

Irreversible Inhibition of Epidermal Growth Factor Receptor Activity by 3-Aminopropanamides

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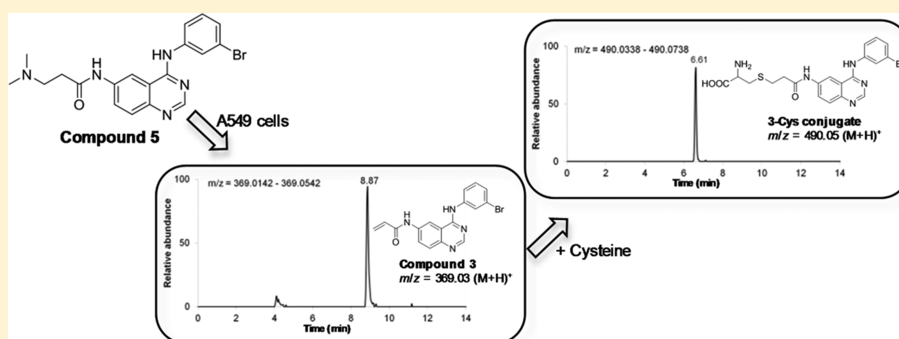
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Supporting Information



ABSTRACT: Irreversible epidermal growth factor receptor (EGFR) inhibitors contain a reactive warhead which covalently interacts with a conserved cysteine residue in the kinase domain. The acrylamide fragment, a commonly employed warhead, effectively alkylates Cys797 of EGFR, but its reactivity can cause rapid metabolic deactivation or nonspecific reactions with off-targets. We describe here a new series of irreversible inhibitors containing a 3-aminopropanamide linked in position 6 to 4-anilinoquinazoline or 4-anilinoquinoline-3-carbonitrile driving portions. Some of these compounds proved to be as efficient as their acrylamide analogues in inhibiting EGFR-TK (TK = tyrosine kinase) autophosphorylation in A549 lung cancer cells. Moreover, several 3-aminopropanamides suppressed proliferation of gefitinib-resistant H1975 cells, harboring the T790M mutation in EGFR, at significantly lower concentrations than did gefitinib. A prototypical compound, *N*-(4-(3-bromoanilino)quinazolin-6-yl)-3-(dimethylamino)propanamide (**5**), did not show covalent binding to cell-free EGFR-TK in a fluorescence assay, while it underwent selective activation in the intracellular environment, releasing an acrylamide derivative which can react with thiol groups.

■ INTRODUCTION

The epidermal growth factor receptor (EGFR, erbB1) is a member of the erbB family of receptor tyrosine kinases (RTKs), also including erbB2/HER2, erbB3/HER3, and erbB4/HER4.^{1,2} Upon ligand binding, EGFR forms homo- or heterodimers with other members of the erbB family and undergoes autophosphorylation of specific tyrosine residues in its intracellular domain. This event activates a series of downstream signals and regulates biological processes such as cell proliferation, apoptosis, angiogenesis, and differentiation.^{3,4} Deregulation of EGFR signaling has been observed in many human cancers, including lung, head and neck, colorectal, ovarian, breast, and bladder cancers,^{5,6} and it has been associated with more aggressive disease and poorer clinical outcome.⁷ In this light, inhibitors targeting the EGFR

have been extensively investigated and employed as antitumor agents.

The EGFR inhibitors **1**⁸ (gefitinib, Figure 1) and 4-(3-ethynylanilino)-6,7-bis(2-methoxyethoxy)quinazoline⁹ (erlotinib, Figure 1) and the EGFR/erbB2 inhibitor **2**¹⁰ (lapatinib, Figure 1) belong to the chemical class of 4-anilinoquinazolines, designed to bind the ATP binding pocket of the kinase domain of the target. Although **1** is effective in the treatment of non-small-cell lung cancer (NSCLC) in patients having activating mutations within the EGFR tyrosine kinase domain,¹¹ accumulating clinical experience indicates that most patients develop resistance after repeated treatments.¹² In half of NSCLC cases,

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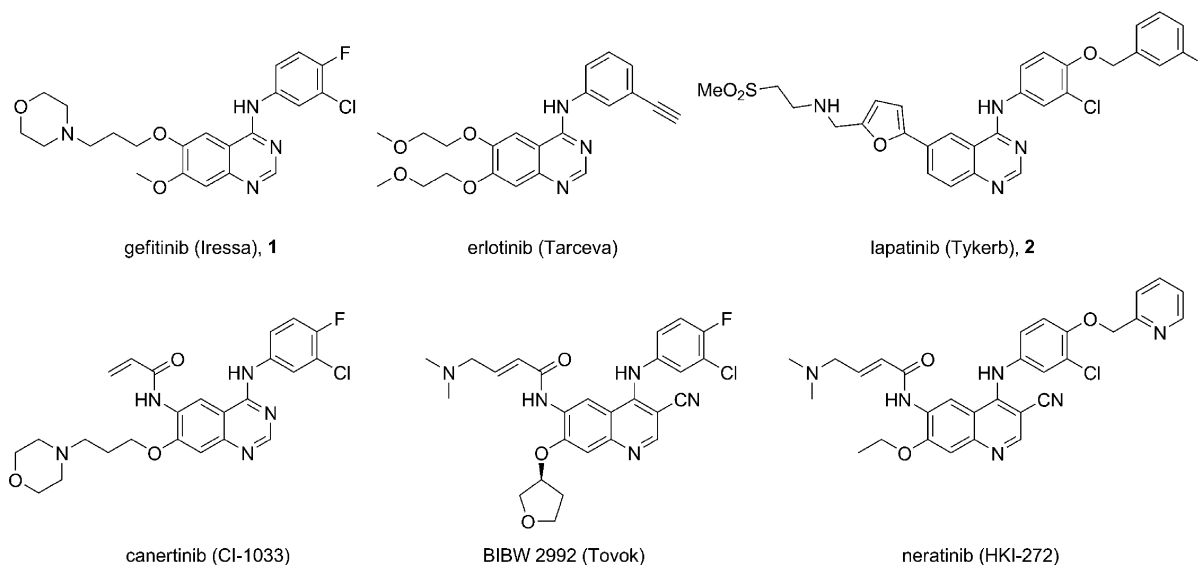


Figure 1. Chemical structures of reversible and irreversible EGFR inhibitors.

resistance was associated with the emergence of a single amino acid substitution in the catalytic domain of EGFR: conversion of the gatekeeper threonine 790 with methionine (T790M).^{12–15}

Covalent alkylation of a cysteine residue (Cys797), positioned at the entrance of the ATP binding site,^{16,17} was the additional mechanism of second-generation irreversible EGFR inhibitors, which had been shown to overcome resistance to gefitinib.^{18–20} Several inhibitors of this class, such as *N*-(4-(3-chloro-4-fluoroanilino)-7-(3-morpholinopropoxy)quinazolin-6-yl)acrylamide²¹ (CI-1033, canertinib, Figure 1), *N*-(4-(3-chloro-4-fluoroanilino)-7-(tetrahydrofuran-3-yloxy)quinolin-6-yl)-4-(dimethylamino)but-2-enamide²² (BIBW-2992, Figure 1), and *N*-(4-(3-chloro-4-(pyridin-2-ylmethoxy)anilino)-3-cyano-7-ethoxyquinolin-6-yl)-4-(dimethylamino)but-2-enamide²³ (HKI-272, neratinib, Figure 1), have progressed to clinical investigation in patients that initially responded to **1** and subsequently relapsed.²⁴ EGFR irreversible covalent inhibitors are characterized by a heterocyclic core structure (driving portion) carrying at a proper position an electrophilic functionality (warhead) that covalently interacts with the specific cysteine residue in the target protein.^{25–29} The ability of the warhead to covalently interact with nucleophiles is responsible for the advantages of EGFR irreversible inhibitors over conventional ATP-competitive ones: (i) overcoming secondary resistance,¹⁹ (ii) selectivity for erbB-family TK,³⁰ (iii) long-lasting effect.¹⁶ On the other hand, the intrinsic reactivity of the warhead is also associated with augmented metabolic degradation and toxicity, due to reactions with nontarget proteins.^{31,32} Systematic analysis of cysteine residues present in the nucleotide binding site of kinases had recently shown that combination of a driving portion and an electrophilic warhead could also be applied in a more general way to target different cysteines in different kinases.³³ To this aim, the availability of a panel of cysteine-reactive groups with different reactivities could be beneficial for the development of new druglike inhibitors.

Recently, our group has reported a systematic exploration of the role and reactivity of warheads for EGFR-TK inhibition by introducing different cysteine-trap portions on a 4-anilinoquinazoline recognition scaffold.³⁴ Compounds with different functional groups that were less reactive toward bionucleophiles

(such as glutathione) than the reference acrylamide **3**¹⁶ (PD168393, Table 1) proved efficient in irreversibly inhibiting EGFR autophosphorylation.

In our ongoing search for functional groups that are able to covalently interact with cysteine residues of EGFR,^{35–37} we focused on a new chemical entity containing a 3-aminopropanamide moiety. Aryl β -aminoethyl ketones (Mannich bases) have been described as irreversible inhibitors of enzymes having a crucial cysteine residue in the active site or at regulatory positions.^{38–40} These compounds possess a particular profile of reactivity, being nonreactive directly, but able to covalently modify their biological target after bioconversion via β -elimination to the corresponding α,β -unsaturated carbonyl compound. Maresso and co-workers³⁸ reported aryl β -aminoethyl ketones as irreversible inhibitors of the bacterial enzyme sortase, a target for the development of antibacterial therapeutics. They demonstrated through mass spectrometry and X-ray analysis that enzyme inhibition proceeds via a reactive olefin intermediate, generated in situ by amine elimination, which forms a covalent adduct with a specific cysteine residue within the protein active site. Aryl β -aminoethyl ketones have also been reported as inhibitors of intracellular kinases, such as Janus kinase 3 (Jak3),⁴¹ interleukin-2 inducible tyrosine kinase (ITK),⁴² and Bruton's tyrosine kinase (BTK),⁴² as well as weak EGFR tyrosine kinase inhibitors in enzymatic assay.⁴³ Recently, β -aminolactones have also been described as novel anticancer agents, endowed with high oral bioavailability in rats. β -Aminolactone derivatives can undergo retro-Michael addition reaction to generate the parent α -methylene lactones that interact with nucleophilic sites on biological macromolecules.⁴⁴ In principle, masking the acrylamide warhead of irreversible inhibitors should reduce the risk of covalent interactions with off-targets, due to limited systemic exposure. Of course, this would result in a specific advantage only if the antiproliferative potency and efficacy of masked agents are equivalent or similar to those of corresponding compounds having a free acrylamide fragment.

In the present study, we describe the synthesis and biological evaluation of new EGFR-TK inhibitors containing a 3-aminopropanamide side chain linked to a 4-anilinoquinazoline (**5–9**, A-series, and **11–13**, B-series, in Table 1) or 4-anilinoquinoline-3-carbonitrile (**15–17** and **19–21**, C- and

Table 1. EGFR Tyrosine Kinase and Autophosphorylation Inhibition in A549 Cells: Viability Inhibition of the H1975 Gefitinib-Resistant Cell Line


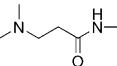
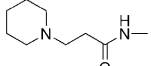
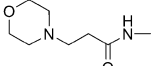
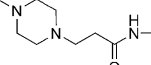
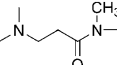

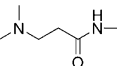
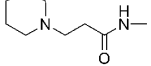
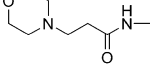

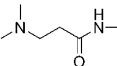
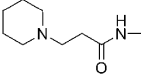
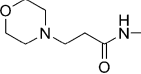

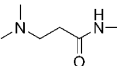
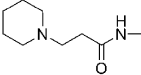
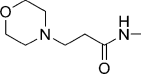
Compd	Series	R	kinase assay ^a	autophosphorylation assay (A549) ^b		H1975 cell line ^c
			IC ₅₀ (nM)	% inhibition 1 μM		IC ₅₀ (μM)
				1 h	8 h	
3	A		1.69 ± 0.16 ^d	98 ± 1.8	86 ± 0.4	0.61 ± 0.05 ^d
4	A	H ₂ N-	n.d.	91 ± 5.9	0.0 ± 0.1	19.5 ± 2.46 ^d
5	A		0.28 ± 0.07	97 ± 1.4	89 ± 1.0	3.69 ± 1.10
6	A		0.27 ± 0.04	95 ± 2.6	93 ± 4.0	6.67 ± 0.75
7	A		0.51 ± 0.06	98 ± 1.8	91 ± 5.4	> 20
8	A		0.23 ± 0.07	99 ± 0.5	79 ± 11	14.3 ± 2.23
9	A		17.4 ± 1.62	47 ± 8.8	10 ± 3.3	13.6 ± 1.66
10	B		0.18 ± 0.04	96 ± 4.0	99 ± 0.6	1.62 ± 0.45
11	B		0.27 ± 0.05	98 ± 1.1	100 ± 0.3	2.21 ± 0.69
12	B		0.51 ± 0.06	98 ± 1.6	92 ± 3.7	1.61 ± 0.07
13	B		0.29 ± 0.06	97 ± 2.3	97 ± 0.4	2.11 ± 0.63
14	C		1.46 ± 0.37	92 ± 4.4	33 ± 11	3.57 ± 1.04

Table 1. continued

Compd	Series	R	kinase assay ^a	autophosphorylation assay (A549) ^b		H1975 cell line ^c
			IC ₅₀ (nM)	% inhibition 1 μM		IC ₅₀ (μM)
				1 h	8 h	
15	C		0.68 ± 0.11	88 ± 2.8	67 ± 8.6	2.37 ± 0.15
16	C		2.21 ± 0.37	96 ± 5.8	82 ± 3.6	2.73 ± 0.28
17	C		2.02 ± 0.44	86 ± 5.2	48 ± 7.4	3.93 ± 0.95
18	D		50.7 ± 1.33	100 ± 0.3	83 ± 7.9	0.63 ± 0.13
19	D		12.5 ± 0.88	61 ± 5.2	59 ± 11	2.03 ± 0.42
20	D		24.8 ± 3.88	71 ± 2.1	58 ± 3.2	0.71 ± 0.14
21	D		37.5 ± 8.99	48 ± 3.3	39 ± 9.5	2.76 ± 0.41

^aConcentration to inhibit by 50% EGFR-wt tyrosine kinase activity. IC₅₀ values were measured by the phosphorylation of a peptide substrate using time-resolved fluorometry (see the Experimental Section). Mean values of three independent experiments ± SEM are reported. ^bInhibition of EGFR autophosphorylation was measured in A549 intact cells by Western blot analysis. The percent inhibition at 1 μM concentration was measured immediately after and 8 h after removal of the compound from the medium (1 h of incubation). Mean values of at least two independent experiments ± SEM are reported. ^cConcentration to inhibit by 50% the proliferation of NSCLC H1975. The cell proliferation was determined by the MTT assay after 72 h of incubation with compounds (0.1–20 μM). Mean values of three independent experiments ± SEM are reported. ^dData from ref 34.

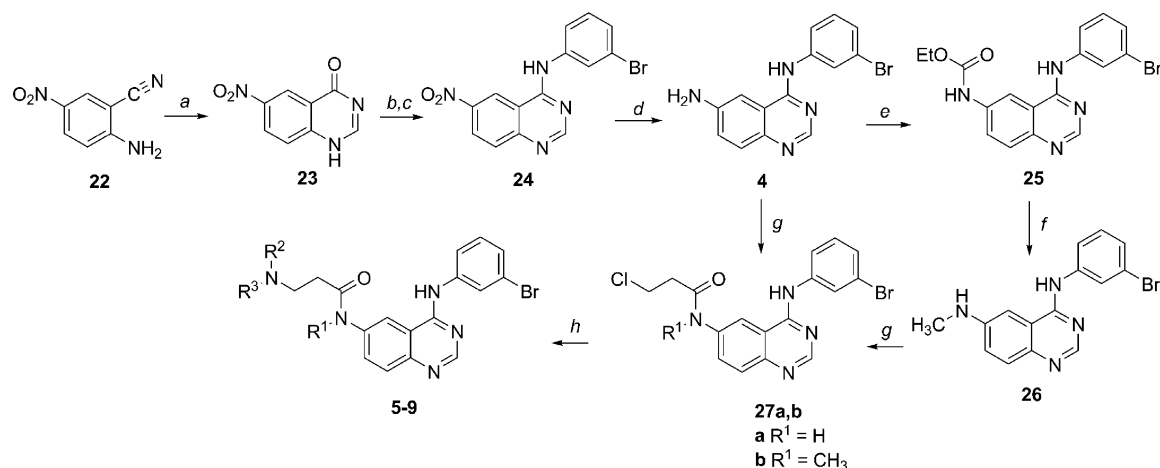
D-series, respectively, in Table 1) driving portion. Reference acrylamide derivatives for each series were also prepared (3, 10, 14, and 18, Table 1).

The new compounds were tested as EGFR tyrosine kinase inhibitors in enzyme-based and cell-based assays. Antiproliferative activity of the new compounds was also investigated in the gefitinib-resistant H1975 NSCLC cell line, harboring the T790M mutation. Moreover, for the most active agents, effects on mutated (T790M) EGFR autophosphorylation and on ErbB2 tyrosine kinase activity were evaluated. We hypothesized that the observed long-lasting effect on EGFR autophosphorylation was the result of an irreversible covalent interaction between the 3-aminopropanamide side chain and Cys797 within the active site of the enzyme. To evaluate this hypothesis, we performed a series of *in vitro* chemical stability assays, reactivity studies in the presence of thiol nucleophiles, and reactivity studies toward EGFR tyrosine kinase. Pharmacological data and reactivity study results were combined and evaluated to identify new irreversible EGFR inhibitors with lower intrinsic reactivity and optimized efficacy/toxicity profile compared to those of the other cysteine-reactive species described to date.

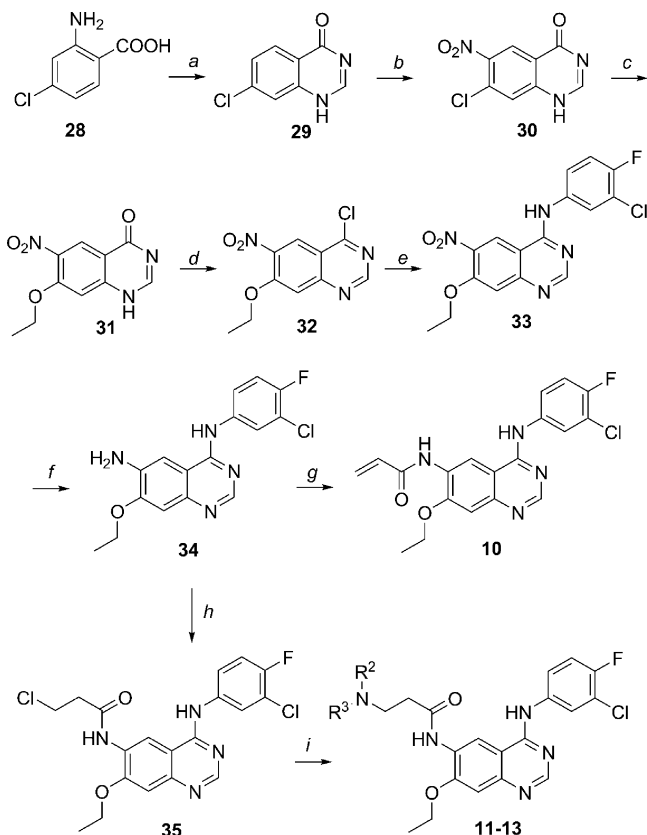
CHEMISTRY

Compounds of series A (5–9) of Table 1 were synthesized by coupling their precursor amines (4 or 26) with the proper carboxylic acid and substituting the terminal chlorine with various amines, as described in Scheme 1. The 6-amino-4-(3-bromoanilino)quinazoline (4) was prepared in three steps from 5-nitroanthranilonitrile (22) as previously described (Scheme 1).^{45,46} Condensation of 4 with 3-chloropropionyl chloride gave the 3-chloropropanamide intermediate 27a, which underwent substitution with the proper amine to obtain the secondary 3-aminopropanamides 5–9. Alternatively, 6-aminoquinazoline 4 was converted to its *N*-methyl analogue 26⁴⁷ by carbamoylation with ethyl chloroformate in pyridine followed by reduction of the resulting carbamate 25 with sodium bis(2-methoxyethoxy)aluminum hydride (Red-Al). The (*N*-methylamino)quinazoline 26 thus generated was first condensed with 3-chloropropionyl chloride to 27b and then substituted with dimethylamine to obtain the tertiary 3-aminopropanamide derivative 9 (Scheme 1).

4-(3-Chloro-4-fluoroanilino)-7-ethoxyquinazolino compounds of series B (10–13) of Table 1 were synthesized as described in Scheme 2. 6-Aminoquinazoline intermediate 34⁴⁸ was obtained following a slightly modified procedure⁴⁹ from

Scheme 1. Synthesis of Compounds 4–9^a

^aReagents and conditions: (a) H₂SO₄, formic acid, reflux; (b) SOCl₂, dioxane, reflux; (c) 3-bromoaniline, *i*-PrOH, 60 °C; (d) Fe, AcOH, EtOH/H₂O, reflux; (e) ClCOOEt, anhydrous pyridine, 0 °C to room temperature; (f) Red-Al, THF, room temperature; (g) ClCH₂CH₂COCl, THF, 50 °C; (h) R²R³NH, KI, absolute EtOH, reflux.

Scheme 2. Synthesis of Compounds 10–13^a

^aReagents and conditions: (a) formamidine acetate, 2-methoxyethanol, 130 °C; (b) H₂SO₄, HNO₃, 100 °C; (c) NaOEt, anhydrous EtOH, reflux; (d) POCl₃, reflux; (e) 3-chloro-4-fluoroaniline, *i*-PrOH, reflux; (f) Fe, AcOH, EtOH/H₂O, reflux; (g) acryloyl chloride, DIPEA, DMF, 0–50 °C; (h) ClCH₂CH₂COCl, 50 °C; (i) R²R³NH, KI, absolute EtOH, reflux.

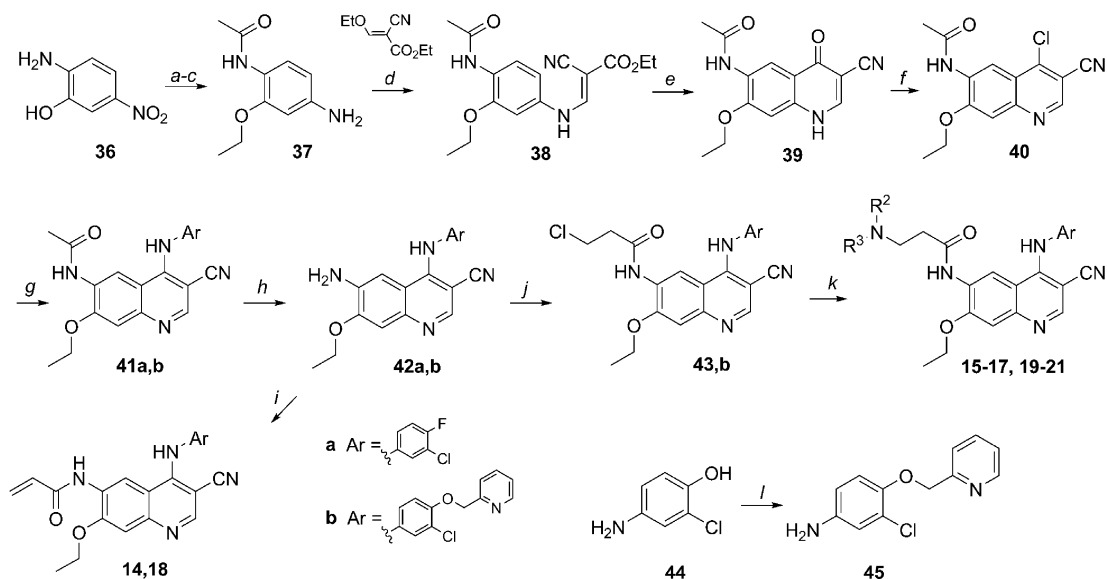
4-chloroanthranilic acid (28). Briefly, cyclization of 28 with formamidine acetate followed by nitration gave a mixture of isomers from which pure 30 was obtained after recrystallization from acetic acid. The 7-chlorine group was substituted with

sodium ethoxide, and the resulting 7-ethoxy derivative 31 was first chlorinated by heating in phosphorus oxychloride and then substituted with 3-chloro-4-fluoroaniline to obtain the intermediate 33. Reduction of the nitro group of 33 with iron and acetic acid gave the 6-aminoquinazolinone 34. The 6-acrylamide derivative 10 was then obtained from 34 with acryloyl chloride in the presence of *N,N*-diisopropylethylamine (DIPEA). Finally, 3-aminopropanamides 11–13 were synthesized from 34 by reaction with 3-chloropropionyl chloride to 35 and then substitution of the chlorine with the proper secondary amine (Scheme 2).

4-Anilinoquinoline-3-carbonitriles of series C and D (14–17 and 18–21, respectively) of Table 1 were synthesized as described in Scheme 3. The key *N*-(4-chloro-3-cyano-7-ethoxyquinolin-6-yl)acetamide intermediate (40) was synthesized in six steps from 2-amino-5-nitrophenol (36), as previously reported.⁵⁰ The quinoline intermediate 40 was reacted with the proper aniline (to 41a and 41b) and deacetylated in aqueous hydrochloric acid to amines 42a⁵¹ and 42b.⁵² Aniline derivative 45 was synthesized by reaction of 4-amino-2-chlorophenol (44) and picolyl chloride in the presence of benzaldehyde as described in Scheme 3.⁵³ The acrylamides 14 and 18 were subsequently synthesized from amine precursors 42a and 42b, respectively, with acryloyl chloride in the presence of a base (DIPEA). The 3-aminopropanamides 15–17 and 19–21 were synthesized in two steps from amines 42 by condensation with 3-chloropropionyl chloride and substitution with a secondary amine (Scheme 3).

RESULTS AND DISCUSSION

Kinase and Cellular Inhibitory Activities. Compounds 3–21 (Table 1) were evaluated in enzyme-based and cell-based assays for their ability to inhibit EGFR tyrosine kinase activity. Their inhibitor potency on human EGFR in a cell-free environment was measured on the phosphorylation of a peptide substrate using time-resolved fluorometry (see the Experimental Section). In these conditions, the reference compound 3 had an IC₅₀ of 1.7 nM.³⁴ Within the series of quinazolinone derivatives with the same driving portion as 3 (series A in Table 1), substitution of the acrylamide warhead with a substituted 3-aminopropanamide (5–8) produced a generalized increase in

Scheme 3. Synthesis of Compounds 14–21^a

^aReagents and conditions: (a) Ac₂O, AcOH, 60 °C; (b) EtBr, DMF, K₂CO₃, 60 °C; (c) H₂, Pd/C, THF, room temperature; (d) toluene, 90 °C; (e) Dowterm, 250 °C; (f) POCl₃, diglyme, 100 °C; (g) 3-chloro-4-fluoroaniline or **45**, pyridine hydrochloride, *i*-PrOH, reflux; (h) HCl, reflux; (i) acryloyl chloride, DIPEA, DMF, 0–50 °C; (j) ClCH₂CH₂COCl, THF, 50 °C; (k) R²R³NH, KI, absolute EtOH, reflux; (l) PhCHO, K₂CO₃, picolyl chloride hydrochloride, DMF, 50 °C.

inhibitor potency, with IC₅₀ values in the subnanomolar range. These values are likely to result from a complex inhibition mechanism, including reversible competition with ATP and covalent interaction with EGFR in different proportions for different compounds. We therefore consider these values as rough measures of the relative propensity to interact with the target. Methylation of the amide nitrogen of **5** to the tertiary amide **9** notably reduced EGFR-TK inhibition potency (IC₅₀ = 17 nM). B-series quinazoline compounds **10**–**13**, carrying 4-(4-fluoro-3-chloroanilino) and 7-ethoxy substituents, did not show significant changes in kinase inhibition potencies when the electrophilic warhead in **10** was replaced with 3-(dimethylamino)propanamide in **11**, 3-piperidinopropanamide in **12**, or 3-morpholinopropanamide in **13**, all compounds having subnanomolar IC₅₀ values. Considering the two series of quinoline-3-carbonitriles C and D, compounds **14**–**17** (C-series) were only slightly less potent in inhibiting EGFR-TK compared to the parent B-series quinazoline derivatives, while compounds **17**–**21** (D-series), carrying a 3-chloro-4-(3-fluorobenzoyloxy)anilino substituent at position 4 similar to that of the EGFR/erbB2 inhibitor **2** (Figure 1), were up to 60-fold less potent than the A- and B-series quinazolines. In view of the similarity between the D-series compounds (Table 1) and the EGFR/erbB2 inhibitor **2**, the effect of **19** and **20** was also evaluated on erbB2-TK. Compound **2** is a slowly reversible, noncovalent inhibitor of tyrosine kinase that binds the enzyme in an inactive-like conformation, thus reducing the rate of inhibitor dissociation, enabling prolonged actions in biological systems.¹⁰ Results of enzyme-based assay for erbB2 inhibition by time-resolved fluorometry were compared to that of the 3-(dimethylamino)propanamide **5**, belonging to the A-series of quinazoline compounds (Supporting Information, Table S2). As expected, while **5** did not inhibit erbB2 up to 10 μM concentration, compounds **19** and **20** showed IC₅₀ values for erbB2 tyrosine kinase inhibition in the submicromolar range (IC₅₀ = 0.97 and 0.98 μM, respectively). These data show that the 3-aminopropanamide is well tolerated at

both the EGFR and erbB2 binding sites of the quinazoline/quinoline-3-carbonitrile driver portions.

The ability of the compounds to inhibit EGFR autophosphorylation was investigated in the A549 human lung cancer cell line by Western blotting. Percent inhibitions at 1 μM concentration are reported in Table 1. Results were compared to those observed for the reference compounds **3** and **4**, recognized as irreversible and reversible EGFR inhibitors, respectively. A549 cells, which express wild-type EGFR, were treated for 1 h with inhibitor and then washed with drug-free medium. The degree of EGFR autophosphorylation was measured either immediately after or 8 h after removal of the inhibitor.¹⁶ As previously reported,⁵⁴ 80% or greater EGFR inhibition 8 h after removal of the inhibitor from the medium was considered a sign of irreversible inhibition. Within the A-series of 4-anilinoquinazolines, the irreversible acrylamide **3** showed 86% inhibition after the 8 h washout. Substitution of the reactive acrylamide warhead with 3-(dimethylamino)propanamide in **5**, 3-piperidinopropanamide in **6**, or 3-morpholinopropanamide in **7** gave similar results on EGFR autophosphorylation, both immediately after and 8 h after washout, with inhibition of EGFR autophosphorylation over 95% and in the range of 89–93% at 1 and 8 h, respectively. The 4-methylpiperazine analogue **8** strongly inhibited EGFR autophosphorylation after 1 h (>99% inhibition), while it showed a slight decrease in inhibitory activity 8 h after removal from the medium (79% inhibition). The tertiary amide **9** poorly inhibited EGFR (47% inhibition at 1 h) with only 10% residual inhibition 8 h after treatment, probably because of reduced affinity at the recognition site. As expected, the 6-amino derivative **4** completely inhibited EGFR activity after 1 h of treatment and did not show any inhibitory effect 8 h after its removal from the medium.

We further introduced the 3-aminopropanamido fragment on quinazoline or quinoline-3-carbonitrile scaffolds with 3-chloro-4-fluoro substitution on the 4-anilino ring and a 7-ethoxy group on the heterocyclic nucleus (B- and C-series, Table 1) since previous work had suggested that this substitution pattern led

to optimal activity.^{25,51} The acrylamide derivative **10**, belonging to the B-series of quinazolines, completely inhibited EGFR autophosphorylation 1 and 8 h after treatment at 1 μM concentration (96% and 99% inhibition, respectively). Substitution of the electrophilic warhead of **10** with a 3-aminopropanamide side chain led to compounds **11** (dimethylamino), **12** (piperidino), and **13** (morpholino), which gave EGFR percent inhibition 1 and 8 h after removal from the medium between 92% and 100%. In the C-series of compounds, the acrylamide **14** proved efficient in inhibiting EGFR at 1 μM concentration after 1 h of treatment (92% inhibition), but produced only partial inhibition 8 h after washout (33% inhibition). This unexpected behavior may be due to the reduced solubility of compound **14** in the reaction medium (data not shown). In fact, among 3-aminopropanamides **15–17**, only the piperidino derivative **16** gave complete and irreversible EGFR inhibition (92% and 82% inhibition 1 and 8 h after removal from the medium, respectively), while dimethylamino (**15**) and morpholino (**17**) compounds inhibited EGFR at 1 h, but produced only a partial, yet significant, irreversible inhibition 8 h after treatment, as demonstrated by 67% and 48% inhibition of autophosphorylation, respectively.

Partial inhibition of EGFR autophosphorylation in A459 cells was also shown by the other series of quinoline-3-carbonitrile derivatives (D-series, **19–21**). In this series, the acrylamide **18** was quite effective as an irreversible inhibitor of EGFR (100% and 83% inhibition 1 and 8 h after treatment, respectively), while 3-(dimethylamino)propanamide **19**, 3-piperidinopropanamide **20**, and 3-morpholinopropanamide **21** produced incomplete EGFR inhibition after 1 h of treatment at 1 μM concentration (inhibitions between 48% and 71%) with only partial inhibition 8 h after removal from the medium (**19**, 59% inhibition at 8 h; **20**, 58% inhibition at 8 h; **21**, 39% inhibition at 8 h).

Activity on Gefitinib-Resistant H1975 Cells. Compounds **5–21** were evaluated for their ability to inhibit the growth of the gefitinib-resistant H1975 NSCLC cell line harboring the T790M mutation by MTT assay. Results were compared with those of the reference compounds **1**, **3**, and **4** in the same assay. As reported in Table 1, the 3-(dimethylamino)propanamide **5** showed the best antiproliferative activity on H1975 cells within the A-series of quinazoline compounds with an IC_{50} of 3.7 μM , being 2 times more potent than **1** (IC_{50} of 8.3 μM)³⁴ and 5-fold more active than **4** (IC_{50} of 19.5 μM).³⁴ Substitution of the dimethylamino group with a piperidine made compound **6** equally as potent as **1** (IC_{50} of 6.7 μM), while 3-morpholinopropanamide **7**, 3-(4-methylpiperazino)propanamide **8**, and 3-(dimethylamino)-*N*-methylpropanamide **9** gave IC_{50} values over 13 μM . B-series 3-aminoamides **11–13** proved 3–4 times more effective than **1** in inhibiting H1975 proliferation and showed IC_{50} values between 1.6 and 2.2 μM , in the same range as that of the corresponding irreversible acrylamide **10** (IC_{50} of 1.6 μM). Compounds characterized by a quinoline-3-carbonitrile driving portion (C- and D-series) were more potent than **1** in inhibiting H1975 cell proliferation. In particular, 3-aminopropanamides **15–17** (C-series) showed IC_{50} values between 2.4 and 4.0 μM , comparable to that of the corresponding acrylamide derivative **14**. Finally, D-series quinoline-3-carbonitriles **19–21** gave similar results, with the compounds displaying IC_{50} values in the low micromolar or submicromolar range. The 3-piperidinopropanamide derivative **20** proved to have the most potent antiproliferative effect on H1975 cells within all the series of 3-aminopropanamide

derivatives (IC_{50} of 0.7 μM), 11 times more potent than **1** in the same test.

We further examined the ability of compounds **5** and **20** to inhibit EGFR autophosphorylation in the NSCLC cell line H1975 harboring the T790M mutation on EGFR. Results of Western blot analysis are shown in Figure 4. The compounds produced dose-dependent inhibition of EGFR autophosphorylation, indicating that the 3-aminopropanamide side chain had a positive effect on EGFR-T790M inhibition (compound **1** had marginal activity on EGFR in H1975 cells up to 10 μM).³⁴ In particular, quinazoline derivative **5** showed an IC_{50} of 0.07 μM , and the potent antiproliferative quinoline-3-carbonitrile derivative **20** had an IC_{50} of 0.15 μM . No correlation was found between enzyme activities and the ability of these compounds to inhibit the growth of the H1975 cell line nor between wild-type EGFR and T790M-mutated EGFR autophosphorylation inhibition. However, this was not an unexpected result, as factors other than EGFR-TK inhibiting potency (e.g., cell permeability of our compounds) may account for the results observed.

Reactivity Studies. Compound **5**, carrying a 3-(dimethylamino)propanamidic side chain at position 6 on a 4-(3-bromoanilino)quinazoline nucleus, was chosen as the prototype of the new series and tested for its chemical (buffered solutions at pH 7.4 and 9.0) and biological (80% (v/v) rat plasma) stability, as well as for its reactivity against low molecular weight (LMW) thiol nucleophiles. Results were compared to those obtained for the acrylamide **3** (Tables 2 and 3).

Both compounds **3** and **5** were stable at pH 7.4, with percentages of remaining compound over 97% after 24 h of incubation at 37 °C in buffer (Table 2). Under alkaline pH con-

Table 2. In Vitro Stability Studies on Compounds 3 and 5

conditions	percentage of compd remaining (1 μM , 24 h) ^a	
	3	5
pH 7.4	102.3 \pm 3.1	97.5 \pm 0.1
pH 9.0	105.2 \pm 3.7	77.0 \pm 0.8
80% (v/v) rat plasma	21.8 \pm 7.4	98.7 \pm 4.6

^aPercentage of parent compound detected by LC–UV and LC–ESI–MS after incubation for the indicated time at 37 °C (see the Experimental Section). Mean \pm SD, $n = 3$.

Table 3. In Vitro Reactivity Studies on Compounds 3 and 5

conditions	percentage of compd remaining (1 μM , 1 h) ^a	
	3	5
DTT	100.0 \pm 2.3	99.2 \pm 8.1
GSH	63.8 \pm 1.9	101.4 \pm 2.8
NAC	85.6 \pm 3.1	100.4 \pm 2.1
cysteamine	20.1 \pm 0.4	100.0 \pm 1.1

^aPercentage of parent compound detected by LC–UV and LC–ESI–MS after incubation in the presence of LMW thiols (2 mM) for the indicated time at pH 7.4, 37 °C (see the Experimental Section). Mean \pm SD, $n = 3$.

ditions (pH 9.0), while **3** was stable over the entire incubation time, 77% of intact 3-aminopropanamide **5** was recovered, with acrylamide **3** (19%) and amine **4** (4%) as the major degradation products (see the Supporting Information, Figure S1, for details). In the presence of rat plasma, compound **5** showed high stability, while only 22% of acrylamide **3** was detected after

24 h of incubation. The reactivity of compounds **3** and **5** against thiol nucleophiles was evaluated according to literature procedures.^{55,56} While for the acrylamide derivative **3** significant formation of conjugates was detected in the presence of cysteamine, glutathione (GSH), and *N*-acetylcysteine (NAC) (Table 3 and Supporting Information, Figure S2), compound **5** gave no measurable adducts with thiol derivatives up to 24 h of incubation.

Intracellular concentrations of **5**, **3**, and **4** were measured by HPLC–ESI-MS in A549 lung cancer cells under the conditions of the EGFR autophosphorylation assay, i.e., immediately after or 8 h after incubation for 1 h with the compound at 1 μ M (see the Experimental Section and Supporting Information, Table S1).⁵⁷ Both **4** and **5** were detected in the intracellular extracts after incubation (0.105 ± 0.010 and 0.050 ± 0.010 nmol/mg of protein, respectively), while their levels dropped below the limit of detection (LOD) of the LC–MS method when measured 8 h after incubation. Intracellular concentrations of **3** were below the limit of detection both 1 and 8 h after incubation. This result is probably due to the reactivity of the acrylamide group in **3**, interacting covalently with cellular components.

The reactivity of **5** was also tested in the presence of purified EGFR-TK by a fluorescence-based assay for evaluation of irreversible kinase inhibition.^{58,59} Fluorescent molecules are very sensitive to solvent polarity and dipolar perturbation from their environments.^{60,61} Moreover, reversible interactions,⁶² such as solvation, hydrogen bonding, charge transfer, and redox, as well as irreversible interactions,⁶³ such as Michael addition of thiols to electron-deficient alkenes, significantly influence the fluorescence spectra of fluorophores. In particular, quinazoline and quinoline fluorophores had been shown to significantly enhance fluorescence emission after covalent reaction with Cys797 of EGFR-TK.⁵⁸ Thus, the 3-aminopropanamide **5** was added to a buffered solution containing EGFR-TK (see the Experimental Section and Figure 2A for details), samples were excited at 390 nm, and fluorescence emission at 420 nm was monitored over time. The results were compared to those of the irreversible **3** and the reversible *N*-(4-(3-bromoanilino)-quinazolin-6-yl)acetamide⁶⁴ (**46**) (Figure 2B). Upon addition of **3** to EGFR-TK, covalent bond formation with the cysteine sulfhydryl group resulted in a time-dependent saturable increase in emission intensity at 420 nm, whereas a significantly lower fluorescence change was observed over 50 min when the reversible 6-acetyl compound **46** or the 3-aminopropanamide **5** was added to EGFR-TK. This result suggests that **5** did not covalently react with EGFR under these experimental conditions, behaving more like the acetamide derivative than like the acrylamide one.

Finally, compounds **3** and **5** were tested for their reactivity in A549 cell lysate. The formation of conjugates with cysteine added in molar excess (2 mM) was evaluated in cell lysate by LC–HRMS employing an LTQ-Orbitrap mass analyzer.⁵⁶ Compound **3** quickly reacted with the thiol derivative to form the corresponding cysteine conjugate (data not shown). When compound **5** was added to the cell lysate containing cysteine, a peak corresponding to the acrylamide derivative **3** was detected after 1 h ($[M + H]^+ = 369.03$, $t_R = 8.87$; Figure 3A), as well as a peak corresponding to the adduct of cysteine to the acrylamide fragment ($[M + H]^+ = 490.05$, $t_R = 6.61$ min; Figure 3B). After 1 h of incubation, 25.5% of the starting acrylamide **3** had been converted to the corresponding cysteine conjugate, compared to a 1.1% conversion observed for **5**. The concentration of

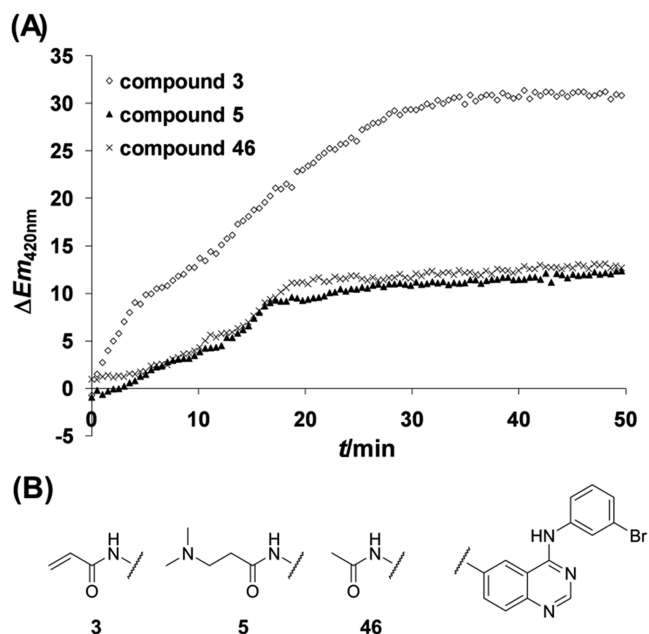


Figure 2. Fluorescence-based assay for irreversible kinase inhibition. (A) Compounds **3**, **5**, and **46** (1 μ M) were added to purified EGFR-TK (0.5 μ M) in the presence of ATP (1 mM), and fluorescence emission was monitored at 420 nm in real time over 50 min (excitation 390 nm). $\Delta Em_{420\text{ nm}} = Em_{\text{compd}+\text{EGFR solution}} - Em_{\text{compd solution}} - Em_{\text{EGFR solution}}$. (B) Chemical structures of compounds **3**, **5**, and **46**.

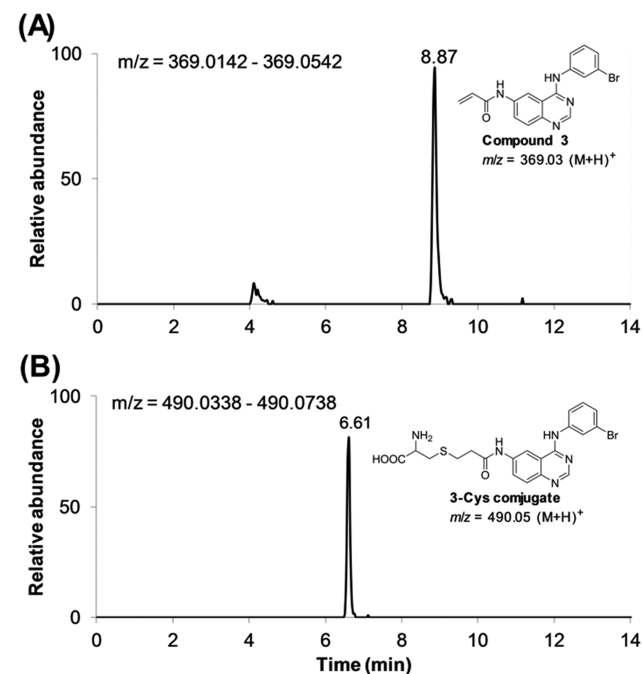


Figure 3. HPLC–HRMS extracted ion chromatograms of A549 cell lysate after 1 h of incubation in the presence of compound **5** (10 μ M) and cysteine (2 mM): (A) formed acrylamide derivative ($m/z = 369.03$, $[M + H]^+$, $t_R = 8.81$ min), (B) acrylamide–cysteine conjugate ($m/z = 490.03$, $[M + H]^+$, $t_R = 6.61$ min).

cysteine conjugate doubled for both **3** and **5** at the second time point ($t = 4$ h), being 54.4% (**3**) and 2.0% (**5**) of the starting concentrations, respectively. The unique identity of the conjugate formed by compounds **3** and **5** with cysteine was further confirmed by high-resolution mass spectrometry (Supporting Information, Figure S4). On the other hand, the

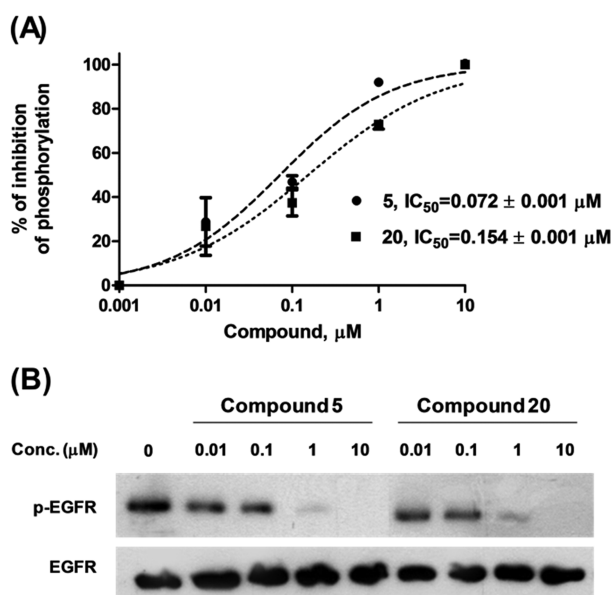


Figure 4. Effect of compounds **5** and **20** on EGFR autophosphorylation in the H1975 cell line harboring the T790M mutation. (A) Percent inhibition of EGFR autophosphorylation for compounds **5** (●) and **20** (■) on H1975 cells. Results are reported as the mean \pm SD of three independent experiments. (B) Representative Western blot analysis for EGFR inhibition by **5** and **20**. Analysis was done using monoclonal antibodies directed to p-Tyr1068 (see the Experimental Section). Total EGFR is shown as the loading control.

effect of acrylamide released by the 3-aminopropanamide derivative can be enhanced by higher persistence of the prodrug **5**, which has detectable intracellular levels in A549 cells 1 h after incubation (see above).

Evidence for Irreversible Binding to EGFR. Mannich bases are versatile synthetic intermediates used in various transformations to prepare Michael acceptors via elimination of the amino moiety.⁶⁵ As reported in the literature, aryl β -aminoethyl ketones can irreversibly inhibit enzymes by covalent interaction with cysteine residues^{38–40} after bioconversion to the corresponding α,β -unsaturated carbonyl compound. The new 3-aminopropanamides, characterized by a quinazoline (**5–7** and **11–13**) or quinoline-3-carbonitrile (**15–17** and **19–21**) driving portion, showed inhibition of EGFR autophosphorylation in A549 cells after 1 h of incubation at 1 μ M concentration, and the effect generally persisted up to 8 h after removal of the compounds from the reaction medium (Table 1). In principle, the long-lasting effect observed on EGFR autophosphorylation could be ascribed to different phenomena: (i) accumulation of the inhibitor in cells, as previously demonstrated for some reversible quinazolines;⁶⁶ (ii) conversion of the competitive inhibitor into an irreversible one at the active site of the enzyme (mechanism-based inhibition), as described for other β -aminocarbonyl compounds;³⁸ (iii) generation of the corresponding reactive acrylamide, as described for aryl β -aminoethyl ketones that have potential application as prodrugs of unsaturated ketones.⁶⁷

As previously described,^{54,66} some reversible quinazoline EGFR inhibitors are sequestered in cells generating false-positive results in the autophosphorylation assay based on the 8 h washout protocol. As an example, the fully reversible compound **1**, which is strongly sequestered in cells, produced $46.4\% \pm 6.7\%$ inhibition of EGFR autophosphorylation (A459 cells) at 1 μ M concentration 8 h after removal from the medium.

We therefore first tested if the long-lasting inhibition observed for the new 3-aminopropanamides of Table 1 could be explained by their accumulation in cells. The absence of a detectable amount of **5** in A549 cells after the 8 h period, and the presence of **1** in the same conditions,⁵⁷ suggested that **5** is not accumulated in the A549 cell line. Moreover, data from fluorescence-based assay for irreversible enzyme inhibition (Figure 2) ruled out direct interaction between the 3-aminopropanamide **5** and purified EGFR-TK in the chosen time period. On the other hand, reactivity studies on **5** indicated that the compound regenerated significant amounts of the acrylamide **3** only in the presence of cell lysate (Figure 3) while it did not under cell-free conditions (Tables 2 and 3). The results demonstrate that **5** can act as a prodrug of **3**, releasing the acrylamide fragment in the intracellular environment of A549 cells.

In principle, activation of 3-aminopropanamides to acrylamides in the intracellular environment could be affected by the nature of the heterocyclic nucleus (i.e., quinazoline or quinoline-3-carbonitrile), since a specific enzymatic transformation is likely to occur. However, the similar behavior of quinazolines (A- and B-series) and quinoline-3-carbonitriles (C- and D-series) on EGFR autophosphorylation at 8 h, as well as previous data on the *in vivo* activity of Mannich bases (see the Introduction), suggests that activation of the β -amino-carbonyl fragment to a Michael acceptor is a rather general process. In this context, masking the electrophilic warhead may provide some improvements in the pharmacokinetic or pharmacodynamic profile of antiproliferative agents. Although not conclusive evidence of specific advantages, the observation that some 3-aminopropanamide derivatives in the quinazoline and quinoline-3-carbonitrile series showed inhibition potencies on H1975 cell lines close to those of the corresponding acrylamides encourages further evaluation of the biological properties of these compounds.

CONCLUSION

We report here a new series of EGFR inhibitors containing a 3-aminopropanamide linked to a 4-anilinoquinazoline (**5–7** and **11–13**) or 4-anilinoquinoline-3-carbonitrile (**15–17** and **19–21**) nucleus. The newly synthesized 3-aminopropanamides proved efficient in inhibiting EGFR-TK activity, showing a long-lasting effect on the enzyme autophosphorylation in A549 lung cancer cells. Notably, several 3-aminopropanamides suppressed proliferation of gefitinib-resistant NSCLC cells (H1975) at significantly lower concentration than **1**. Furthermore, compounds **5** and **20** blocked mutated (T790M) EGFR-TK activity on the resistant cellular model.

Finally, a combined approach, based on (i) *in vitro* chemical stability assays, (ii) reactivity studies in the presence of thiol nucleophiles, and (iii) reactivity studies toward EGFR tyrosine kinase and in the presence of cell lysate, showed that 3-(dimethylamino)propanamide **5** acts as a prodrug, releasing the acrylamide derivative **3** in the intracellular environment, although it is stable in other conditions.

In conclusion, these findings expand the chemical diversity of irreversible inhibitors of EGFR, and similar strategies might be applied to the design of compounds able to form a covalent bond with a peripheral cysteine residue within a biological target.

EXPERIMENTAL SECTION

Reagents were obtained from commercial suppliers and used without further purification. Solvents were purified and stored according to standard procedures. Anhydrous reactions were conducted under a

positive pressure of dry N₂. Reactions were monitored by TLC, on Kieselgel 60 F 254 (DC-Alufolien, Merck). Final compounds and intermediates were purified by flash chromatography (SiO₂ 60, 40–63 μm). Microwave reactions were conducted using a CEM Discover synthesis unit (CEM Corp., Matthews, NC). Melting points were not corrected and were determined with a Gallenkamp melting point apparatus. The ¹H NMR spectra were recorded on a Bruker 300 MHz Avance or on a Bruker 400 MHz Avance spectrometer; chemical shifts (δ scale) are reported in parts per million relative to the central peak of the solvent. ¹H NMR spectra are reported in the following order: multiplicity, approximate coupling constant (*J* value) in hertz, and number of protons; signals are characterized as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), dt (doublet of triplets), q (quartet), m (multiplet), and br s (broad signal). Mass spectra were recorded using an API 150 EX instrument (AB/SCIEX, Toronto, Canada). Compounds **1**,⁶⁸ **3**,⁵⁴ and **4**⁶⁹ were synthesized according to literature methods. The final compounds were analyzed on a ThermoQuest (Italia) FlashEA 1112 elemental analyzer for C, H, and N. Analyses were within ±0.4% of the theoretical values (Supporting Information, Table S3). All tested compounds were >95% pure by elemental analysis.

N-(4-(3-Bromoanilino)quinazolin-6-yl)-3-(dimethylamino)propanamide (5). A 33% (v/v) solution of dimethylamine in absolute EtOH (0.8 mL, 4.46 mmol) was added over 15 min to a stirred suspension of *N*-(4-(3-bromoanilino)quinazolin-6-yl)-3-chloropropanamide (**27a**) (145 mg, 0.36 mmol) and KI (42 mg, 0.25 mmol) in absolute EtOH (10 mL), and the resulting mixture was refluxed for 8 h. After being cooled to 0 °C, the mixture was basified with KOH pellets (0.74 g) and stirred for 1 h at 0 °C. The solvent was evaporated under reduced pressure, and the solid residue was dissolved with EtOAc and washed with brine. The organic phase was dried, the solvent evaporated, and the residue purified by silica gel chromatography (CH₂Cl₂/MeOH, 99:1 to 70:30) to give **5** as a pale yellow solid (86%): mp (EtOAc/*n*-hexane) 170–172 °C; MS (atmospheric pressure chemical ionization, APCI) *m/z* 414.4, 416.4; ¹H NMR (CD₃OD, 300 MHz) δ 2.35 (s, 6H), 2.65 (t, *J* = 6.5 Hz, 2H), 2.78 (t, *J* = 6.5 Hz, 2H), 7.29–7.31 (m, 2H), 7.74 (m, 3H), 8.12 (br s, 1H), 8.53 (s, 1H), 8.66 (br s, 1H). Anal. (C₁₉H₂₀BrN₅O) C, H, N.

N-(4-(3-Bromoanilino)quinazolin-6-yl)-3-piperidin-1-ylpropanamide (6). **27a** was reacted with anhydrous piperidine according to the procedure described for compound **5**. The product was purified by silica gel chromatography (CH₂Cl₂/MeOH, 95:5) to give **6** as a white solid (78%): mp (Et₂O) 184–186 °C; MS (APCI) *m/z* 454.1, 456.2; ¹H NMR (DMSO-*d*₆, 300 MHz) δ 1.61 (m, 2H), 1.74–1.82 (m, 10H), 2.68–2.73 (m, 2H), 7.18–7.32 (m, 3H), 7.67 (d, *J* = 6.8 Hz, 1H), 7.83 (d, *J* = 8.8 Hz, 1H), 7.97 (s, 1H), 8.08 (s, 1H), 8.71 (s, 1H), 8.89 (s, 1H), 12.04 (s, 1H). Anal. (C₂₂H₂₄BrN₅O·³/₄H₂O) C, H, N.

N-(4-(3-Bromoanilino)quinazolin-6-yl)-3-morpholinopropanamide (7). **27a** was reacted with morpholine according to the procedure described for compound **5**. The product was purified by silica gel chromatography (CH₂Cl₂/MeOH, 95:5) to give **7** as a yellow solid (70%): mp (Et₂O/*n*-hexane) 196–198 °C; MS (APCI) *m/z* 456.2, 458.4; ¹H NMR (CDCl₃, 300 MHz) δ 2.59–2.77 (m, 8H), 3.89 (m, 4H), 7.17–7.25 (m, 3H), 7.62 (d, *J* = 7.4 Hz, 1H), 7.76 (d, *J* = 8.9 Hz, 1H), 7.90 (s, 1H), 8.16 (br s, 1H), 8.67 (s, 1H), 8.93 (d, *J* = 1.9 Hz, 1H), 11.40 (s, 1H). Anal. (C₂₁H₂₂BrN₅O₂·¹/₃H₂O) C, H, N.

N-(4-(3-Bromoanilino)quinazolin-6-yl)-3-(4-methylpiperazin-1-yl)propanamide (8). **27a** was reacted with *N*-methylpiperazine according to the procedure described for compound **5**. The product was purified by silica gel chromatography (CH₂Cl₂/MeOH, 95:5) to give **8** as a white solid (77%): mp (Et₂O) 196 °C; MS (APCI) *m/z* 469.3, 471.3; ¹H NMR (CDCl₃, 300 MHz) δ 2.33 (s, 3H), 2.42–2.49 (m, 12H), 6.94–7.07 (m, 3H), 7.44–7.51 (m, 2H), 7.58 (s, 1H), 8.50 (s, 1H), 8.68 (m, 1H), 8.88 (s, 1H), 11.55 (s, 1H). Anal. (C₂₂H₂₃BrN₆O·¹/₂H₂O) C, H, N.

N-(4-(3-Bromophenylamino)quinazolin-6-yl)-3-(dimethylamino)-*N*-methylpropanamide (9). *N*-(4-(3-bromophenylamino)quinazolin-6-yl)-3-chloro-*N*-methylpropanamide (**27b**) was reacted with dimethylamine according to the procedure described for compound **5**.

Silica gel chromatography purification (CH₂Cl₂/MeOH, 98:2 to 90:10) afforded **9** as a white solid (68%): mp (CH₂Cl₂/*n*-hexane) 169–172 °C; MS (APCI) *m/z* 430.4, 431.4, 432.4; ¹H NMR (CD₃OD, 300 MHz) δ 2.14 (s, 6H), 2.39 (br s, 2H), 2.67 (br s, 2H), 3.40 (s, 3H), 7.34 (m, 2H), 7.79 (m, 1H), 7.85 (dd, *J* = 8.6, 2.3 Hz, 1H), 7.93 (d, *J* = 8.0 Hz, 1H), 8.17 (s, 1H), 8.42 (s, 1H), 8.66 (s, 1H). Anal. (C₂₀H₂₂BrN₅O) C, H, N.

N-(4-(3-Chloro-4-fluoroanilino)-7-ethoxyquinazolin-6-yl)-acrylamide (10). Acryloyl chloride (25 μL, 0.3 mmol) in anhydrous THF (0.5 mL) was added dropwise to a solution of 6-aminoquinazolin-3-ylamine (**34**) (100 mg, 0.3 mmol) and *N,N*-diisopropylethylamine (DIPEA) (52 μL, 0.3 mmol) in anhydrous DMF (2.5 mL) at 0 °C. The reaction mixture was stirred for 1 h, then the solvent was removed under reduced pressure, and the residue was purified by silica gel chromatography (CH₂Cl₂/MeOH, 95:5) to afford **10** as a white solid (25%): mp (EtOH/H₂O) 228.5–230 °C; MS (APCI) *m/z* 387.3, 389.3; ¹H NMR (acetone-*d*₆, 400 MHz) δ 1.52 (t, *J* = 6.9 Hz, 3H), 4.35 (q, *J* = 6.9 Hz, 2H), 5.83 (dd, *J* = 10.1, 1.7 Hz, 1H), 6.45 (dd, *J* = 16.8, 1.7 Hz, 1H), 6.70 (dd, *J* = 16.8, 10.1 Hz, 1H), 7.29 (s, 1H), 7.32 (t, *J* = 9.0 Hz, 1H), 7.86 (ddd, *J* = 9.0, 4.1, 2.8 Hz, 1H), 8.25 (dd, *J* = 6.8, 2.6 Hz, 1H), 8.57 (s, 1H), 9.04 (s, 1H). Anal. (C₁₉H₁₆ClFN₄O₂) C, H, N.

N-(4-(3-Chloro-4-fluoroanilino)-7-ethoxyquinazolin-6-yl)-3-(dimethylamino)propanamide (11). 3-Chloropropanamide (**35**) was reacted with dimethylamine according to the procedure described for compound **5**. The product was purified by silica gel chromatography (AcOEt/MeOH, 99:1 to 85:15) to obtain **11** as a white solid (85%): mp (EtOH/H₂O) 182–183 °C; MS (APCI) *m/z* 432.5, 434.3; ¹H NMR (CD₃OD, 300 MHz) δ 1.54 (t, *J* = 7.0 Hz, 3H), 2.37 (s, 6H), 2.69 (m, 4H), 4.26 (q, *J* = 7.0 Hz, 2H), 7.11 (s, 1H), 7.21 (t, *J* = 9.0 Hz, 1H), 7.62 (ddd, *J* = 9.0, 4.1, 2.7 Hz, 1H), 7.95 (dd, *J* = 6.7, 2.6 Hz, 1H), 8.41 (s, 1H), 8.83 (s, 1H). Anal. (C₂₁H₂₃ClFN₅O₂·²/₃H₂O) C, H, N.

N-(4-(3-Chloro-4-fluoroanilino)-7-ethoxyquinazolin-6-yl)-3-piperidin-1-ylpropanamide (12). 3-Chloropropanamide (**35**) was reacted with anhydrous piperidine according to the procedure described for compound **5**. The product was purified by silica gel chromatography (AcOEt/MeOH, 99:1 to 93:7) to give **12** as a white solid (60%): mp (EtOH/H₂O) 106.5–108 °C; MS (APCI) *m/z* 472.2, 474.2; ¹H NMR (DMSO-*d*₆, 400 MHz) δ 1.54 (t, *J* = 6.9 Hz, 3H), 1.68 (4H, m), 2.56 (s, 4H), 2.75 (m, 4H), 4.38 (q, *J* = 6.9 Hz, 2H), 7.22 (s, 1H), 7.25 (t, *J* = 8.9 Hz, 1H), 7.65 (ddd, *J* = 9.0, 3.7, 2.8 Hz, 1H), 7.99 (dd, *J* = 6.6, 2.5 Hz, 1H), 8.47 (s, 1H), 8.83 (s, 1H). Anal. (C₂₄H₂₇ClFN₅O₂·H₂O) C, H, N.

N-(4-(3-Chloro-4-fluorophenylamino)-7-ethoxyquinazolin-6-yl)-3-morpholinopropanamide (13). 3-Chloropropanamide (**35**) was reacted with morpholine according to the procedure described for compound **5**. The product was purified by silica gel chromatography (AcOEt/MeOH, 99:1 to 96:4) to obtain **13** as a light yellow solid (96%): mp (EtOH/H₂O) 108–110 °C; MS (APCI) 474.0, 476.3 °C; ¹H NMR (DMSO-*d*₆, 400 MHz) δ 1.54 (t, *J* = 7.0 Hz, 3H), 2.60 (m, 4H), 2.74 (t, *J* = 6.0, 2H), 2.81 (t, *J* = 5.8 Hz, 2H), 3.77 (t, *J* = 4.6 Hz, 4H), 4.37–4.41 (m, 2H), 7.18–7.26 (m, 2H), 7.63–7.65 (m, 1H), 7.97–7.99 (m, 1H), 8.46 (d, *J* = 2.9 Hz, 1H), 8.83 (d, *J* = 4.7 Hz, 1H). Anal. (C₂₃H₂₅ClFN₅O₃·²/₃H₂O) C, H, N.

N-(4-(3-Chloro-4-fluoroanilino)-3-cyano-7-ethoxyquinolin-6-yl)acrylamide (14). The product was obtained by coupling the amino intermediate **42a** with acryloyl chloride as described for compound **10**. Silica gel chromatography purification (EtOAc/*n*-hexane, 60:40) afforded **14** (40%) as a white solid: mp (EtOH/H₂O) > 230 °C; MS (APCI) *m/z* 411.0; ¹H NMR (DMSO-*d*₆, 300 MHz) δ 1.48 (t, *J* = 6.9 Hz, 3H), 4.33 (q, *J* = 6.9 Hz, 2H), 5.81 (d, *J* = 10.7 Hz, 1H), 6.29 (d, *J* = 16.5 Hz, 1H), 6.77 (dd, *J* = 16.5, 9.7 Hz, 1H), 7.25 (m, 1H), 7.41 (m, 3H), 8.53 (s, 1H), 8.98 (s, 1H), 9.60 (s, 1H), 9.75 (br s, 1H). Anal. (C₂₁H₁₆ClFN₄O₂·¹/₂H₂O) C, H, N.

N-(4-(3-Chloro-4-fluoroanilino)-3-cyano-7-ethoxyquinolin-6-yl)-3-(dimethylamino)propanamide (15). 3-Chloropropanamide (**43a**) was reacted with dimethylamine according to the procedure described for compound **5**. Silica gel chromatography (CH₂Cl₂/MeOH, 99:1 to 97:3) gave **15** as a light yellow solid (69%): mp (EtOH) 168–171 °C; ¹H NMR (DMSO-*d*₆, 300 MHz) δ 1.48

(t, $J = 7.0$ Hz, 3H), 2.29 (s, 6H), 2.57 (m, 4H), 4.32 (q, $J = 7.0$ Hz, 2H), 7.19 (m, 1H), 7.40 (m, 3H), 8.54 (s, 1H), 9.06 (s, 1H), 9.68 (br s, 1H), 10.98 (s, 1H). Anal. ($C_{23}H_{23}ClFN_3O_2 \cdot H_2O$) C, H, N.

***N*-(4-(3-Chloro-4-fluoroanilino)-3-cyano-7-ethoxyquinolin-6-yl)-3-piperidin-1-ylpropanamide (16).** 3-Chloropropanamide **43a** was reacted with piperidine according to the procedure described for compound **5**. Silica gel chromatography ($CH_2Cl_2/MeOH$, 96:4) gave **16** as a white solid (45%): mp (EtOH) 180–184 °C; MS (APCI) m/z 496.4, 497.3, 499.4; 1H NMR (DMSO- d_6 , 400 MHz) δ 1.46 (t, $J = 7.0$ Hz, 3H); 1.49 (m, 2H), 1.56 (m, 4H), 2.44 (m, 4H), 2.60 (s, 4H), 4.39 (t, $J = 7.0$ Hz, 2H), 7.22 (m, 1H), 7.41 (m, 3H), 8.54 (s, 1H), 8.97 (s, 1H), 9.68 (br s, 1H), 10.24 (s, 1H). Anal. ($C_{26}H_{27}ClFN_5O_2 \cdot 1/2 C_2H_6O$) C, H, N.

***N*-(4-(3-Chloro-4-fluoroanilino)-3-cyano-7-ethoxyquinolin-6-yl)-3-morpholinopropanamide (17).** 3-Chloropropanamide **43a** was reacted with morpholine according to the procedure described for compound **5**. Silica gel chromatography ($CH_2Cl_2/MeOH$, 98:2) afforded **17** as a white solid (52%): mp (EtOH/ H_2O) 195–198 °C; MS (APCI) m/z 498.3, 499.2, 500.3; 1H NMR (DMSO- d_6 , 400 MHz) δ 1.45 (t, $J = 7.0$ Hz, 3H), 2.46 (m, 4H), 2.63 (m, 4H), 3.62 (t, $J = 4.2$ Hz, 4H), 4.36 (q, $J = 7.1$ Hz, 2H), 7.20 (br s, 1H), 7.39 (m, 3H), 8.53 (br s, 1H), 8.94 (s, 1H), 9.69 (br s, 1H), 9.89 (s, 1H). Anal. ($C_{25}H_{25}ClFN_5O_3 \cdot 1/2 C_2H_6O$) C, H, N.

***N*-(4-(3-Chloro-4-(pyridin-2-ylmethoxy)anilino)-3-cyano-7-ethoxyquinolin-6-yl)acrylamide (18).** The product was obtained by coupling the amino intermediate **42b** with acrylic acid as described for compound **10**. Silica gel chromatography purification ($CH_2Cl_2/MeOH$, 98:2) gave **18** as a white solid (35%): mp (EtOH/ H_2O) > 230 °C; MS (APCI) m/z 500.1; 1H NMR (DMSO- d_6 , 300 MHz) δ 1.53 (t, $J = 6.9$ Hz, 3H), 4.39 (q, $J = 6.9$ Hz, 2H), 5.32 (s, 2H); 5.79 (dd, $J = 10.1$, 1.9 Hz, 1H), 6.39 (dd, $J = 16.9$, 1.9 Hz, 1H), 6.68 (dd, $J = 16.9$, 10.1 Hz, 1H), 7.30 (m, 3H), 7.41 (s, 1H), 7.48 (d, $J = 2.3$ Hz, 1H), 7.68 (d, $J = 7.8$ Hz, 1H), 7.87 (td, $J = 7.8$, 1.8 Hz, 1H), 8.50 (s, 1H), 8.60 (d, $J = 4.5$ Hz, 1H), 9.05 (s, 2H), 9.16 (s, 1H). Anal. ($C_{27}H_{22}ClN_5O_3$) C, H, N.

***N*-(4-(3-Chloro-4-(pyridin-2-ylmethoxy)anilino)-3-cyano-7-ethoxyquinolin-6-yl)-3-(dimethylamino)propanamide (19).** 3-Chloropropanamide **43b** was reacted with dimethylamine according to the procedure described for compound **5**. Silica gel chromatography ($CH_2Cl_2/MeOH$, 99:1 to 90:10) gave **19** as a white solid (73%): mp (EtOH) 235–237 °C; MS (APCI) m/z 545.4, 547.3, 502.7, 500.2; 1H NMR (DMSO- d_6 , 300 MHz) δ 2.29 (s, 6H), 2.54–2.61 (m, 4H), 4.31 (q, $J = 7.0$ Hz, 2H), 5.28 (s, 2H), 7.17 (dd, $J = 7.7$, 1.7 Hz, 1H), 7.25 (d, $J = 8.8$ Hz, 1H), 7.38–7.40 (m, 3H), 7.59 (d, $J = 7.9$ Hz, 1H), 7.88 (dt, $J = 7.7$, 1.7 Hz, 1H), 8.47 (s, 1H), 8.60 (d, $J = 4.0$ Hz, 1H), 9.06 (s, 1H), 9.58 (br s, 1H), 10.92 (br s, 1H). Anal. ($C_{29}H_{29}ClN_6O_3 \cdot 1/2 H_2O$) C, H, N.

***N*-(4-(3-Chloro-4-(pyridin-2-ylmethoxy)anilino)-3-cyano-7-ethoxyquinolin-6-yl)-3-piperidin-1-ylpropanamide (20).** 3-Chloropropanamide **43b** was reacted with piperidine according to the procedure described for compound **5**. Silica gel chromatography ($CH_2Cl_2/MeOH$, 97:3 to 95:5) gave **20** as a light yellow solid (69%): mp (EtOH) 190–193 °C; MS (APCI) m/z 585.2, 587.5, 588.5; 1H NMR (DMSO- d_6 , 400 MHz) δ 1.45 (t, $J = 6.8$ Hz, 3H), 1.50 (m, 2H), 1.56 (m, 4H), 2.44 (m, 4H), 2.60 (s, 4H), 4.36 (q, $J = 7.0$ Hz, 2H), 5.28 (s, 2H), 7.18 (d, $J = 8.2$ Hz, 1H), 7.25 (d, $J = 8.4$ Hz, 1H), 7.39 (m, 3H), 7.58 (d, $J = 7.2$ Hz, 1H), 7.88 (t, $J = 7.3$ Hz, 1H), 8.47 (s, 1H), 8.60 (d, $J = 4.3$ Hz, 1H), 8.95 (s, 1H), 9.59 (s, 1H), 10.21 (s, 1H). Anal. ($C_{32}H_{33}ClN_6O_3 \cdot 3/2 H_2O$) C, H, N.

***N*-(4-(3-Chloro-4-(pyridin-2-ylmethoxy)anilino)-3-cyano-7-ethoxyquinolin-6-yl)-3-morpholinopropanamide (21).** 3-Chloropropanamide **43b** was reacted with morpholine according to the procedure described for compound **5**. Silica gel chromatography ($CH_2Cl_2/MeOH$, 99:1 to 97:3) gave **21** as a light yellow solid (74%): mp (EtOH) 224–228 °C; MS (APCI) m/z 587.3, 589.5; 1H NMR ($CDCl_3$, 400 MHz) δ 1.49 (t, $J = 6.9$ Hz, 3H), 2.55 (m, 4H), 2.67 (m, 4H), 3.77 (s, 4H), 4.30 (q, $J = 6.8$ Hz, 2H), 5.19 (s, 2H), 6.72 (s, 2H), 7.61 (d, $J = 7.8$ Hz, 2H), 7.75 (t, $J = 7.7$ Hz, 1H), 8.16 (s, 1H), 8.32 (s, 1H), 8.58 (d, $J = 4.4$ Hz, 1H), 9.19 (s, 1H), 10.08 (s, 1H). Anal. ($C_{31}H_{31}ClN_6O_4$) C, H, N.

In Vitro Stability and Reactivity Assays. Chemical (pH 7.4, pH 9.0) and rat plasma stability was investigated at 37 °C according to procedures reported previously.⁷⁰ (See the Supporting Information for details.) The reactivity of **3** and **5** toward cysteamine, NAC, dithiothreitol (DTT), and reduced GSH was evaluated by adding a freshly prepared thiol solution (2 mM) to PBS (pH 7.4) containing the test compound (1 μ M). Formation of conjugates with LMW thiols after 1 h of incubation at 37 °C was measured by HPLC–UV and HPLC–ESI-MS. The reactivity of compounds **3** and **5** in A549 cell lysate was tested in the presence of cysteine, to trap thiol-reactive compounds. A549 cells were seeded at 1 000 000 cells/dish (66 cm²); after 2 days, the cells were lysed mechanically by the freeze/thaw method. Stock solutions (1 mM) of **3** and **5** were prepared in DMSO immediately before use and added to cell lysate at a final concentration of 10 μ M (equal percentage of DMSO in controls), where 2 mM cysteine had been previously added. Incubations were carried out at 37 °C. At stated time points ($t = 0, 1,$ and 4 h) aliquots were sampled, a double volume of acetonitrile was added, and the resulting solutions were centrifuged (4 °C, 10000g, 10 min) and directly analyzed by LC–HRMS employing an LTQ–Orbitrap mass analyzer, working in positive ion mode (PIM). (See the Supporting Information for details.)

Determination of Intracellular Concentrations. A549 cells were plated at a 1 000 000 cells/dish (25 cm²) density. After 24 h, test compounds (**3**–**5**) were added to the medium (final concentration 1 μ M, final DMSO concentration 0.1%). the cells were incubated for 1 h at 37 °C and tested either immediately after or 8 h after removal of the compound from the extracellular medium by washing the cells three times with 1 mL aliquots of fresh medium. Compounds were then extracted from the cells with absolute EtOH (1.1 mL at 4 °C), and the cell extracts were centrifuged (4 °C, 10000g, 5 min) and collected. A fixed volume of ethanolic extract was evaporated to dryness, dissolved in HPLC eluent, and injected into the LC–MS system. Calibration curves were built in the concentration range from 5 μ M to 50 nM for compounds **3**–**5** (limit of quantification (LOQ) of 50 nM, LOD = 20 nM). Cell proteins were quantified after solubilization in NaOH (0.5 N) (2 mL/25 cm² dish) by the Bradford method.⁷¹

Analytical Method. A linear gradient elution was set up to evaluate in the same chromatographic run the disappearance of compounds **3** and **5** and the formation of corresponding degradation products. An Agilent 1100 HPLC gradient system (Agilent Technologies, Santa Clara, CA) with an RP-C18 Supelco Discovery (Supelco, Bellefonte, PA), 5 μ m, 150 \times 4.6 mm i.d. column and an AB/SCIEX API 150-EX single-quadrupole mass spectrometer equipped with an ESI ion source acquiring in positive ion mode were employed. (See the Supporting Information for details.)

Real-Time Detection of Covalent Bond Formation in Microtiter Plates. The assay was carried out in black 384-well small-volume plates, and fluorescence emission was measured over time by using a multilabel plate reader (Victor, PerkinElmer), as previously described.⁵⁸ EGFR-TK was purchased from Sigma-Aldrich. The sample wells were excited at 390 nm, and the changing emission intensity was monitored at 420 nm. The reaction was monitored immediately after combination of equal volumes (7 μ L) of EGFR-TK (0.5 μ M) in buffer (50 mM Hepes, 200 mM NaCl, 0.05% Triton-X100, pH 7.4) containing ATP (1 mM) and inhibitor (1 μ M, 0.2% DMSO) solution in each well. Data were reported as $\Delta Em_{420\text{ nm}}$ calculated as the difference in emission intensity between the samples (inhibitor + EGFR) and controls (inhibitor in buffer solution and EGFR in buffer solution).

Kinase Assay. Evaluation of the effects of compounds on the kinase activity of human EGFR was performed by measuring the phosphorylation of the substrate Ulight-CAGAGAIETDKEYTVDK (JAK1) using a human recombinant enzyme expressed in insect cells⁷² and the LANCE detection method,⁷³ employing the Cerep EGFR kinase assay⁷⁴ as previously described.³⁴

Cell Culture. The human NSCLC cell lines A549 and H1975 were cultured as recommended. Medium was supplemented with L-glutamine (2 mmol/L) and fetal calf serum (FCS) (10%), and the cells were

maintained under standard cell culture conditions at 37 °C in a water-saturated atmosphere of 5% CO₂ in air. The A549 cell line was from the American Type Culture Collection (Manassas, VA), and H1975 was kindly provided by Dr. E. Giovannetti (Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands).

Antibodies and Reagents. Medium was from Euroclone, and fetal bovine serum (FBS) was purchased from Gibco-BRL (Grand Island, NY). Monoclonal anti-EGFR and polyclonal anti-phospho-EGFR (Tyr1068) antibodies were from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Pierce. The enhanced chemiluminescence (ECL) system was from Millipore (Millipore, MA). Reagents for electrophoresis and blotting analysis were obtained from, respectively, BIO-RAD Laboratories and Millipore.

Western Blot Analysis. Procedures for protein extraction, solubilization, and protein analysis by 1-D polyacrylamide gel electrophoresis (PAGE) are described elsewhere.⁷⁵ Samples of 40–50 µg of proteins from lysates were resolved by 7.5% sodium dodecyl sulfate (SDS)-PAGE and transferred to poly(vinylidene fluoride) (PVDF) membranes (Millipore). The membranes were then incubated with primary antibody, washed, and then incubated with HRP-antirabbit antibody. Immunoreactive bands were visualized using an enhanced chemiluminescence system.

Autophosphorylation Assay. Inhibition of EGFR autophosphorylation was determined as previously described using specific anti-phosphotyrosine and anti total EGFR antibodies by Western blot analysis.³⁴

Cell Growth Inhibition. Cell viability was assessed after 3 days of treatment by tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sigma, Dorset, U.K.] assay as previously described.⁷⁵ Representative results of at least three independent experiments were used for evaluation of dose–response curves, calculated from experimental points using Graph Pad Prism5 software. The concentration that inhibits 50% (IC₅₀) (e.g., the point at which viability is 50%) was extrapolated from the dose–response curves.

■ ASSOCIATED CONTENT

● Supporting Information

Experimental procedures for compounds 25–27a,b, 29–35, 41b, 42b, 43a,b, and 45, combustion analytical data for compounds 3–21, experimental procedures for in vitro stability assays and HPLC–ESI-MS analysis on compounds 3–5, HPLC–MS chromatogram of 5 incubated at pH 9.0 for 24 h, HPLC–MS chromatograms of 3 incubated in the presence of LMW thiols, HR–MS chromatograms of conjugates formed by 3 and 5 with cysteine in A549 cell lysate, quantification of the intracellular concentration of compounds 3–5 in A549 cells, and ErbB2 tyrosine kinase inhibition for compounds 3, 5, 19, and 20. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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Forensic Medicine at the University of Parma) for support in the fluorescence-based kinase assay.

■ ABBREVIATIONS USED

EGFR, epidermal growth factor receptor; erbB2, human epidermal growth factor receptor 2; TK, tyrosine kinase; ATP, adenosine triphosphate; NSCLC, non-small-cell lung cancer; GSH, glutathione; NAC, N-acetylcysteine; DTT, dithiothreitol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Red-Al, sodium bis(2-methoxyethoxy)aluminum hydride; DIPEA, N,N-diisopropylethylamine; PBS, phosphate-buffered saline

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