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Radioiodination of new EGFR inhibitors as potential SPECT agents for molecular imaging of breast cancer

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Abstract—In our search for the development of novel SPECT radioligands for EGFR positive tumours, new potentially irreversible tyrosine kinase (TK) inhibitors are being explored. The radioiodination of *N*-{4-[(3-chloro-4-fluorophenyl) amino]quinazoline-6-yl}-3-bromopropionamide, a novel EGFR-TK inhibitor synthesised in our laboratory, was accomplished via halogen exchange. Purification by RP-HPLC gave [^{125}I]-*N*-{4-[(3-chloro-4-fluorophenyl)amino]quinazoline-6-yl}-3-iodopropionamide with a radiochemical purity higher than 95% and a high specific activity. In vitro studies indicate that both iodinated quinazoline and its bromo precursor inhibit A431 cell growth and also possess higher potency than the parent quinazoline to inhibit the EGFR autophosphorylation. In vivo stability studies suggest metabolization of the radioiodinated quinazoline indicating a short biological half-life. The in vitro results point out that these quinazoline derivatives could be promising candidates for SPECT imaging of EGFR positive tumours provided that they are selectively modified in order to achieve better in vivo radiochemical stability.

1. Introduction

The epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases (TK) involved in the proliferation of normal and malignant cells. EGFR is often overexpressed in many tumour cells, namely in breast and prostate cancer and non-small cell lung carcinoma.[1] Therefore, the EGFR has become an attractive target for the design and development of potential anticancer drugs. Several compounds that selectively bind the receptor and inhibit its tyrosine kinase activity have been reported. The most active tyrosine kinase inhibitors are quinazoline-based small molecules that compete with adenosine triphosphate (ATP) and prevent its binding to the intracellular tyrosine kinase region. Such compounds can serve for therapeutics and, when labelled, as targeted diagnostic agents. The two compounds that are at the most advanced stage of development are the 4-(phenylamino)quinazolines, Gefitinib[2] and Erlotinib,[3] both of which reversibly target EGFR. The Gefitinib (ZD1839, Iressa®)[2] was approved by the FDA as a third-line treatment of chemotherapy-refractory non-small cell lung carcinomas. The Erlotinib (OSI-774, Tarceva©)[3] is presently undergoing phase 3 clinical trials (Fig. 1).

A predictive marker that will help to select patients for treatment with EGFR inhibitors is needed to optimise the therapeutic potential of EGFR inhibition in cancer. Recently, there has been a growing interest in the use of EGFR-TK inhibitors, such as quinazoline derivatives, as radiotracers for molecular imaging. Several reversible and irreversible inhibitors, particularly from the 4-anilinoquinazoline class, were labelled with positron emitters such as fluorine-18, carbon-11 and iodine-124 and their

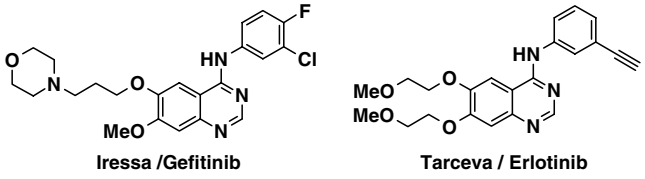

Figure 1. Some successful examples of EGFR-TK inhibitors that have progressed to clinical trials and patient care.

Keywords: EGFR; Tyrosine kinase inhibitors; Quinazoline; SPECT; Cancer.
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potential as PET biomarkers was evaluated.[4–6] In our search for the development of novel SPECT radioligands for early detection and staging of EGFR positive tumours, and having in mind that to date there are no promising SPECT imaging agents for targeting the EGFR, novel ^{125}I probes based on quinazoline inhibitors of tyrosine kinase activity are being explored with the ultimate goal of labelling the molecules with ^{123}I, the most adequate radioisotope of iodine for SPECT imaging.

In this work, we report the synthesis and characterization of the novel bioactive precursor, *N*-{4-[(3-chloro-4-fluorophenyl)amino]quinazoline-6-yl-3-bromopropionamide, as well as the synthesis of the corresponding iodinated and radioiodinated analogues. The in vitro biological behaviour of the halogenated quinazolines was studied. In vivo stability studies of the radioiodinated compound, [^{125}I]-*N*-{4-[(3-chloro-4-fluorophenyl)amino]quinazoline-6-yl}-3-iodopropionamide, are also reported in this study.

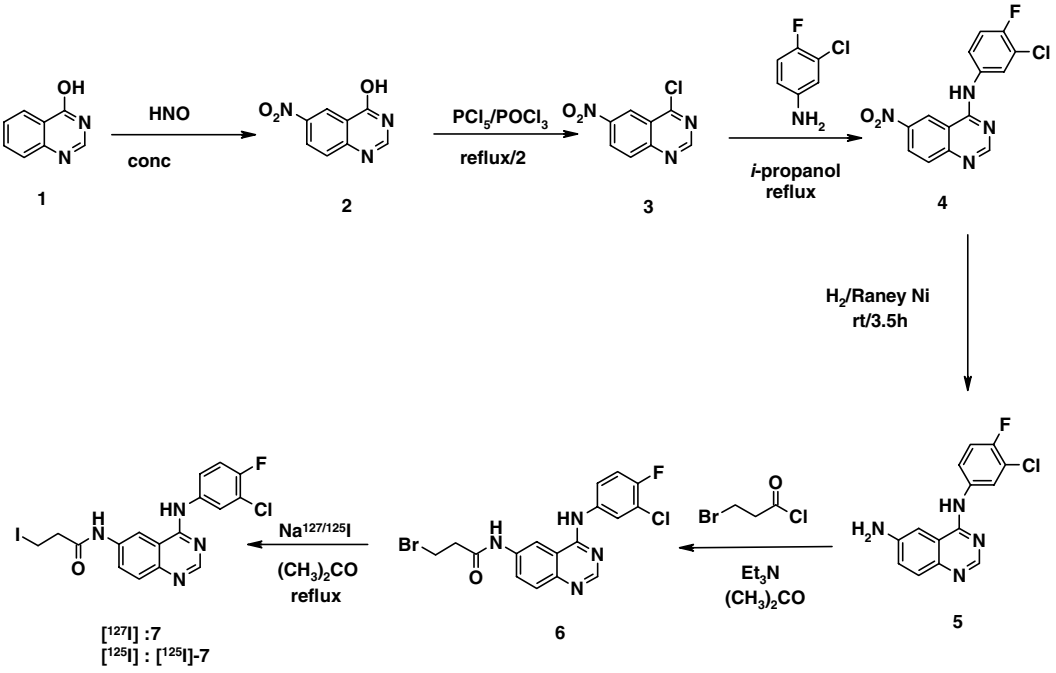
2. Results and discussion

2.1. Chemistry

N-{4-[(3-Chloro-4-fluorophenyl)amino]quinazoline-6-yl}-3-iodopropionamide **7** was prepared from 4-hydroxyquinazoline **1** as depicted in Scheme 1.

4-Hydroxy-6-nitroquinazoline **2**, prepared by nitration of 4-hydroxyquinazoline **1**, was used as the starting material for the preparation of 4-chloro-6-nitroquinazoline **3**. Hence the synthesis of **3** was accomplished according to a slightly modified reported procedure by treatment of the 4-hydroxy-6-nitroquinazoline **2** with $PCl_5/POCl_3$ at 160 °C.[7] 4-[(3-Chloro-4-fluorophenyl)amino]-6-nitroquinazoline, **4**, was obtained by coupling 3-chloro-4-fluoroaniline to the chlorinated compound **3**. In the next step, the nitro group at the 6-position of the quinazoline ring in **4** was reduced to amine by hydrogenation with H_2 over Raney/Ni at room temperature affording 6-amino-4-[(3-chloro-4-fluorophenyl)amino]quinazoline **5**.[8] The reduction of this nitro group was confirmed in the ^1H NMR spectrum by the presence of a singlet (5.61 ppm) corresponding to the amine protons. The amine **5** was subsequently reacted with 3-bromopropanoylchloride to give *N*-{4-[(3-chloro-4-fluorophenyl) amino]quinazoline-6-yl}-3-bromopropionamide **6**. The structure of this bromo precursor was elucidated throughout its ^1H NMR spectrum by absence of the amine protons and by the occurrence of two triplets, integrating for two protons each, at 2.96 and 3.93 ppm corresponding to the bromopropionamide chain methylene protons.

Synthesis of *N*-{4-[(3-chloro-4-fluorophenyl)amino]quinazoline-6-yl}-3-iodopropionamide **7** was carried out by a halogen exchange reaction of **6** with sodium iodide in dry acetone at reflux during 11 h. The iodinated quinazoline **7** was obtained in high chemical purity after semi-preparative reversed-phase HPLC purification with CH_3CN/TFA 0.1% (35:65) as eluent. This mobile phase was selected in order to allow an efficient separation of the iodinated quinazoline from its bromo precursor. The substitution of bromine by an iodine atom in the propionamide chain was confirmed in the ^1H NMR spectrum of **7**. Actually, in this spectrum the methylene protons are relatively shifted appearing at 3.13 and 3.52 ppm, respectively, while the methylene protons of **6** appear at 2.96 and 3.93 ppm.



Scheme 1. Synthetic pathway of *N*-{4-[(3-chloro-4-fluorophenyl)amino]quinazoline-6-yl}-3-iodopropionamide **7** and its radioiodinated analogue [^{125}I]-**7**.

2.2. Radiochemistry

[^{125}I]-*N*-{4-[(3-Chloro-4-fluorophenyl)amino]quinazoline-6-yl}-3-iodopropionamide [^{125}I]-**7** was prepared by halogen exchange reaction of the bromo precursor **6** in the presence of [^{125}I]-NaI at 70 °C for 2–2.5 h (Scheme 1). The radioiodinated quinazoline [^{125}I]-**7**, obtained in 48% radiochemical yield, was purified by analytical reversed-phase HPLC with simultaneous UV (254 nm) and radioactivity detection. The experimental HPLC conditions described above for the purification of the non-radioiodinated quinazoline **7** have been used to allow an efficient separation of [^{125}I]-**7** from other chemical and radiochemical species as well as from the bromo precursor, leading to compound [^{125}I]-**7** with a high specific activity (Fig. 2). Since no UV absorption at the most sensitive detector setting was observed for the purified radioiodinated quinazoline it was then assumed that its specific activity is in the same range as that of the starting [^{125}I]-NaI, 2200 Ci/mmol. From a biological perspective, high specific activity is an important requirement to image receptor positive tumours in order to prevent saturation of targeted receptors.

The authenticity of the radioiodinated quinazoline [^{125}I] -**7** was established by comparing its chromatographic behaviour with that of the unlabelled analogue **7** (Fig. 3).

As shown in the radiochromatogram of Figure 3 the radiolabelled compound was obtained in radiochemical purity higher than 98%. High radiochemical stability in the elution solvent was found up to two weeks. The in vitro stability of a radioiodinated compound under physiological conditions is also a very important factor to be taken into account in the evaluation of its clinical potential, since dehalogenation leads to free iodide, which may result in undesirable biodistribution of radioactivity. In vitro stability studies have demonstrated that the radioiodinated quinazoline [^{125}I]-**7** is radiochemically stable in saline solution up to 4 days at 6 °C and 37 °C and also in fresh human serum up to 4 h of incubation at 37 °C. However, 28% of free iodine was observed after 21 h incubation time.

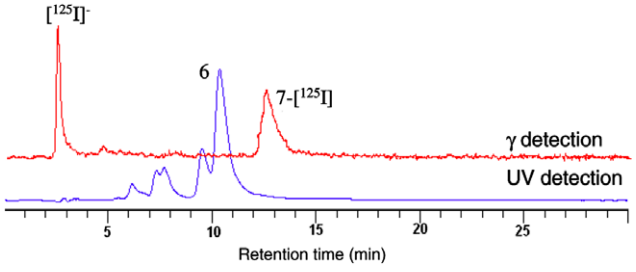


Figure 2. HPLC purification of radioiodinated quinazoline [^{125}I]-**7**. Isolation was carried out on a Nucleosil C_{18} reversed phase column (250 × 4 mm, 10 μm) eluted with a mixture of CH_3CN and 0.1% TFA (35:65) at 1 mL/min. The eluent was simultaneously monitored by a UV detector (254 nm) and a radioactivity detector. The peaks eluting between 6 and 10 min correspond to unidentified chemical impurities.

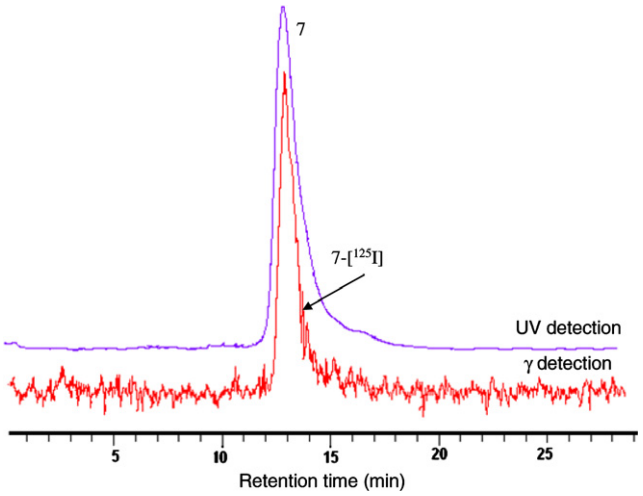


Figure 3. HPLC analysis of radioiodinated quinazoline [^{125}I]-**7** co-injected with its unlabelled analogue **7**. Analysis was carried out on a Nucleosil C_{18} reversed phase column (250 × 4 mm, 10 μm) eluted with a mixture of CH_3CN and 0.1% TFA (35:65) at 1 mL/min. The eluent was simultaneously monitored by a UV detector (254 nm) and a radioactivity detector.

2.3. In vitro assays

2.3.1. Biological evaluation. As quinazoline is a relatively small biomolecule its radioiodination should be performed in such a way that its affinity for the EGFR-associated tyrosine kinase (EGFR-TK) will not be disturbed. In order to assess the EGFR affinity of the novel quinazoline analogues both the cell proliferation inhibition and the EGFR autophosphorylation inhibition were evaluated in this study.

2.3.1.1. Inhibition of cell proliferation by MTT assay. To evaluate the effect of introducing an β-halopropionamide moiety at the 6-position of the quinazoline ring the ability of the halogenated analogues **6** and **7** to inhibit intact A431 cell growth was evaluated by means of a MTT assay. The results were compared to the parent quinazoline **5**. Both halogenated quinazoline derivatives **6** and **7** inhibit cell growth at lower concentrations (IC_{50} values 3–12 μM and 2–7 μM, respectively) compared to the parent compound **5** (IC_{50} values 25–30 μM).

2.3.1.2. Inhibition of EGFR autophosphorylation. The inhibitory effect of the halogenated quinazolines **6** and **7** and the parent quinazoline **5** upon EGFR autophosphorylation was assessed by incubation of the compounds, at various concentrations ranging from 0.1 to 5 μM, with intact A431 cells stimulated for 2 h with EGFR, as described in the experimental section. Cell lysates were loaded onto 8% SDS–PAGE and antiphosphotyrosine content was measured. Controls were run in the same experimental conditions without compounds but with EGF (+) or without EGF (−). Band quantification has been performed using Band leader ver. 3.00 program and the percentage of EGFR autophosphorylation inhibition at 1 μM has been found to be 95%, 100% and 100% for compounds **5**, **6** and **7**, respectively. As an example, the results obtained for the parent quinazoline

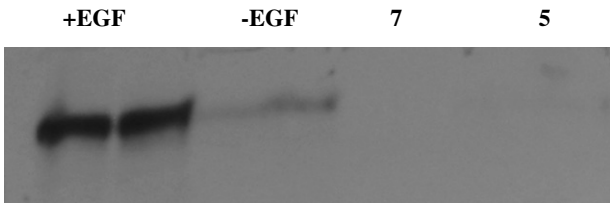


Figure 4. Inhibitory effect of compounds **5** and **7** upon EGFR autophosphorylation.

5 and for the iodinated quinazoline **7** are presented in Figure 4. At the lowest concentration tested (0.1 μM) the halogenated derivatives (**6** and **7**) showed almost complete inhibition of phosphorylation, suggesting that the IC_{50} values are in the low nM range.

2.3.2. Lipophilicity. The lipophilicity is a fundamental physicochemical property that plays an important role in the tissue permeability of a ligand affecting its ability to enter target tissues. The lipophilicity of [^{125}I]-**7** was assessed using the octanol/PBS distribution coefficients ($\log P_{o/w}$) defined as the concentration ratio of the compounds in octanol phase and in aqueous phase at physiological pH. The $\log P_{o/w}$ found was 2.25 at pH 7.4. This value is in the range (1.5–3) usually described as optimal for a receptor binding radiotracer to cross the cellular membrane by passive diffusion and reach the target tissue without leading to high nonspecific binding, which could compromise image contrast.[9]

2.3.3. Human serum protein binding. Protein binding affects the tissue distribution and blood clearance of a radiopharmaceutical and its uptake by the target organ. Determination of the extent of this non-specific binding at various time intervals (15 min, 1, 2 and 4 h) has been accomplished in vitro by incubation of the radiolabelled quinazoline [^{125}I]-**7** in fresh human serum at 37 °C followed by precipitation of the plasmatic proteins with ethanol and comparison of the radioactivity in the precipitate with the radioactivity in the supernatant to give the percentage of radiolabelled compound bound to proteins. Data from these studies indicated a low percentage of radiolabelled compound bound to the plasmatic proteins (6–7%) suggesting a low non-specific binding. By HPLC analysis only the radiolabelled quinazoline was detected in the supernatant confirming its high in vitro stability.

2.4. In vivo stability studies

In vivo studies of compound [^{125}I]-**7** were performed in CD-1 normal female mice, to evaluate its stability. From the tissue distribution a general decrease of the radiotracer over time in all organs was found, excluding intestine and thyroid. The rapid decrease of the radioactivity in the liver (t = 1 h, 6.1% ID/organ; t = 4 h, 2.3% ID/organ) and stomach (t = 1 h, 35.2% ID/organ; t = 4 h, 3.9% ID/organ) may indicate that this compound is deiodinated in these organs. The free iodide formed justifies the increase in thyroid uptake (t = 1 h, 5.7% ID/organ; t = 4 h, 13.2% ID/organ) and the radiochemical species found in urine which has been identified as iodine, based on HPLC analysis. The low in vivo stability of compound [^{125}I]-**7**, which has not been anticipated by in vitro studies, may be related with the low chemical stability of the aliphatic carbon–iodine bond.[10] This finding has discouraged us to proceed with in vivo studies in tumour bearing animal models.

3. Conclusions

In order to find a potential SPECT biomarker for molecular imaging of EGFR positive tumours the novel quinazoline derivative [^{125}I]-*N*-{4-[(3-chloro-4-fluorophenyl)amino]quinazoline-6-yl}-3-iodopropionamide, [^{125}I]-**7**, has been synthesised and characterized by comparing its chromatographic behaviour with that of its non-radioiodinated analogue **7** which has been fully characterized by multinuclear NMR spectroscopy, elemental analysis and HPLC.

The radiolabelled quinazoline [^{125}I]-**7** was obtained with high radiochemical purity (>98%) and high specific activity. In vitro assays indicate that the halogenated quinazolines **6** and **7** inhibit intact A431 cell growth and also inhibit completely autophosphorylation of the receptor in intact A431 cells at the concentration of 0.1 μM. These results demonstrate that the introduction of a β-halopropionamide chain at the 6 position of the quinazoline moiety has improved the capability of inhibition of A431 cell growth and autophosphorylation of EGFR when compared to the parent quinazoline (**5**). However, preliminary in vivo studies have shown a low stability for [^{125}I]-**7**, due to the deiodination of the compound, certainly due to the low chemical stability of the aliphatic carbon-iodine bond. These results may indicate that [^{125}I]-**7** is not a good candidate to image EGFR in breast cancers. Some other quinazoline derivatives labelled in different positions are being explored as radiotracers for imaging EGFR positive tumours.

4. Experimental

4.1. General

All commercial reagents and solvents were of analytical grade and used as supplied from Sigma–Aldrich, Merck or Lancaster. Carrier-free [^{125}I]-NaI was obtained from Amersham Biosciences, UK.

Proton and carbon nuclear magnetic resonance spectra (^{1}H and ^{13}C) were performed on a Varian Unity 300 MHz spectrometer using DMSO or CD_3OD as solvents. Infrared analyses were performed on a Bruker Tensor 27 spectrometer. Elemental analyses were performed on an EA-1110, CE Instruments Equipment. High performance liquid chromatography (HPLC) analyses were performed on a Perkin-Elmer system equipped with a biocompatible quaternary pump (series 200), a UV/vis detector (LC 290, Perkin-Elmer; UV detection at 254 nm) and a radioactivity detector (LB 509, Berthold). Purification of iodinated compound **7** was carried out by preparative HPLC on a BondaPack C_{18} column

(150 × 19 mm, 10 μm) with a flow rate of 5 mL/min using acetonitrile/0.1% TFA in water (35/65) as eluent. Analyses of the labelled and unlabelled compounds as well as the purification of radioiodinated compound [^{125}I]-**7** were achieved on a reverse phase EC-Nucleosil C_{18} column (250 × 4 mm, 10 μm) eluted with a flow rate of 1.0 mL/min and using the same binary isocratic system.

4.2. Chemistry

4.2.1. 6-Nitro-4-hydroxyquinazoline (2). Preparation of this compound followed the general literature procedure.[7] 4-Hydroxyquinazoline **1** (2.0 g, 0.014 mol) was added during 30 min to a mixture of concentrated H_2SO_4 (4 mL) and fuming HNO_3 (4 mL) and heated for 1 h at 90–95 °C. After cooling the solution was poured into ice-water (60 mL) to give **2** in almost pure form (2.3 g, 88%). Pure 4-hydroxyquinazoline was obtained after purification by silica gel column chromatography eluted with CH_3CN/CH_2Cl_2 (1/3). mp 276–277 °C; ^{1}H NMR [δ, ppm, $(CD_3)_2SO$]: 12.8 (s, 1 H, OH), 8.80 (d, 1H, H-5), 8.54 (dd, 1H, H-7), 8.32 (s, 1H, H-2), 7.86 (d, 1H, H-8); ^{13}C NMR [δ, ppm, $(CD_3)_2SO$]:160.24, 152.95, 148.98, 145.11, 129.14, 128.43, 122.77, 122.02.

4.2.2. 4-Chloro-6-nitroquinazoline (3). To a mixture of 6-nitro-4-hydroxyquinazoline (0.4 g, 2.1 mmol) and pentachlorophosphorane (1 g) was added phosphoric trichloride (2 mL). The suspension was heated under reflux for 3 h. After cooling, the excess of PCl_5 and $POCl_3$ was removed under reduced pressure and the residue was washed with ligroin. The residue was dissolved in aqueous Na_2CO_3 solution and extracted with CH_2Cl_2. The organic layer was dried under $MgSO_4$, filtered and the solvent removed to give **3** (0.35 g, 80%): ^{1}H NMR [δ, ppm, $(CD_3)_2SO$]: 8.80 (d, 1H, H-5), 8.54 (dd, 1H, H-7), 8.34 (s, 1H, H-2), 7.87 (d, 1H, H-8); ^{13}C NMR [δ, ppm, $(CD_3)_2SO$]: 160.05, 152.53, 149.04, 145.10,128.80, 128.39, 122.68, 121.70.

4.2.3. 4-[(3-Chloro-4-fluorophenyl)amino]-6-nitroquinazoline (4). A solution of 3-chloro-4-fluoroaniline (482 mg, 3.32 mmol) in isopropanol (5 mL) was added to a suspension of 4-chloro-6-nitroquinazoline (348 mg, 1.66 mmol) in the same solvent. Concentrated hydrochloric acid (5 μL) was added and the mixture refluxed during 30 min. A yellow precipitate was obtained. After cooling, a few drops of NH_3 were added to basify the solution and the precipitate was isolated by filtration, washed with isopropanol and dried affording **4** as a yellow solid (450 mg, 85%). ^{1}H NMR [δ, ppm, $(CD_3)_2SO$]: 10.6 (s, 1H, NH), 9.63 (d, 1H, H-5), 8.76 (s, 1H, H-2), 8.56 (dd, 1H, H-7), 8.15 (dd, 1H, H-8), 7.95 (d, 1H, H-2′), 7.85–7.80 (m, 1H, H-6′), 7.49 (t, 1H, H-5′).

4.2.4. 6-Amino-4-[(3-chloro-4-fluorophenyl)amino]quinazoline (5). Preparation of this compound followed a slightly modified method described in the literature.[8] The free base of 4-[(3-chloro-4-fluorophenyl)amino]-6-nitroquinazoline (620 mg, 1.95 mmol) in THF:MeOH (2:1, 80 mL) was hydrogenated over Raney nickel (approximately 1.0 g) flushing the solution with H_2 at room temperature. After 3.5 h, the mixture was filtered through Celite and the solvent removed under reduced pressure to give **5** as an orange solid and in quantitative yield. ^{1}H NMR [δ, ppm, $(CD_3)_2SO$]: 9.6 (s, 1H, NH), 8.38 (s, 1H, H-2), 8.20 (dd, 1H, H-2′), 7.95-7.80 (m, 1H, H-6′), 7.53 (d, 1H, H-8), 7.39 (t, 1H, H-5′), 7.31 (d, 1H, H-5), 7.24 (dd, 1H, H-7), 5.61 (s, 2H, NH_2). ^{13}C NMR ($(CD_3)_2SO$) δ (ppm): 156.9, 153.9 (d, $J_{F\text{-}C}$ = 243.2 Hz), 148.6, 147.8, 135.9, 124.9, 124.8, 124.6, 123.4 (d, $J_{F\text{-}C}$ = 6,7 Hz), 119.0, 118.8, 116.7 (d, $J_{F\text{-}C}$ = 21,7 Hz), 115.9, 101.1.

4.2.5. *N*-{4-[(3-Chloro-4-fluorophenyl)amino]quinazoline-6-yl}-3-bromopropionamide (6). 3-Bromopropanoyl chloride (446 mg, 2.6 mmol) was added slowly to a solution of 6-amino-4-[(3-chloro-4-fluorophenyl)amino]quinazoline (**5**) (342 mg, 1.2 mmol) in dry acetone (15 mL). A yellow precipitate was formed and the reaction mixture was left under stirring for 2 h. The precipitate was separated by filtration and dried under reduced pressure to give **6** as a yellow solid. Yield: (394 mg, 79%). ^{1}H NMR ($(CD_3)_2SO$) δ (ppm): 3.09 (t, 2H, –CH$_2$–CH$_2$–Br); 3.78 (t, 2H, –CH$_2$–CH$_2$–Br); 7.55 (t, 1H, H-5′, J = 8.7 Hz); 7.65 (m, 1H, H-6′); 7.91 (d, 1H, H-8); 7.96 (m, 1H+1H, H-2′, H-7); 8.88 (s, 1H, H-2); 9.02 (s, 1H, H-5); 10.76 (s, 1H, NH), 11.46 (s, 1H, CONH). ^{1}H NMR (CD_3OD) δ (ppm): 2.96 (t, 2H, –CH$_2$–CH$_2$–Br); 3.93 (t, 2H, –CH$_2$–CH$_2$–Br); 7.37 (t, 1H, H-5′); 7.71–7.66 (m, 1H, H-6′); 8.04–7.84 (m, 3H, H-8, H-2′, H-7); 8.76 (s, 1H, H-2); 9.01 (d, 1H, H-5). ^{13}C NMR (CD_3OD) δ (ppm): 171.5 (CONH), 161.7, 157.9 (d, $J_{F\text{-}C}$, 247 Hz), 150.8, 140.7, 136.3, 134.7, 130.7, 128.4, 126.4, 126.5, 117.9 (d, $J_{F\text{-}C}$, 22.5 Hz), 115.6, 113.5, 40.8 (CH_2), 27.8 (CH_2). IR (KBr) (cm^{-1}): 1681.32 (vs); 1498.64 (s); 1446.54 (s); 1205.33 (vs). HPLC rt = 10.6 min using the conditions described above.

4.2.6. *N*-{4-[(3-Chloro-4-fluorophenyl)amino]quinazoline-6-yl}-3-iodopropionamide (7). To a solution of *N*-{4-[(3-chloro-4-fluorophenyl) amino]quinazoline-6-yl}-3-bromopropionamide (100 mg, 0.24 mmol) in dry acetone (20 mL) was added sodium iodide (71 mg, 0.47 mmol). The mixture was stirred at 80 °C for 11 h. The solvent was evaporated and the residue was purified by preparative reverse-phase HPLC according to the conditions described above. The collected fractions were analysed by analytical HPLC and evaporated. The residue was washed several times with water and lyophilized to give **7** as a white powder (46.8 mg, 32%).

^{1}H NMR ($(CD_3)_2SO$) δ (ppm): 3.09 (t, 2H, –CH$_2$–CH$_2$–I); 3.47 (t, 2H, –CH$_2$–CH$_2$–I); 7.44 (t, 1H, H-5′, J = 9.1 Hz); 7.82 (m, 1H, H-6′); 8.09 (m, 3H, arom); 8.57 (s, 1H, arom); 8.72 (s, 1H, arom); 10.08 (s, 1H, –NH$_2$); 10.40 (s, 1H, –NH). ^{1}H NMR (CD_3OD) δ (ppm): 3.13 (t, 2H, –CH$_2$–CH$_2$–Br); 3.52 (t, 2H, –CH$_2$–CH$_2$–Br); 7.30 (t, 1H, H-5′); 7.73–7.67 (m, 1H, H-6′); 8.01–7.78 (m, 3H, H-8, H-7, H-2′); 8.76 (s, 1H, H-2); 9.01 (d, 1H, H-5). Anal. ($C_{17}H_{13}ClFN_4OI\cdot CF_3COOH$) calcd: C, 39.03; H, 2.41; N, 9.58. Found: C, 39.25; H, 2.51; N, 9.80. IR (KBr) (cm^{-1}): 1678.7 (vs); 1498.4 (s); 1204.2(s). HPLC: rt = 12.6 min.

4.3. Radiochemistry

4.3.1. [^{125}I]-*N*-{4-[(3-chloro-4-fluorophenyl)amino]quinazoline-6-yl}-3-iodopropionamide ([^{125}I]-7). To a solution of *N*-{4-[(3-chloro-4-fluorophenyl) amino]quinazoline-6-yl}-3-bromopropionamide (200 μg, 0.47 μmol) in dry acetone was added [^{125}I]-NaI (25 MBq) and the mixture was stirred for 2–2.5 h at 70 °C. The radioiodinated quinazoline [^{125}I]-**7** was then isolated by analytical reverse phase HPLC, with simultaneous UV (254 nm) and radioactivity detection. The radiochemical yield of [^{125}I]-**7** was 48%. HPLC: rt = 12.6 min.

4.4. In vitro assays

4.4.1. Biological evaluation. The human epidermoid carcinoma A431 cell line, kindly provided by Dr. E. Mishani (Hadassah Hebrew University, Israel), was maintained in D-MEM (high glucose) supplemented with 10% foetal calf serum and penicillin/streptomycin 100 UI/100 μg/mL, in 5% CO_2 incubator at 37 °C.

4.4.1.1. Inhibition of cell proliferation by MTT assay. The A431 cells (3×10^3 cells/well) were seeded in 96-well plates and incubated for 24 h. Then the exponentially growing cells were incubated with various concentrations of quinazoline analogues **5**, **6** and **7**, respectively, (ranging from 1 to 50 μM in 4 replicates) during 24, 48 and 72 h. Controls consisted of wells without drugs. The medium was removed and the cells were incubated for 4 h in the presence of 1 mg/mL MTT in RPMI without phenol red at 37 °C. The MTT solution was removed and 100 μL of isopropanol per well was added. After thorough mixing, absorbance of the wells was read in an ELISA reader at test and at reference wavelengths of 540 and 620 nm, respectively. The mean of the optical density of different replicates of the same sample and the percentage of each value were calculated (mean of the OD of various replicates/OD of the control). The percentage of the optical density against drug concentration was plotted in a semi-log chart and the IC_{50} from the dose–response curve was determined.

4.4.1.2. Inhibition of autophosphorylation of receptor. A431 cells (5×10^5) were seeded in 6-well plates and grown to about 70% confluence, and then incubated in fresh medium without foetal bovine serum for 24 h. The day after, cells were treated for 2 h with various concentrations of the compounds ranging from 0.1 to 5 μM. The cells were then stimulated for 5 min with EGF (20 ng/mL). Then, the medium was removed, cells were washed with PBS and cell lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 50 μg/mL aprotinin, 2 mM sodium orthovanadate, 50 μg/mL leupeptine and 5 mM EDTA) was added. Controls consisted of wells without drugs and with or without EGF. The total amount of protein was estimated by a BCA assay in ELISA plates, using a BSA standard curve. A volume of lysis mixture, corresponding to 30 mg of total protein, were loaded onto polyacrilamide gel (8%), proteins were separated by electrophoresis and transferred to PVDF membrane. For visualization of molecular weight bands, the membrane was immersed in Ponceau reagent (0.5% Ponceau in 1% acetic acid) for 5 min. The membrane was washed in H_2O, blocked overnight in TTN with 5% milk (1% fat) and incubated for 2 h in antiphosphotyrosine antibody diluted 1/2000 (PY20, Santa Cruz Biotechnology). Then, the membrane was washed 3 times with TTN and incubated in a horseradish peroxidase-conjugated secondary anti mouse antibody diluted 1/3000 (Sigma) for 2 h and finally washed 3 times in TTN. Detection was performed using chemiluminescent detection system according to manufacturer's instructions (ECL kit, Amersham). Band quantification has been performed using Band leader ver. 3.00 programm.

4.4.2. Lipophilicity. Log $P_{o/w}$ was determined by the 'shakeflask' method using the back-extraction technique.[11] The radiolabelled compound was added to a mixture of octanol (1 mL) and PBS, pH 7.4, (1 mL) previously saturated in each other by stirring the mixture. The mixture was vortexed and centrifuged (3000 rpm, 10 min, rt) to allow for phase separation. Aliquots of both octanol and PBS, pH 7.4, were counted for radioactivity in a gamma counter. A 0.5 mL aliquot of the octanol layer was withdrawn and added to an equal volume of PBS, pH 7.4, and the extraction and counting repeated as above. The log $P_{o/w}$ was calculated by dividing the counts in the octanol by those in the buffer and the results expressed as log $P_{o/w}$.

4.4.3. Human serum protein binding. The radioiodinated quinazoline [^{125}I]-**7** (100 μL, ≈370 kBq) was incubated at 37 °C with human serum (1 mL). At appropriate periods of time (15 min, 1 h, 2 h, 4 h and 21 h) 100 μL aliquots (in duplicate) were sampled and treated with 200 μL of ethanol to precipitate the proteins.[12] Samples were then cooled at 4 °C and centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant was separated from the precipitate, the sediment washed with ethanol (2×1 mL) and counted in a gamma counter. The activity in the sediment was compared with the total activity used and the percentage of [^{125}I]-**7** bound to proteins was calculated. The supernatant was analysed by HPLC, using the experimental conditions described above.

4.4.4. Stability studies. The radiochemical stability of [^{125}I]-**7** was evaluated by HPLC analysis at several time points after incubation in saline at 6 °C and 37 °C. Radiochemical stability in physiological medium was also determined at 37 °C at 15 min, 1 h, 2 h, 4 h and 21 h after incubation in human serum. For this analysis the supernatants collected in the human serum protein binding assay were examined by HPLC for radiochemical purity evaluation at the same time intervals.

In order to assess the radiochemical stability in physiological medium the supernatants collected in the human serum protein binding assay were examined by HPLC for radiochemical purity evaluation at the same time intervals.

4.5. In vivo stability studies

In vivo studies were carried out in groups of 5 female CD-1 mice (randomly bred, obtained from IFFA, CREDO, Spain) weighing approximately 20–25 g according the EU guidelines for Animal Care and Ethic for Animal Experiments. Animals were injected into the tail vein with 175–200 kBq of the radioiodinated compound in saline (100 μL) containing 0.1% Tween 20. Injected dose (ID) was assumed to be the difference between the measured radioactivity in the syringe before and after injection. Animals were maintained on normal diet *ad libitum* and were sacrificed by cervical dislocation at 1 and 4 h post-injection with the radiotracer. Tissue samples of the main organs were removed, weighed and the activity measured in a gamma counter (Berthold LB2111, Germany). Results were expressed as percent of injected dose per total organ (% ID/organ) and presented as mean values ± SD. For blood, bone and muscle, this value was calculated assuming that these organs constitute 6%, 10% and 40% of the total weight, respectively. Whole animal body radioactivity excretion was not quantified. The in vivo stability was evaluated by RP-HPLC analysis of urine samples collected at sacrifice in the experimental conditions described above for the radiochemical purity determination.

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