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Novel iodine-124 labeled EGFR inhibitors as potential PET agents for molecular imaging in cancer

Mazal Shaul,[a] Galith Abourbeh,[a,b] Orit Jacobson,[a] Yulia Rozen,[a] Desideriu Laky,[a] Alexander Levitzki[b] and Eyal Mishani[a,*]

[a]*Department of Medical Biophysics and Nuclear Medicine, Hadassah Hebrew University, Hadassah Hospital, Jerusalem 91120, Israel*
[b]*Unit of Cellular Signaling, Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University, Jerusalem 91904, Israel*

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Abstract—The in vivo results with our previously reported irreversible labeled inhibitor [^{11}C]-ML03 suggested that more chemically stable inhibitors, labeled with a longer-lived radioisotope, could be better candidates for molecular imaging of epidermal growth factor receptor (EGFR) positive tumors. On the basis of this hypothesis we synthesized three new irreversible tyrosine kinase (TK) inhibitors with various chemical reactivities. The three new inhibitors were successfully labeled on the anilino moiety with [^{124}I], starting with the 6-amino-4-[(3-tributylstannylphenyl)amino]-quinazoline (**9**) precursor. The cell-free results, obtained with these new irreversible inhibitors, indicated that compounds **5** (α-chloro-acetamide derivative) and **6** (4-dimethylamino-but-2-enoic amide derivative) possessed high potencies toward the EGFR with an irreversible inhibition effect. Compound **4** (α-methoxy-acetamide derivative) was found to be less potent, with only a partially irreversible effect. The high potency of compounds **5** and **6** toward the EGFR establishes their potential as PET agents for molecular imaging of EGFR positive tumors. Their prospect as PET biomarkers is further being investigated.

1. Introduction

The epidermal growth factor receptor (EGFR, Erb-B1) belongs to a family of proteins, involved in the proliferation of normal and malignant cells.[1] Overexpression of the EGFR is a hallmark of many human tumors, such as breast cancer, glioma, laryngeal cancer, carcinoma of the head and neck, and prostate cancer.[2–5] Thus, the EGFR is an attractive target for the design and development of compounds that can specifically bind the receptor, and inhibit its tyrosine kinase (TK) activity and its signal transduction pathway in cancer cells. Such compounds may serve as therapeutic or diagnostic agents. The EGFR reversible inhibitor, Iressa® (Fig. 1), was recently approved by the FDA for treatment of NSCLC and prostate cancer, and several other anti-EGFR targeted molecules, such as Tarceva© (Fig. 1) and the anti-EGFR antibody Erbitux©, are presently undergoing clinical Phase 3 trials. Consequently, there has been a growing interest in the use of EGFR-TK inhibitors as radiotracers for molecular imaging of EGFR overexpressing tumors by nuclear medicine modalities[6–9] such as positron emission tomography (PET). PET is based on the use of short half-life positron-emitting isotopes, such as ^{11}C ($t_{1/2}$ 20.39 min), ^{18}F ($t_{1/2}$ 109.8 min), ^{15}O ($t_{1/2}$ 2.037 min), and ^{13}N ($t_{1/2}$ 9.965 min) produced by an in house cyclotrons. Following injection of a suitable biomarker, the PET scan provides a mapping of the biomarker distribution and hence of a specific receptor, transporter or enzyme in the human body.

Our research endeavors have been devoted to the development of PET bioprobes that would target the EGFR-TK for molecular imaging purposes.[10–12] During the course of this research several reversible and irreversible inhibitors, such as ML01 and ML03[13–15] (Fig. 1) were labeled with fluorine-18 and carbon-11, respectively, and their potential as PET biomarkers was investigated both in vitro and in vivo. In the case of reversible inhibitors, such as [^{18}F]-ML01, the high

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* Corresponding author. Tel.: +972-2-6777931; fax: +972-2-642-1203; e-mail: mishani@md2.huji.ac.il

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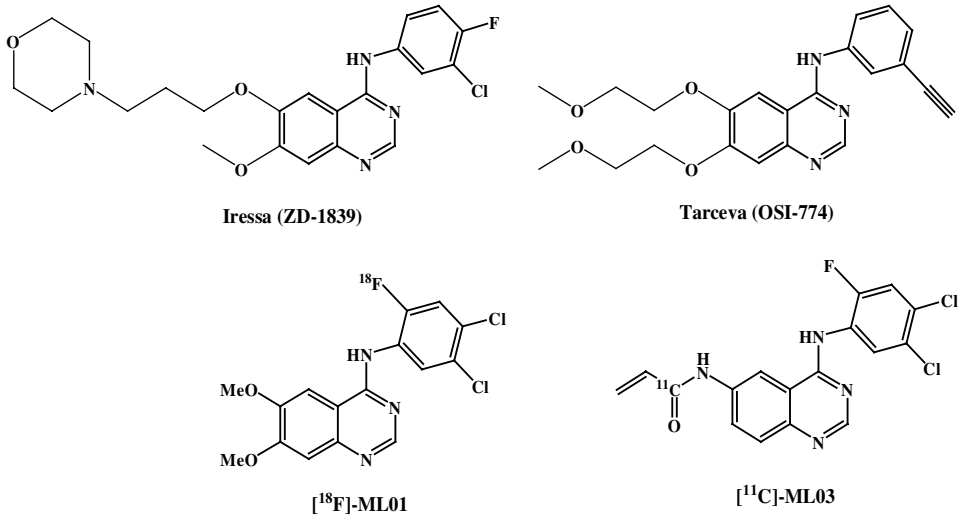

Figure 1. Chemical structure of reversible and irreversible EGFR inhibitors.

cellular concentration of competitive ATP molecules brought about the fast washout of the labeled inhibitor from the receptor's ATP-binding pocket.[13] However, the rapid washout was abrogated using the irreversible inhibitor, [^{11}C]-ML03, most probably, due to covalent binding between the double bond of the acryl-amide group at the 6-position of the quinazoline ring and Cys-773 at the receptor's TK domain. Then again, this unsaturated, chemically reactive bond led to rapid in vivo metabolism, low bioavailability and, consequently, low accumulation of the labeled compound in the tumor, resulting in low tumor/blood uptake ratios.[14] In order to decrease the chemical reactivity of this irreversible inhibitor, minimize the metabolic rate and increase the tumor uptake of the labeled biomarkers we modified the functional group attached at the 6-position of the quinazoline ring of ML03. The acryl-amide group of ML03 was replaced with α-methoxy-acetamide, α-chloro-acetamide and 4-dimethylamino-but-2-enoic amide. Our previous results with [^{11}C]-ML03 indicated that despite the low initial tumor/blood uptake ratio, it moderately increased with time. Based on these findings we chose to label the new inhibitors with the longer-lived radioisotope, [^{124}I], at the anilino moiety. This particular radioisotope is becoming increasingly significant in PET diagnostic use.[16–20] It can be produced by an in house moderate energy cyclotron however required a special target chamber. It decays ($t_{1/2} = 4.2$ days) simultaneously by positron emission (25.6%) and by electron capture (74.4%). Due to its quantity of short-range Auger electrons (9.2/decay) it has also been discussed as a potential therapeutic nuclide.

2. Results and discussion

The in vivo studies, performed with our previously reported irreversible labeled inhibitor, [^{11}C]-ML03, revealed a high metabolic rate and a low tumor availability of the compound, resulting in moderate tumor/blood uptake ratios of the radiotracer.[14] These findings suggested that the development of more chemically stable inhibitors, which would be less reactive, and therefore less susceptible to metabolic modifications, may improve the in vivo performance of these compounds, and set them as better candidates for molecular imaging of EGFR positive tumors. The major drawback of labeling ML03 with ^{11}C is the short half-life of this isotope ($t_{1/2} = 20.3$ min), limiting the in vivo studies to rather short time periods, of 1–2 h post injection into the rats. Hence, this set of new, more chemically stable inhibitors was designated for labeling with ^{124}I. The substantially longer half-life of this isotope would enable a prolonged follow up after injection of the radiotracer. This assumption relies on the fact that following autophosphorylation of the receptor, it is degraded with a half-life of 20 h,[21] thus allowing sufficient receptor-inhibitor binding time for imaging. In addition, our previously reported studies with A431 cells revealed that the inhibitory effect of ML03 upon EGFR autophosphorylation was sustained (98% inhibition, 8 h post removal of the compound from the medium),[14] implying that the compound is covalently bound to the receptor, thus leaving a broad range of time for imaging.

On the basis of these premises we synthesized three new EGFR inhibitors, all of which belong to the 6-anilino-quinazoline family. The three derivatives,[11] α-methoxy-acetamide (**4**), α-chloro-acetamide (**5**) and 4-dimethyl-amino-but-2-enoic amide (**6**) were labeled with iodine-124 at the anilino moiety. These groups of inhibitors are capable of maintaining the irreversible binding nature since the α carbon (compound **5**) and the β carbon (compound **6**) are partially positively charged, and thus sufficiently reactive to nucleophilic attack by the cysteine moiety at the receptor's ATP-binding site.[11] The synthesis of the nonlabeled compounds is outlined in Figure 2. Compound **3** was used as a basic starting material for the preparation of the three different groups of inhibitors. It was reacted with α-methoxy-acetylchloride to

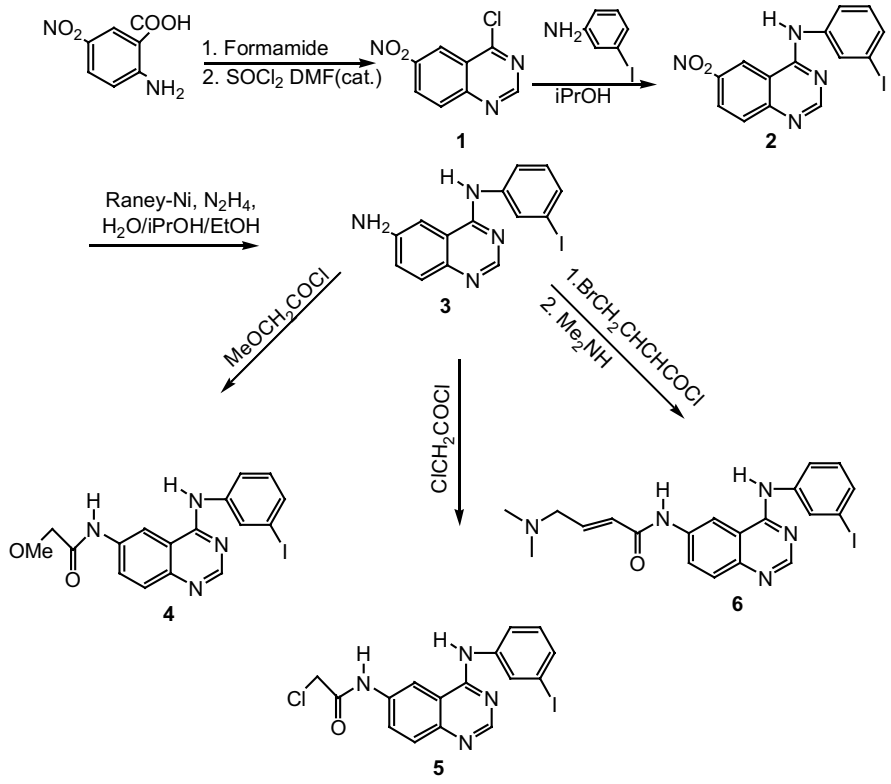

Figure 2. Synthesis of new irreversible EGFR inhibitors.

yield compound **4** with 65% yield, and with α-chloroacetylchloride to yield compound **5** with 54% yield. Compound **6** was obtained after a two-step synthesis (Fig. 2). Compound **3** was reacted with bromo/chlorocrotonyl chloride followed by a reaction with dimethylamine to obtain **6** with a 20% yield. Since the crotonyl chloride was obtained from the bromocrotonic acid by a reaction with oxalyl chloride, we obtained a mixture of inseparable bromo/chlorocrotonyl chloride.[22]

The three nonlabeled compounds, **4**–**6**, were evaluated in a cell-free system by means of ELISA in order to determine their EGFR autophosphorylation IC_{50} values. The assays were performed with A431 cell lysates, as described in Section 4, and the IC_{50} values of the three compounds are summarized in Table 1. Compounds **5** and **6** appeared to be more potent than the α-methoxyacetamide derivative (**4**), with compound **6** being as potent as ML03 with regard to EGFR inhibition.

In order to assess the irreversible effect of the compounds on EGFR autophosphorylation, the inhibitors were incubated with intact A431 cells for 1 h, as described in Section 4, and the degree of EGFR phosphorylation was measured either immediately after or 8 h post removal of the inhibitor from the medium. As previously described,[23] if 80% inhibition, or more, is achieved after 8 h, the compound is considered to be irreversible, while 20–80% inhibition classifies the compound as 'partially irreversible'. The results in Table 1 indicate that compounds **5** and **6** possess similar potencies with regard to their inhibitory effect upon EGFR phosphorylation. Inhibition of phosphorylation by these two compounds was retained 8 h post removal of the inhibitors from the medium, reflecting the irreversible effect of these inhibitors, which is most likely, due to covalent binding at the ATP-binding site. The ability of compound **5** to bind irreversibly indicates that a chain of four atoms attached to the 6-position at the

Table 1. IC_{50} values in the ELISA screen with A431 cell lysates and IC_{80} values for inhibition of EGFR autophosphorylation in intact A431 cells immediately after and 8 h post removal of the compound from the medium

Structure	A431 lysate IC_{50} app	Intact A431 cells	
		IC_{80}[a] range (immediately after removal of the inhibitor)	IC_{80}[a] range (8 h post removal of the inhibitor)
ML01	0.208 nM	3–5 nM	—
ML03	0.037 nM	5–10 nM	5–10 nM
4	72 ± 50 nM	<1 μM	>80 μM
5	0.1–20 nM	4–10 nM	10–50 nM
6	0.05–5 nM	2–10 nM	2–10 nM

[a] IC_{80} is the inhibitory concentration required to inhibit 80% of EGFR phosphorylation.

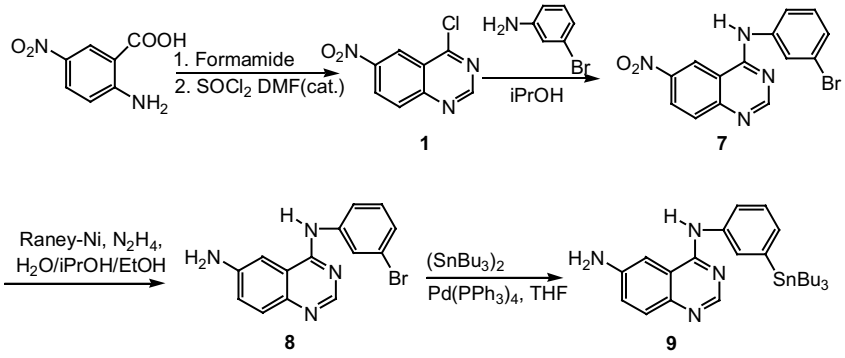

Figure 3. Synthesis of the starting material for radiolabeling with ^{124}I.

quinazoline moiety is not essential. Structurally, a chain of three atoms, as in compound **5**, is sufficient to achieve covalent binding at the receptor's kinase domain. With compound **4**, which is much more chemically stable, partial irreversible binding was observed.

Compound **9**, the starting material for the radiolabeling, was prepared by the reaction of 4-chloro-6-nitroquinazoline (**1**) with 6-bromoaniline to yield compound **7** (Fig. 3). The nitro group at the 6-position of the quinazoline ring was reduced using hydrazine hydrate and RaNi to yield 6-amino-4-[(3-bromophenyl)amino]-quinazoline (**8**). In the last step compound **9** was obtained by the reaction of **8** with $(SnBu_3)_2$ in the presence of a catalytic amount of $Pd(PPh_3)_4$ under dry conditions. For the preparation of the starting material, **9**, we chose to work with the bromoaniline rather than with the iodoaniline derivative since the nitro reduction step with the bromo derivative gave better yields than the iodo derivative (deiodination). In addition, had we chosen to work with the iodo derivative, the specific activity of the final labeled compounds might have been lower.

The radiosynthesis route is outlined in Figure 4. Compound **9** was reacted with a solution of [^{124}I] NaI in 0.1 M NaOH[24] at room temperature to yield the [^{124}I]-6-amino-4-[(3-iodophenyl)amino]-quinazoline **[^{124}I]-3** with 50% radiochemical yield. Reaction of the latter with bromo/chlorocrotonylchloride at 0 °C was followed by reaction with dimethylamine, which provided **[^{124}I]-6** (**[^{124}I]-ML06**) with 23% yield and a total radiosynthesis time, including HPLC purification, of 90 min. Reaction of **[^{124}I]-3** with α-methoxyacetylchloride or α-chloroacetylchloride at 0 °C provided the desired **[^{124}I]-4**(**[^{124}I]-ML07**), and **[^{124}I]-5** (**[^{124}I]-ML08**) with 28% and 36% overall radiochemical yields, respectively, and a total radiosynthesis time of 70 min.

3. Conclusion

In order to achieve a better tumor uptake of the irreversible EGFR inhibitors and to afford in vivo analyses at later time points post injection, we successfully designed and synthesized three new irreversible EGFR inhibitors with varying chemical reactivities. These inhibitors were radiolabeled with the longer-lived radionuclide, iodine-124, on the anilino moiety of the molecule. The inhibitory potency of compounds **5** (α-chloroacetamide) and **6** (4-dimethylamino-but-2-enoic

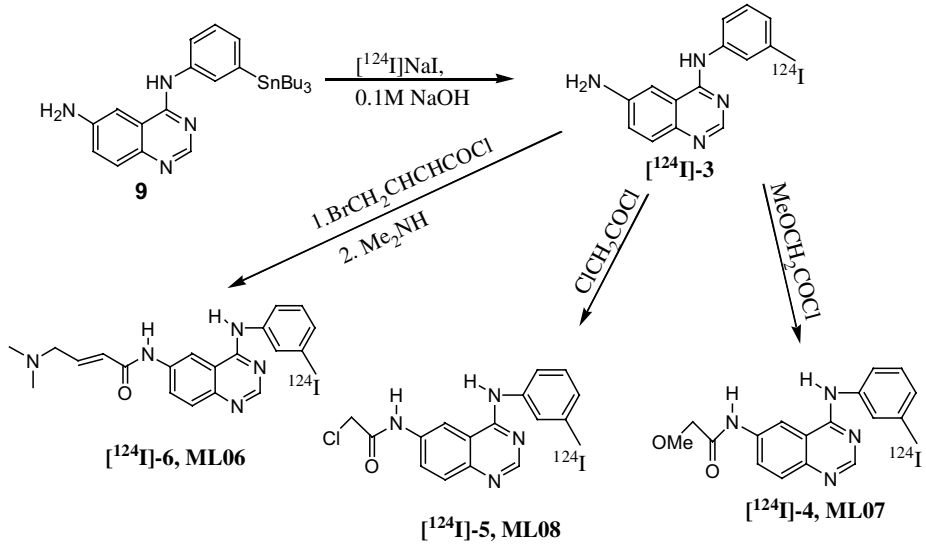

Figure 4. ^{124}I labeling of irreversible EGFR inhibitors.

amide) toward the EGFR in intact A431 cells was found to be in the nM range, and their inhibitory effect was demonstrated to be sustained. These data suggest that a chain of four atoms, attached at the 6-position of the quinazoline moiety, is not essential for covalent binding. Structurally, a chain of three atoms is sufficient to achieve irreversible binding. With compound **4** (α-methoxyacetamide), the methoxy group at the α position of the acetamide group was found to be insufficient to serve as a leaving group so as to form a covalent bond between Cys-773 at the receptor's kinase domain and the inhibitor, resulting in a partially irreversible effect. Altogether, these in vitro data reflect the promising potential of compounds **5** and **6** as future biomarkers for molecular imaging of EGFR positive tumors. The feasibility of their use as PET biomarkers is currently investigated.

4. Materials and methods

4.1. General

All chemicals were purchased from Sigma–Aldrich, Fisher Scientific, Merck or J. T. Baker. Chemicals were used as supplied, excluding THF, which was refluxed over sodium and benzophenone, and freshly distilled prior to use. [^{124}I]-NaI was purchased as a 0.02 M solution from Ritverc GmbH, Russia.

Mass spectrometry was performed in EI mode on a Thermo Quest—Finnigan Trace MS—mass spectrometer at the Hadassah-Hebrew University Mass Spectroscopy facility. ^{1}H NMR spectra were obtained on a Bruker AMX 300 MHz instrument. Elemental analysis was performed at the Hebrew University Microanalysis Laboratory. HPLC separations were carried out using a Varian 9012Q pump, a Varian 9050 variable wavelength detector operating at 254 nm and a Bioscan Flow-Count radioactivity detector with a NaI crystal. Analyses of the labeled and unlabeled compounds were performed on a reversed-phase system using Waters γ-Bondapack C18 analytical column (10 μm, 300 × 3.9 mm) with mobile phase systems, composed of CH_3CN/acetate buffer or 47% CH_3CN/53% 0.1 M ammonium formate buffer.

For the preparation of the labeled [^{124}I]-amine-quinazoline we followed the general procedure of John et al.[24] 6-Nitroquinazolone was prepared according to the published procedure.[25]

A431 human epidermoid vulval carcinoma cells were grown in Dulbecco's modified Eagle medium (DMEM) (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal calf serum and antibiotics (penicillin 10^5 units/L, streptomycin 100 mg/L) at 37 °C, 5% CO_2.

4.1.1. 4-Chloro-6-nitroquinazoline (1). 6-Nitroquinazolone (2 g, 0.01 mmol) and $SOCl_2$ (20 mL) were placed in a two-necked flask and DMF (100 μL) was added. The mixture was refluxed for 1 h, and then additional quantities of $SOCl_2$ (10 mL) and DMF (50 μL) were added. After a 3 h reflux the thionyl chloride was distilled out, and the purity of the product, 4-chloro-6-nitroquinazoline (**1**), was determined using a reversed-phase C18 analytical HPLC column (96–98% purity). The compound was kept at 0 °C, and used without any further purification for the next step. Mp = 130 °C, ^{1}H NMR (DMSO-d_6) δ 8.78 (1H, d, $J = 2$ Hz), 8.555 (1H, dd, $J1 = 6.7$ Hz, $J2 = 2$ Hz), 8.432 (1H, s), 7.883 (1H, d, $J = 6.7$ Hz). HPLC conditions: C18 analytical column, 40% acetate buffer pH = 3.8/60% acetonitrile, flow = 1 mL/min; rt = 4.95 min.

4.1.2. 6-Nitro-4-[(3-iodophenyl)amino]-quinazoline (2). 4-Chloro-6-nitroquinazoline (**1**) (4 g, 23 mmol) and 3-iodo-aniline (12.57 g, 57 mmol) were dissolved, and stirred in *i*-PrOH (40 mL) at 25 °C for 10 min, yielding a bright-yellow precipitate. The mixture was then refluxed, stirred for an additional 3 h, and cooled. The solid was filtered, rinsed with *i*-PrOH (12 mL), and dried in a vacuum oven at 80 °C to yield **2** (5.99 g, 78%). MS (m/z) 393.2 $(MH)^+$; ^{1}H NMR (DMSO-d_6) δ 10.56 (1H, s), 9.664 (1H, d, $J = 2.4$ Hz), 8.784 (1H, s), 8.578 (1H, dd, $J1 = 11.4$ Hz, $J2 = 2.1$ Hz), 8.270 (1H, br s), 7.955 (2H, m), 7.543 (1H, d, $J = 8.1$ Hz), 7.228 (1H, t, $J = 7.8$ Hz); HPLC conditions: C18 analytical column, 45% acetate buffer pH = 3.8/55% acetonitrile, flow = 1 mL/min; rt = 17.8 min.

4.1.3. 6-Amino-4-[(3-iodophenyl)amino]-quinazoline (3). Compound **2** (620 mg, 1.58 mmol) was placed in a flask, and a solution of H_2O–EtOH–IPA, 5:45:50 (107 mL) was added. The mixture was heated to 95 °C, and an additional 50 mL of solvent was added until complete dissolution. The mixture was cooled to 65 °C, and RaNi (1/2 Pasteur pipette) and hydrazine hydrate (153 μL, 3.16 mmol) were added successively until a green solution was obtained. The reaction was heated to 80–85 °C, and more RaNi (1/2 Pasteur pipette) and hydrazine hydrate (38 μL, 0.8 mmol) were added. Reflux was maintained for 15–20 min. The solution was cooled, and filtered through a layer of Celite (prepared as slurry in EtOH). The mixture was evaporated to yield compound **3** (180 mg, 31.4%). MS (m/z) 363.0 $(MH)^+$; ^{1}H NMR (DMSO-d_6) δ 9.365 (1H, s), 8.347 (1H, s), 8.323 (1H, t, $J = 2.4$ Hz), 7.918 (1H, dd, $J1 = 10$ Hz, $J2 = 2.4$ Hz), 7.524 (1H, d, $J = 11.6$ Hz), 7.388 (1H, d, $J = 7.2$ Hz), 7.318 (1H, d, $J = 2.8$ Hz) 7.235 (1H, dd, $J1 = 11.6$ Hz, $J2 = 2.8$ Hz), 7.134 (1H, t, $J = 10.4$ Hz) 5.595 (2H, br s); HPLC conditions: C18 analytical column, 55% acetate buffer pH = 3.8/45% acetonitrile, flow = 1 mL/min; rt = 8.3 min.

4.1.4. N-{4-[(3-Iodophenyl)amino]-quinazoline-6-yl}-2-methoxyacetamide (4). 6-Amino-4-[(3-iodophenyl)-amino]-quinazoline (**3**) (100 mg, 0.276 mmol) was dissolved in dry THF (20 mL), and cooled to 0 °C. Triethylamine (65 μL, 48 mg; 0.469 mmol, 1.7 equiv) was

added, and the mixture was kept at 0 °C for an additional 15 min. Methoxyacetylchloride (43 μL, 51 mg, 0.469 mmol, 1.7 equiv) was added, and the mixture was stirred at 0 °C for 30 min. Then the temperature was raised to room temperature, and a solution of saturated $NaHCO_3$ (30 mL) was added. THF was evaporated, and the residue was extracted with ethyl acetate (3 × 30 mL). The organic layer was dried with Na_2SO_4, and evaporated. The product was purified on a silica gel flash chromatography column with MeOH–CH_2Cl_2, 3:97 as eluent to yield **4** (102 mg, 64.5%). Mp = 159–163 °C; MS (*m*/*z*) 435.0 $(MH)^+$; ^1H NMR (DMSO-d_6) δ 10.06 (1H, s), 9.83 (1H, s), 8.697 (1H, d, $J = 3$ Hz), 8.571 (1H, s), 8.277 (1H, dd, $J1 = 5.7$ Hz, $J2 = 2.7$ Hz), 7.991 (1H, dd, $J1 = 16.8$ Hz, $J2 = 3.3$ Hz), 7.909 (1H, dd, $J1 = 16.5$ Hz, $J2 = 0.9$ Hz), 7.781 (1H, d, $J = 16.8$ Hz), 7.46 (1H, d, $J = 16.5$ Hz), 7.183 (1H, t, $J = 14.43$ Hz), 4.09 (1H, s), 3.43 (3H, s). Anal. Calcd: C, 46.97; H, 3.45; N, 12.89. Found: C, 46.19; H, 3.65; N, 12.59. HPLC conditions: C18 analytical column, 55% acetate buffer pH = 3.8/45% acetonitrile, flow = 1 mL/min; rt = 10.7 min.

4.1.5. N-{4-[(3-Iodophenyl)amino]-quinazoline-6-yl}-2-chloroacetamide (5). Compound **5** was prepared in the same manner as **4**, starting with compound **3** (138 mg, 0.38 mmol) and chloroacetyl chloride (76 μL, 0.94 mmol). The crude product was purified by silica gel flash chromatography with MeOH–CH_2Cl_2, 3:97 as eluent to yield **5** (90 mg, 54%). Mp>300 °C; MS (*m*/*z*) 439.0 $(MH)^+$; ^1H NMR (DMSO-d_6) δ 10.6 (1H, s), 9.9 (1H, s), 8.71 (1H, s), 8.57 (1H, s), 8.25 (1H, m), 7.82 (2H, m), 7.45 (1H, d, $J = 7.8$ Hz), 7.19 (2H, m), 4.34 (2H, s). Anal. Calcd: C, 43.81; H, 2.76; N, 12.77. Found: C, 43.52; H, 3.18; N, 12.21. HPLC conditions: C18 analytical column, 55% acetate buffer pH = 3.8/45% acetonitrile, flow = 1 mL/min; rt = 13.1 min.

4.1.6. 4-Dimethylamino-but-2-enoic acid {4-[(3-iodophenyl)-amino]-quinazoline-6-yl}-amide (6). (2*E*)-4-Bromo/chloro-N-4-[(3-iodophenyl)amino]-quinazoline-6-yl-2-butenamide was prepared in the same manner as **5**, starting with **3** (382 mg, 1.055 mmol) and Br/Cl-crotonylchloride (221 mg, 1.213 mmol). The crude material was purified by silica gel flash chromatography (MeOH–CH_2Cl_2 5:95) to yield (2*E*)-4-bromo/chloro-N-{4-[(3-iodophenyl)amino]-quinazoline-6-yl}-2- butenamide (149 mg, ~30%) as an inseparable mixture of the bromide and chloride in a ~1:1 ratio. MS (*m*/*z*) 509 $(M)^+$, 465 $(MH)^+$; ^1H NMR (DMSO-d_6) δ 10.6 (1H, s), 10.24 (1H, s), 8.8 (1H, s), 8.58 (1H, s), 8.27 (1H, s), 7.8 (3H, m), 7.47 (1H, d, $J = 8$ Hz), 7.19 (1H, t, $J = 8$ Hz), 6.96 (1H, br dt, $J1 = 15$ Hz, $J2 = 7.2$ Hz), 6.47 (1H, d, $J = 15$ Hz), 4.38 (2H, d, $J = 7.2$ Hz). Anal. Calcd (for a 1:1 ratio): C, 43.27; H, 2.82; N, 11.21; Found: C, 43.60; H, 2.81; N, 11.12; HPLC condition: C18 analytical column, 55% acetate buffer/45% acetonitrile, flow = 1.0 mL/min; rt = 21.69 min (first peak), 24.08 min (second peak).

A solution of (2*E*)-4-bromo/chloro-N-4-[(3-iodophenyl)amino]-quinazoline-6-yl-2-butenamide (320 mg, 0.662 mmol) in dry THF (70 mL) was stirred, and dimethylamine (2 M in THF, 10 mL) was added dropwise. The reaction was heated to 80 °C for 1 h, cooled, and EtOAc (50 mL) and saturated $NaHCO_3$ (50 mL) were added. The layers were separated, and the organic layer was washed with brine, dried with $MgSO_4$, and evaporated. The crude material was purified by silica gel flash chromatography (MeOH–CH_2Cl_2 5:95) to yield **6** (180 mg, 58%). Mp = 212–216 °C; MS (*m*/*z*) 474 $(MH)^+$; ^1H NMR (DMSO-d_6) δ 10.4 (1H, s), 9.8 (1H, s), 8.8 (1H, s), 8.57 (1H, s), 8.32 (1H, s), 7.8 (3H, m), 7.47 (1H, d, $J = 8$ Hz), 7.19 (1H, t, $J = 8$ Hz), 6.85 (1H, dt, $J1 = 15$ Hz, $J2 = 7.2$ Hz), 6.37 (1H, d, $J = 15$ Hz), 3.1 (2H, d, $J = 7.2$ Hz), 2.19 (6H, s). Anal. Calcd: [$C_{20}H_{20}IN_5O{\cdot}0.5H_2O$]: C, 49.79; H, 4.35; N, 14.52. Found: C, 49.51; H, 4.36; N, 13.89. HPLC conditions: C18 analytical column, 55% acetate buffer pH = 3.8/45% acetonitrile, flow = 1 mL/min; rt = 7.54 min.

4.1.7. 6-Nitro-4-[(3-bromophenyl)amino]-quinazoline (7) . This compound was prepared in the same manner as **2**, according to Tsou et al.,[22] starting from 4-chloro-6-nitroquinazoline (**1**). Mp = 267–270 °C; MS (*m*/*z*) 345 $(MH)^+$; HPLC conditions: C18 column, 55% acetate buffer pH = 3.8/45% acetonitrile, flow = 1 mL/min; rt = 7.54 min.

4.1.8. 6-Amino-4-[(3-bromophenyl)amino]-quinazoline (8). This compound was prepared in the same manner as **3** starting from 6-nitro-4-[(3-bromophenyl) amino]-quinazoline (**7**) (590 mg, 1.7 mmol) to yield **8** (332 mg, 62%).Mp = 204 °C; MS (*m*/*z*) 315 $(MH)^+$; HPLC conditions: C18 column, 45% acetate buffer pH = 3.8/55% acetonitrile, flow = 1 mL/min; rt = 6.41 min.

4.1.9. 6-Amino-4-[(3-tributylstannylphenyl)amino]-quinazoline (9). 6-Amino-4-[(3-bromophenyl)-amino]-quinazoline (**8**) (300 mg, 0.95 mmol) was dissolved in dry THF (20 mL), and $(SnBu_3)_2$ (1.92 mL, 3.78 mmol) was added, followed by the addition of $Pd(PPh_3)_4$ (547.8 mg, 0.474 mmol) in dry THF (0.5 mL). The mixture was refluxed for 16 h, and the solvent was evaporated. The crude product was purified over an aluminium oxide 90 column (70–230 mesh) with hexane–dichloromethane 20:80 followed by 100% dichloromethane as eluents to yield **9** (85 mg, 20%); MS (*m*/*z*) 527 $(M+2H)^+$; ^1H NMR δ ($CDCl_3$): 8.592 (1H, s), 7.75 (1H, d, $J = 8.7$ Hz), 7.64 (2H, m), 7.58 (1H, m), 7.47 (3H, m), 1.567 (6H, m), 1.308 (6H, m), 1.077 (6H, t, $J = 5.7$ Hz), 0.919 (9H, t, $J = 7.2$ Hz); HPLC conditions: Normal-Phase analytical column, 100% acetonitrile, flow = 1.0 mL/min; rt = 13.59 min.

4.2. Radiochemistry

4.2.1. [^{124}I]-6-Amino-4-[(3-iodophenyl)amino]-quinazoline ([^{124}I]-3). 6-Amino-4-[(3-tributylstannylphenyl)amino]-quinazoline (**9**) (4 mg) was placed in a conical vial, EtOH (1.2 mL) was added, followed by addition of 0.1 M [^{124}I] NaI (1 mL). 0.1 N HCl (1 mL) and Chloramine-T (1 mg/mL) (1 mL) were added, and the vial was

sealed. The reaction was stirred at room temperature for 15 min, after which, sodium metabisulfite (200 mg/mL) (3 mL), a saturated solution of $NaHCO_3$ (6 mL) and saline solution (6 mL) were added. After the aqueous solution was vortexed, and loaded onto a C18 Sep-pak, the latter was rinsed with water (2.5 mL), dried under nitrogen for 10 min, and the product was eluted with dry THF (4 mL). The THF was dried with Na_2SO_4, filtered through 0.45 μm filter into a v-vial, and used without any further treatment for the next step. The purity of the product was analyzed by a reversed-phase C18 analytical column (10 μm, 300 × 3.9 mm), eluted with 55% acetate buffer/45% acetonitrile, flow = 1.0 mL/min; rt = 8.3 min. In order to measure the radiochemical yield of this step the THF solution, which contained the reaction product, was evaporated to a volume of 200 μL, and injected onto a reversed-phase C18 preparative column. The average radiochemical yield of **[^{124}I]-3** was 50% ($n = 7$). HPLC conditions: C18 preparative column, eluted with 60% acetate buffer/40% acetonitrile, flow = 3.0 mL/min; rt = 10.6 min.

4.2.2. [^{124}I]-4-Dimethylamino-but-2-enoic acid {4-[(3-iodophenyl)amino]-quinazoline-6-yl}amide ([^{124}I]-6). **[^{124}I]-3** in THF (4 mL) was cooled to 0 °C for 10 min, and a 0.5 mL solution of Br/Cl-crotonylchloride[22] in dry THF (182 mg/3 mL) was added. The reaction mixture was stirred for 30–40 min at 0 °C, and used without any further treatment for the next step. For measurement of the radiochemical yield of this step the THF solution, which contained the reaction product, was evaporated to a volume of 200 μL, and injected onto a reversed-phase C18 preparative column. [^{124}I]-(2*E*)-4-bromo/chloro-N-{4-[(3-iodophenyl)amino]-quinazoline-6-yl}-2-butenamide was obtained with an average of 58% radiochemical yield ($n = 4$). HPLC conditions: C18 analytical column, 55% acetate buffer/45% acetonitrile, flow = 1.0 mL/min; rt = 21.69 min (first peak), 24.08 min (second peak). HPLC conditions: C18 preparative column, 55% acetate buffer/45% acetonitrile, flow = 4.0 mL/min; rt = 27.17 min (first peak), 31.29 min (second peak).

[^{124}I]-(2E)-4-Bromo/chloro-N-{4-[(3-iodophenyl)amino]-quinazoline-6-yl}-2-butenamide in THF (4 mL) was cooled to 0 °C for 10 min, and 1 mL of 2.0 M dimethylamine in THF was added. After 30–40 min of stirring at 0 °C a mixture of ACN–H_2O (1:1) (200 μL) was added, and the solution was evaporated under nitrogen, while cooling in an iced-water bath, to a volume of 400 μL. The crude product was purified using an HPLC reversed-phase C18 preparative column to yield **[^{124}I]-6** with an average 45% radiochemical yield, specific activity of >6 Ci/mmol (our detection limit) and 99% radiochemical purity ($n = 4$). HPLC conditions: C18 preparative column, 60% acetate buffer/40% acetonitrile, flow = 3.0 mL/min; rt = 6.95 min. HPLC conditions: C18 analytical column, 55% acetate buffer/45% acetonitrile, flow = 1.0 mL/min; rt = 7.54 min.

4.2.3. [^{124}I]-N-{4-[(3-Iodophenyl)amino]-quinazoline-6-yl}-2-methoxyacetamide ([^{124}I]-4). **[^{124}I]-3** in THF (4 mL) was cooled to 0 °C for 10 min, and methoxyacetylchloride (200 μL) in dry THF (300 μL) was added. The reaction mixture was stirred for 30–40 min at 0 °C. A mixture of ACN–H_2O (1:1) (200 μL) was added, and the solution was evaporated under nitrogen, while cooling in an iced-water bath, to a volume of 400 μL. The crude product was purified using an HPLC reversed-phase C18 preparative column to yield **[^{124}I]-4**, with an overall radiochemical yield of 28%, specific activity of >6 Ci/mmol (our detection limit) and 99% radiochemical purity ($n = 4$). HPLC conditions: C18 preparative column, 60% acetate buffer/40% acetonitrile, flow = 4.0 mL/min; rt = 22.31 min; HPLC conditions: C18 analytical column, 55% acetate buffer/45% acetonitrile, flow = 1.0 mL/min; rt = 10.78 min.

4.2.4. [^{124}I]-N-{4-[(3-Iodophenyl)amino]-quinazoline-6-yl}-2-chloroacetamide ([^{124}I]-5). **[^{124}I]-5** was obtained in the same manner as **[^{124}I]-4** by the reaction of compound **[^{124}I]-3** with chloroacetylchloride (200 μL) in dry THF (300 μL) with an overall radiochemical yield of 36% specific activity of >6 Ci/mmol (our detection limit) and 99% radiochemical purity ($n = 4$). HPLC conditions: C-18 analytical column, 55% acetate buffer/45% acetonitrile, flow = 1.0 mL/min; rt = 13.16 min; HPLC conditions: C18 preparative column, 55% acetate buffer/45% acetonitrile, flow = 3.0 mL/min; rt = 20.39 min; HPLC conditions: C18 analytical column, 55% acetate buffer/45% acetonitrile, flow = 1.0 mL/min; rt = 13.16 min.

4.3. Biology

4.3.1. Production of A431 cell lysates as an EGFR source. A431 cells were grown in 14 cm Petri dishes to about 90% confluence. The dishes were washed twice with cold phosphate buffered saline (PBS) pH 7.4, and placed on ice, prior to the addition of 3.25 mL cold, freshly prepared lysis buffer (50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride (AEBSF), 1 μg/mL aprotinin, 300 μg/mL benzamidine, 10 μg/mL leupeptin, 10 μg/mL soy-trypsin inhibitor) for 10 min. The cells were scraped from the plates with a rubber policeman, homogenized with a dounce homogenizer, and centrifuged (Sorvall centrifuge, rotor 5, 10,000 rpm, 10 min, 4 °C). The supernatant, which contained the EGFR, was collected and frozen at −70 °C in aliquots.

4.3.2. ELISA screen. EGFR autophosphorylation IC_{50} values were obtained by means of an ELISA screen. All the following incubations were performed at 25 °C with constant shaking. The final volume for each well was 150 μL. After each step, the plate was washed with 200 μL of double distilled water (×4) and 200 μL TBST buffer (10 mM Tris–HCl pH 7.4 (TBS), 0.2% Tween 20, 170 mM NaCl) (×1).

A Corning 96-well ELISA plate was coated with monoclonal anti-EGFR antibody (mAb108) by incubating overnight (4 °C) in a solution of mAb108 diluted in PBS pH 8.5. Total mAb108 content per well was 0.75 μg. The plate was washed to remove unbound mAb108, and PBS containing 5% low-fat milk was added to block the unbound sites in the plate for 30 min. An aliquot of A431 cell lysate was thawed, diluted in PBS pH 7.4, and added to the plate at a final total protein concentration of 10 μg/well.

After 30 min each inhibitor was added at a range of concentrations, and for each series, one well was left with no inhibitor, as a zero inhibition control. All inhibitors were diluted in TBS/dimethylsulfoxide (DMSO) to a final DMSO concentration of 0.5% in each well. After an additional 25 min, and without washing the plate, ATP–$MnCl_2$ solution was added to each well to start the reaction, the final concentration being 5 μM ATP, 5 mM $MnCl_2$. The reaction was allowed to proceed for exactly 5 min.

To stop the phosphorylation reaction an ethylenediamine tetra-acetic acid (EDTA) solution (pH 8.0, 100 mM final concentration) was added to each well, and after 10 min the plate was washed. Polyclonal antiphosphotyrosine serum (Sugen Inc.) diluted 1:2000 in TBST containing 5% low-fat milk (blocking TBST) was added, and the plate was incubated for 45 min.

For the colorimetric detection of phosphotyrosine content in EGFR commercial TAGO anti-rabbit peroxidase-conjugated antibody diluted 1:10,000 in blocking TBST was added to the wells, and allowed to react for 45 min. After washing with TBST only the colorimetric reaction was initiated by adding 100 μL/well ABTS–H_2O_2 in citrate–phosphate buffer pH 4.0 (7.5 mg 2-2′-azino-bis(3-ethylbenzethiazoline-6-sulfonic acid) (ABTS), 2 μL 30% H_2O_2, 15 mL citrate–phosphate buffer pH 4.0). After 5–10 min, the plate was read on a Dynaytec MR 5000 ELISA reader at 405 nm. Analysis of the data was performed with GraphPad Prism, version 2.01 (GraphPad Software, Inc.). For each compound, at least two independent assays that gave similar results were performed. Each assay was carried out using duplicate samples.

4.3.3. Irreversibility test protocol. A431 cells (5×10^5) were grown in six-well plates (35 mm diameter, Nalge Nunc) for 24 h to ≈90% confluence, and then incubated in serum-free medium for 18 h. Duplicate sets of cells were treated with compounds to be tested as irreversible inhibitors at varying concentrations for 1 h. The final concentration of the vehicle in the medium was 0.05% DMSO, 0.1% EtOH. After removal of the inhibitor from the medium, PBS wash (×2) and addition of serum-free medium to the wells, one set of cells was stimulated with 20 ng/mL human EGF (Sigma) for 5 min at 37 °C, and then washed with cold PBS. Cell extracts were made by adding 0.4 mL boiling Laemmli buffer (10% glycerol, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 62.5 mM Tris·HCl pH 6.8), containing 0.001% bromophenol blue, to the cells, scraping them with a rubber policeman and heating to 100 °C for 10 min. The other set of cells was incubated for 2 h, washed with PBS, incubated for another 2 h, washed again, incubated a further 4 h, and then stimulated with EGF. Cell lysates were prepared similar to the first set of cells.

4.3.4. Western blot analysis. Protein lysates (30 μg) were separated by SDS-PAGE (8% polyacrylamide), and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in blocking TBST for 30 min, and incubated overnight with PY20 anti phosphotyrosine antibody (Santa Cruz Biotechnology Inc., Santa Cruz, USA) diluted 1:2000 in blocking TBST. The membrane was washed thoroughly with TBST, and incubated for 1 h with a horseradish peroxidase-conjugated anti-mouse IgG (Jackson Immuno Research, 1:10,000 dilution in blocking TBST). Finally, the membrane was washed in TBST (4×5 min), and immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) detection reagent. Quantification of each band was performed using Adobe Photoshop 5.0 ME and NIH image 1.61/ppc programs.

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