acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI). Compounds **1**, **2**, and **8** were prepared as previously reported.[8] Purity

Table 1
EGFR tyrosine kinase inhibition, EGFR autophosphorylation inhibition and intracellular concentrations of compounds **1**–**8** in the A549 cell line



2-8

Compd	R	Kinase assay[a]	Autophosphorylation assay (A549)[b]				Intracell concentration[c] (μM)	
		IC_{50} (nM)	% inhibition (1 μM)		IC_{50} (nM)			
			1 h	8 h	1 h	8 h	1 h	8 h
1	Gefitinib	0.60[d]	96 ± 1.3[e]	46 ± 6.7[e]	39.9 ± 1.09	498 ± 1.10	271 ± 34	2.3 ± 0.2
2		1.69 ± 0.16[e]	98 ± 3.2[e]	86 ± 0.4[e]	3.94 ± 1.21	17.9 ± 1.23	<0.05[f]	<0.05[f]
3	H_2N–	n.d.[g]	91 ± 5.9[e]	0.0 ± 0.1[e]	21.45 ± 1.39	>20000	13 ± 2.4	<0.05[f]
4		0.28 ± 0.07[e]	97 ± 1.4[e]	89 ± 1.0[e]	8.59 ± 1.23	38.2 ± 1.35	26 ± 2.5	<0.05[f]
5		0.63 ± 0.13	98 ± 1.7	96 ± 2.5	5.70 ± 1.05	13.5 ± 1.08	147 ± 1.9	7.2 ± 0.3
6		0.41 ± 0.08	95 ± 3.5	90 ± 3.4	1.70 ± 1.11	14.1 ± 1.22	324 ± 24	105 ± 9.3
7		19.0 ± 4.04	97 ± 5.1	96 ± 3.4	21.7 ± 1.08	38.4 ± 1.07	72 ± 16	0.7 ± 0.1
8		23.6 ± 1.75	96 ± 4.3	90 ± 2.6	49.8 ± 3.69	118 ± 10.1	240 ± 9.8	3.6 ± 2.3

[a] Concentration to inhibit by 50% EGFR-wt tyrosine kinase activity measured by the phosphorylation of a peptide substrate using time-resolved fluorometry. Mean values of three independent experiments ± SEM are reported.

[b] Inhibition of EGFR autophosphorylation was measured in A549 intact cells by Western blot analysis. Percent inhibition at 1 μM and IC_{50}s were measured immediately after and 8 h after removal of the compound from the medium (1 h incubation). Mean values of at least two independent experiments ± SEM are reported.

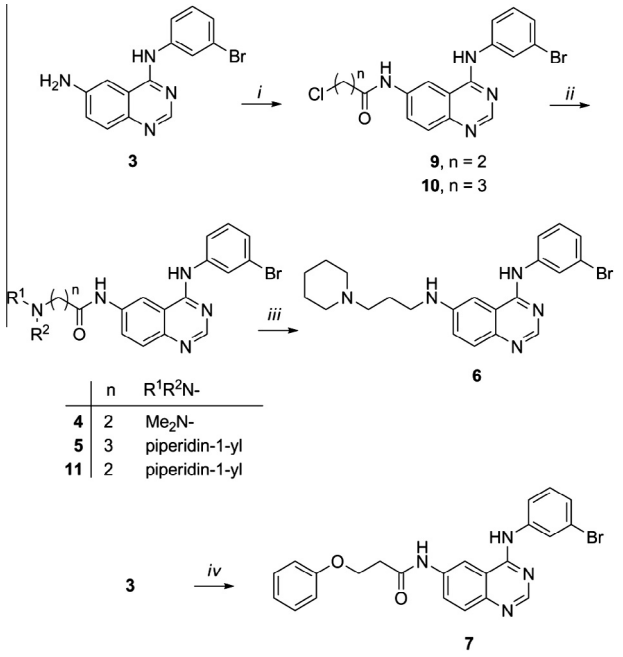
[c] A549 cell lines were incubated with 1 μM extracellular concentration of test compound for 1 h. Intracellular content was quantified immediately after and 8 h after removal of the compound from the medium as pmol/mg of protein in each sample. Reported μM concentrations were calculated dividing the compound content (in pmol) by an average value of cell volume per mg protein (4 μL/mg protein) estimated by measurements of the equilibrium distribution in A549 cells of 3-*O*-methyl-D-[1-^{3}H]glucose using the method of Kletzien et al.[19]

[d] Data from Ref. 20.

[e] Data from Ref. 9.

[f] Below the limit of detection of the analytical method.

[g] Not determined.



Scheme 1. Reagents and conditions: (i) 3-chloropropionylchloride (to **9**), or 4-chlorobutanoylchloride (to **10**), 50 °C, 16 h, yields: 86% (**9**) and 82% (**10**); (ii) Me_2NH (to **4**), or piperidine (to **5** and **11**), rt, 24 h, yields: 86% (**4**), 41% (**5**) and 78% (**11**); (iii) sodium bis(2-methoxyethoxy)aluminumhydride (Red-Al), anhydrous THF, 0 °C to rt, 16 h, yield 48%; (iv) 3-phenoxypropanoic acid, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), anhydrous THF, rt, 16 h, yield 45%.

of all compounds, fully characterized by NMR and MS spectrometry, was assessed by elemental analysis (see Supplementary data for details).

Compounds **1**–**2** and **4**–**8** were primarily evaluated for their inhibitory potency (IC_{50}) on human EGFR-TK in a cell-free environment by monitoring the phosphorylation of a peptide substrate using time-resolved fluorometry (see Table 1).[8] Under these conditions, reference reversible **1** and irreversible **2** compounds showed comparable IC_{50}s (0.6 and 1.7 nM, respectively), indicating that the value mainly reflects the affinity of the compounds for EGFR-TK and the fast reversible component of the interaction. The presence of a basic dimethylamino (**4**) or piperidino (**5**, **6**) group on the side chain at the 6 position on the quinazoline nucleus significantly improved the inhibitory potency with IC_{50} values in the subnanomolar range, since amino groups could act both as water-solubilizing groups and as additional sites for hydrogen bond recognition.[15,16] On the other hand, the introduction of the neutral phenoxy (**7**) and perfluorophenoxy (**8**) groups reduced the inhibitory potency on EGFR-TK up to 80-fold compared to the most active **4**.

The ability of compounds **1–8** to inhibit EGFR autophosphorylation in cells was then investigated in the A549 human lung cancer cell line by Western blotting (Table 1). A549 cells, which express wild-type EGFR, were treated for 1 h with the inhibitor and then washed with drug-free medium. The extent of EGFR autophosphorylation was measured both immediately after and 8 h after the removal of the inhibitor from the cell medium.[6,8] In principle, inhibition of autophosphorylation at 1 h can depend on: (i) the intrinsic potency of the inhibitor on EGFR-TK, (ii) how efficiently it is transported across the cell membrane, either by passive diffusion or by active transport, and (iii) its clearance by metabolic enzymes or efflux transport mechanisms. On the other hand, persistent EGFR inhibition 8 h after wash-out of the compound, which has commonly been attributed to irreversible inhibition of the target, might be also due to the persistence of high concentrations of inhibitor within cells.

In a previous work, we had reported that inhibition of EGFR autophosphorylation in A549 cells was observed both at 1 h and 8 h after drug contact for the acrylamide **2** and for the 3-dimethylaminopropanamide **4**. The last behaves as a prodrug giving a reactive species, by β-elimination, in the intracellular environment. Conversely, the corresponding amino derivative **3**, lacking any chemical function able to covalently interact with Cys797, inhibits autophosphorylation only 1 h, but not 8 h after administration. The reversible inhibitor gefitinib (**1**) gives, 8 h after wash-out, partial inhibition of EGFR autophosphorylation.[9] We then measured IC_{50} values for these inhibitors at 1 h and 8 h, to have a quantitative assessment of potency. While compounds **2** and **4** showed four or five fold decreases of potency at 8 h, **3** showed a complete loss of inhibition potency, consistent with reversible inhibition. Again, **1** showed a potency decrease (approximately 10 fold) that, although larger than those of irreversible inhibitors, pointed toward an additional mechanism to explain long-lasting inhibition.

Classification of EGFR inhibitors based on this single assay became more complex with compounds **5**–**8**, being the prototypes of different chemical classes with different potential mechanisms of target inhibition. Data in Table 1 show that compounds **5**–**8** are all characterized by percentages of inhibition of EGFR autophosphorylation at 8 h between 90% and 96% and potency ratios 8 h/1 h ranging from 2 to 8. Thus, the activity-based assay appeared to be insensitive to the structural modulations applied within the set of compounds and was unable to differentiate inhibitors lacking any reactive portion at position 6 (**5**, **6**) from those having potentially pro-reactive or reactive chains (**7** and **8**, respectively).

A set of in vitro assays was therefore setup to interpret these results and to discriminate irreversible covalent inhibitors from reversible ones. The ability of compounds **4**–**5** and **7**–**8** to covalently modify the kinase domain of recombinant EGFR was tested by real-time fluorescence (see Fig. 2),[17] employing **1** and **2** as negative and positive controls, respectively. Covalent bond formation between **2** and the Cys797 of EGFR led to a time-dependent saturable increase in fluorescence emission intensity at 420 nm. Conversely, no increase in fluorescence emission was observed for the reversible inhibitor **1**. None of the tested inhibitors **4**[9]**–5** and **7–8** produced a significant increase in fluorescence emission during the assay incubation time. This indicated that, differently from the acrylamide derivative (**2**), none of them covalently reacted with EGFR-TK in the time period of the assay (i.e., ~60 min).

The reactivity of compounds **5** and **7–8** was next tested in the presence of a molar excess of cysteine, as a trapping reagent for

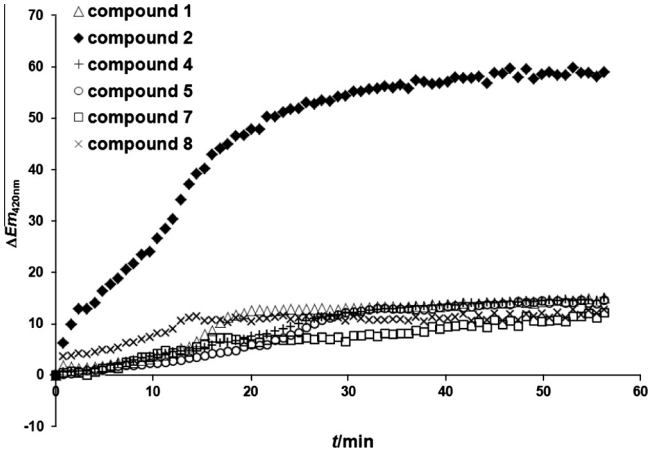


Figure 2. Fluorescence-based assay for irreversible kinase inhibition. Test compounds **1**–**2**,**4**–**5**, **7**–**8** (1 μM) were added to purified EGFR-TK (0.5 μM) in the presence of ATP (1 mM) and fluorescence emission was monitored at 420 nm in real time over 60 min (excitation: 390 nm). $\Delta Em_{420\ nm} = Em_{Compd+EGFR\ solution} - Em_{Compd\ solution} - Em_{EGFR\ solution}$.

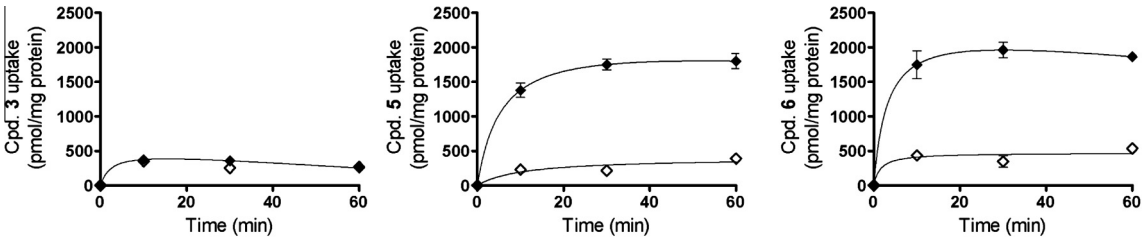


Figure 3. Time course of A549 cell uptake for compds **3**, **5**–**6** at 37 °C (black diamonds) and 4 °C (white diamonds). Each point represents the mean ± SD of three independent experiments.

chemically- or metabolically-driven reactive species.[18] We had previously monitored the formation of conjugates with cysteine in the presence of A549 cell lysate by HPLC–MS for both the acrylamide **2** and the 3-dimethylaminopropanamide **4**, which generates acrylamide **2** by a retro-Michael reaction. In that test, both compounds gave the same cysteine-conjugate at $m/z = 490.05$.[9] For compounds **5** and **7–8**, no conjugate was detected up to 24 h of incubation under the same experimental conditions. This result rules out any bioconversion to electrophilic species able to react with cysteine.

Intracellular concentrations of compounds **5**–**8** were then measured in A549 lung cancer cells by HPLC-ESI-MS/MS under conditions reproducing those of the EGFR autophosphorylation assay, that is, immediately after 1 h exposure to the inhibitor (1 μM) or after a following period (8 h) of drug-free incubation. Results, reported in Table 1, were compared with those obtained for compounds **2**–**4**.[9] Intracellular concentrations of **2** were below the limit of detection (LOD) of the bioanalytical method at both 1 h and 8 h. This is probably due to the high reactivity of the acrylamide, able to covalently interact with different cell components. Compounds **3** and **4** were detected in cells after 1 h incubation, while their concentrations dropped below LOD after 8 h. The disappearance from the intracellular environment of compound **4** is consistent with the intracellular conversion into the reactive acrylamide **2**, previously shown by us.[9] All the other EGFR inhibitors under evaluation (**5**–**8**) showed high intracellular concentrations 1 h after compound administration, well above their IC_{50} values on recombinant EGFR. Compound **6** was the most concentrated in A549 cells at 1 h (324 μM) and concentrations in the micromolar range were also maintained at 8 h (105 μM). Despite compound **6** had been designed as a reversible inhibitor, it showed 90% inhibition of EGFR autophosphorylation at 8 h. This can be fully explained by its intracellular concentration at 8 h, which largely exceeds the reported IC_{50} value. The other reversible inhibitor **5**, where homologation of the side chain was intended to avoid β-elimination and acrylamide generation, had an intracellular concentration of 147 μM at 1 h, which dropped to 7.2 μM 8 h after. In spite of the reduction in intracellular concentration, this still exceeded the reported IC_{50} and is thus sufficient to explain the observed 96% inhibition of the enzyme. Thus, reversible **5** and **6** demonstrated to be highly accumulated in cells at 8 h. Compound **6**, which is as potent as the acrylamide **2** in inhibiting EGFR autophosphorylation both at 1 h and 8 h, shows with particular evidence the possibility of getting long-lasting EGFR autophosphorylation inhibition in A549 cells by the intracellular accumulation of the inhibitor, even in the absence of reactive groups able to covalently inhibit the enzyme.

A similar behavior has also been reported for the reference reversible inhibitor gefitinib (**1**), bearing a basic morpholine in the side chain.[9,10] In fact, the calculated intracellular concentration of **1** at 8 h (2.3 μM) was consistent with the observed percentage of inhibition of autophosphorylation at the same time (46% of inhibition, 1 μM).

To further characterize the mechanism by which reversible inhibitors **5** and **6** appear to accumulate in A549 cells, we examined the time-course of their uptake at the concentration of 1 μM at 37 °C versus 4 °C (see Supplementary data for experimental details). The observed temperature dependency (see Fig. 3) indicated that A549 cell uptake of **5** and**6** was predominantly an active process. In fact, the passive influx component at 4 °C never exceeded 17.9(±3.3)% or 25.2(±3.5)% of the total influx for **5** or**6,** respectively. Moreover, cell uptake increased in a time-dependent manner reaching a plateau for both compounds, so it was a saturable process. On the contrary, cell uptake of compound **3** which does not accumulate within cells, showed no temperature dependency, suggesting the predominance of a passive diffusion mechanism. We also evaluated if the high intracellular concentrations of **5–6** could be explained by a high affinity of these basic compounds for intracellular components. Equilibrium dialysis experiments on compound-spiked A549 cell homogenates (see Supplementary data for experimental details) returned a percentage of unbound drug equal to 15.7(±2.2)% for **5**, 7.7(±0.3) for**6**, if compared to a 32.2(±0.8)% for gefitinib (**1**). The high and long-lasting intracellular accumulation of **5** and **6** can therefore also be related to their high binding to intracellular components.

Compounds **7** and **8**, having side chains in 6 without a basic group, showed intracellular concentrations at 1 h of 72 and 240 μM, respectively. At 8 h, their intracellular concentrations dropped of a 103-fold (**7**) and 67-fold (**8**) factor, even if their IC_{50}s under the same conditions showed only a limited decrease (1.8-fold for **7** and 2.4-fold for **8**). On the other hand, in the case of the reversible inhibitor **1**, comparable decrease in intracellular concentrations led to a 12.5-fold drop in IC_{50} 1 h versus 8 h. Meanwhile, for compound **5**, a 2.4-fold decrease in IC_{50} was related to the maintenance of higher intracellular concentrations. Compounds **7** and **8** were also the least potent of the series in the kinase assay on recombinant EGFR. Therefore, even if a rapid covalent interaction with Cys797 of EGFR was ruled out by fluorescence-based and reactivity assays, a reversible inhibition of EGFR followed by a slower covalent inactivation in cells during the 8 h cannot be excluded.

In conclusion, with our selected set of EGFR inhibitors we proved that the long-lasting inhibition of EGFR autophosphorylation in cells, if taken as a single indicator, is not adequate to classify irreversible covalent inhibitors from reversible inhibitors which accumulate within cells. Cell-based autophosphorylation assay need to be complemented by other experimental data to shed light on the mechanism of action of newly synthesized EGFR inhibitors. The combination of: (i) a fluorescence-based assay on EGFR kinase for covalent bond formation, (ii) a reactivity assay in the presence of a trapping agent for reactive species in cell lysate, and (iii) HPLC–MS/MS dosing of intracellular concentrations at different time points can be efficiently employed to this aim. Moreover, the proposed approach could be exploited to improve the potency and to better understand the mechanism of action of inhibitors targeting non catalytic cysteines by covalent bond formation.

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Supplementary data

Supplementary data (experimental synthetic procedures for compounds **5**–**7** and **10**; experimental conditions for the measurement of intracellular drug concentrations in A549 lung cancer cells, for cell uptake assays and for the determination of the unbound drug fractionin A549 cell homogenate; HPLC-ESI-MS/MS analytical method and operating conditions for compounds **1**, **3** and **5**–**8**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.08.008.

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