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Nuclear Medicine and Biology 31 (2004) 469–476

NUCLEAR MEDICINE — AND — BIOLOGY

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Novel carbon-11 labeled 4-dimethylamino-but-2-enoic acid [4-(phenylamino)-quinazoline-6-yl]-amides: potential PET bioprobes for molecular imaging of EGFR-positive tumors

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Abstract

We have previously reported of labeled reversible and irreversible EGFR inhibitors, such as 4-(3,4-dichloro-6-fluoroanilino)-6,7-dimethoxyquinazoline (ML01) and 6-acrylamido-4-(3,4-dichloro-6-fluoroanilino)quinazoline (ML03), to be suboptimal as imaging agents. On the basis of these studies, a new generation of novel, more chemically stable irreversible inhibitors was labeled with carbon-11 as potential positron emission tomography (PET) biomarkers for molecular imaging of epidermal growth factor receptor (EGFR)–positive tumors. In these new labeled, irreversible inhibitors the acryl-amide group at the 6-position of the quinazoline ring was replaced with a 4-dimethylamino-but-2-enoic amide. The nonlabeled compounds were evaluated *in vitro* to determine their EGFR autophosphorylation IC_{50} values. The IC_{50} values indicated that these new irreversible compounds possess similar potencies towards the EGFR, as the parent compound, ML03. These compounds were labeled with carbon-11 at the dimethylamine moiety, using the well known labeling reagent C-11 MeI. The labeling procedure was automated using a commercial module. The final products were obtained with 10% decay corrected radiochemical yield, 99% radiochemical purity, 96% chemical purity, and a high specific activity of 2.7 Ci/μmol EOB. The high potency of these new labeled bioprobes towards the EGFR establishes their potential to serve as PET agents for molecular imaging of EGFR-positive tumors.

Keywords: PET; carbon-11; EGFR-TK; C-11 MeI

1. Introduction

The understanding that cancer cells differ from normal cells in their aberrant signal transduction has given impetus to cancer researchers to target them for cancer therapy and, more recently, for cancer diagnosis. Overexpression of the epidermal growth factor receptor (EGFR) and its enhanced signaling are a hallmark of epithelial human cancers, and it has been suggested to contribute to the initiation, progression and/or invasiveness of human cancers [1-4]. The correlation between high EGFR levels and poor prognosis or short survival time has been established in patients with various types of cancers [1]. There are several Food and Drug Administration–approved anticancer drugs that target the EGFR: namely, Iressa, and several others that are presently undergoing clinical Phase 3 trials, such as, Tarceva (OSI Pharmaceuticals, Melville, NY, USA) and the anti-EGFR antibody Erbitux (ImClone Systems Inc., New York, NY, USA). Based on the findings from these drugs, to optimize the therapeutic potential of EGFR inhibition in cancer, specific analysis of EGFR expression in tumor should be established, before EGFR-targeted therapy. Indeed, recently, there has been a growing interest in the use of tyrosine kinase inhibitors as radiotracers for imaging human tumors that overexpress the EGFR by nuclear medicine modality [5–11]. Our research interest is associated with the development of positron emission tomography (PET) bioprobes, particularly the 4-anilinoquinazoline class, as a tool for molecular imaging of malignant tumors that overexpress the EGF receptor [9–11]. During the course of this study, several reversible and irreversible [12–14] inhibitors were labeled with fluorine-18 and carbon-11 (Fig. 1), and their potential as PET biomarkers was examined *in vitro* and *in vivo*. In the case of labeled reversible

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0969-8051/04/$ – see front matter
doi:10.1016/j.nucmedbio.2003.12.005

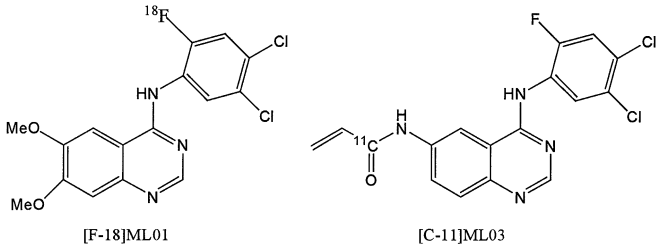

Fig. 1. Chemical structure of labeled reversible and irreversible epidermal growth factor receptor inhibitors.

inhibitors, the presence of high cellular concentrations of competitive ATP molecules brought to a fast washout of our labeled lead, ML01, from the target tumor cells, and thus disqualified it for further development [12]. With irreversible compounds such as ML03, the fast washout was abrogated because of the formation of covalent bonding between the double bond of the acryl-amide group at the 6-position of the quinazoline ring and CYS-773 at the receptor's tyrosine kinase domain. However, the unsaturated acryl-amide bond of ML03 is chemically reactive and leads to rapid metabolism, low bioavailability, and low accumulation of the labeled compound in the tumor [13]. To reduce the chemical reactivity of these irreversible labeled inhibitors and increase the tumor uptake of the labeled biomarkers, we labeled a new, more stable generation of EGFR-TK irreversible inhibitors, as reported recently by Tsou et al. [15].

2. Methods and materials

All chemicals were purchased from Sigma-Aldrich (Tel Aviv, Israel), Fisher Scientific (Pittsburgh, PA, USA), Merck (Darmstadt, Germany), or J.T. Baker (New Jersey, USA). Chemicals were used as supplied, excluding THF, which was refluxed over sodium and benzophenone, and was freshly distilled before use. Mass spectroscopy 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 400 MHz instrument. Elemental analysis was performed at the Hebrew University Microanalysis Laboratory. Radiosyntheses were carried out on a [^{11}C]CH_3I module (Nuclear-Interface, Munster, Germany). Specific radioactivities were determined by HPLC, using cold mass calibration curves. [^{11}C]carbon dioxide was produced by the ^{14}N(p, α)^{11}C nuclear reaction on nitrogen containing 1% oxygen, using an 18/9 IBA-cyclotron. Bombardment was carried out for 15-30 minutes with a 26 μA beam of 16 MeV protons. At the end of bombardment the target gas was delivered and trapped by a cryogenic trap in the [^{11}C]CH_3I module.

A431 human epidermoid vulval carcinoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Biological industries, Kibbuts 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.

2.1. Chemistry

For the preparation of the nonlabeled compounds, we followed the general outlines of Tsou et al. [15], with minor modifications, and prepared the known compound (5b) and the two new compounds (5a and 5c).

2.1.1. 4-Dimethylamino-but-2-enoic acid [4-(3,4-dichloro-6-fluoro-phenylamino)-quinazolin-6-yl]-amide (**5a**),ML04.

4-chloro-6-nitroquinazoline (3) [16] (0.687 g, 3.28 mmol) and 3,4-dichloro-6-fluoroaniline [12] (1.477 g, 8.2 mmol) were dissolved in 10 mL isopropanol (i-PrOH) and refluxed for 3–4 hours. The precipitate was collected, washed with i-PrOH and dried in a vacuum oven at 80°C to give (4a) (709 mg, 60% yield) Mp = 270-271°C 6-nitro-4-(3,4-dichloro-6-fluoro-phenylamino)quinazoline; MS (*m/z*) 353.2, 355.2 (M^+) [14].

6-Nitro-4-(3,4-dichloro-6-fluoro-phenylamino)quinazoline (4a, 710 mg, 2.2 mmol) was dissolved in 200 mL of EtOH: H_2O (9:1) by heating to reflux. The temperature was reduced to 60°C, and hydrazine hydrate (250 μL, 5.15 mmol) and 0.5 mL Raney-Ni suspension were added [17]. Reflux was then maintained for 15 minutes. The cold mixture was filtered over Celite, and the solvent evaporated. The crude product was purified by silica gel chromatography (eluent 4-7% MeOH in CH_2Cl_2) to yield pure 6-amino-4-(3,4-dichloro-6-fluoro-phenylamino)quinazoline with 83% yield, Mp = 268-270°C; MS (*m/z*) 323.4, 325.4 (M^+) [14].

A solution of 6-amino-4-(3,4-dichloro-6-fluoro-phenylamino)quinazoline (765 mg, 2.37 mmol) in THF (80 mL) was cooled in an iced bath, and Br/Cl-crotonyl chloride [15] (3.55 mmol) in THF (5 mL) was added dropwise followed by dropwise addition of N,N-diisoprophylethylamine 620 μL (3.55 mmol). The reaction was stirred for an additional 1.5 hours, and ethylacetate (EtOAc) (80 mL) and water (100 mL) were added. The aqueous layer was extracted with EtOAc, and the combined organic layers were washed with brine, dried with Na_2S0_4, and evaporated. The crude material was purified by flash chromatography on silica gel (MeOH: CH_2Cl_2, 5%: 95%) to yield 720 mg (66% yield) of an inseparable mixture of the (2E)-4-bromo/chloro-N-{4-[(3,4-dichloro-6-fluoro-phenyl)amino]-quinazolin-6-yl}-2-butenamide (6a) in a 4:1 ratio, respectively. MS (*m/z*) 425 $(M+)^+$(Cl derivative), 471 $(MH+)^+$(Br derivative), ^{1}H NMR (DMSO-d_6) δ 10.87 (1H, s), 10.24 (1H, s), 9.08 (1H, s), 8.72 (1H, s), 8.10 (4H, mt), 7.19 (1H, dt, *J1* = 15Hz, *J2* = 7.5Hz), 6.69 (1H, d, *J* = 15 Hz), 4.62 (2H, d, *J* = 7 Hz). Anal. Calcd. (for ratio 9:1): C, 46.47; H, 2.60; N, 12.04. Found: C, 47.31; H, 3.2; N, 11.57; HPLC condition: C-18 column, 55% Acetate buffer/ 45% Acetonitrile, flow=1.5 mL/min; Rt =19.5 minutes (1st peak), 21.9 min (2nd peak).

Dimethylamine (2 mol/L in tetrahydrofuran (THF), 38

mL) was added dropwise to a solution of (*2E*)-4-bromo-N-{4-[(3,4-dichloro-6-fluoro-phenyl)amino]-quinazolin-6-yl}-2-butenamide (6a, 564 mg, 1.21 mmol) in dry THF. The reaction was heated to 80°C for 15 minutes, and cooled; EtOAc (50 mL) and saturated $NaHCO_3$ (50 mL) were then 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 flash chromatography on silica gel (MeOH: CH_2Cl_2, 5%: 95%) to yield 317 mg (73%) of 4-dimethylamino-but-2-enoic acid [4-(3,4-dichloro-6-fluoro-phenylamino)-quinazolin-6-yl]-amide (5a, ML04). MS (*m/z*) 434.1 (MH+); ^{1}H NMR (DMSO-d_6) δ 10.47 (1H, s), 9.95 (1H, s), 8.83 (1H, s), 8.4 (1H, s), 7.92 (1H, dd, *J*1 = 18 Hz, *J*2 = 3.8 Hz), 7.78 (1H, bd, *J* = 20 Hz), 7.22 (2H, mt), 6.86 (1H, dt, *J*1 = 30Hz, *J*2 = 11Hz), 6.36 (1H, d, *J* = 30 Hz), 3.08 (2H, d, *J* = 11 Hz), 2.18 (6H, s). ^{19}F NMR -117.1 ppm (bs). Anal. calculated [$C_{20}H_{18}Cl_2FN_5O{\cdot}0.5H_2O$]: C, 54.2; H, 4.29; N, 15.8. Found: C, 54.48; H, 4.85; N, 15.36.

*2.1.2. 4-Dimethylamino-but-2-enoic acid [4-(3-bromo-phenylamino)-quinazolin-6-yl]-amide (***5b***)*

This compound was prepared according to the general route described by Tsou et al. [15], and similarly to compound 5a (ML04) described above.

MS (*m/z*) 428.1 (M+); ^{1}H NMR (DMSO-d_6) δ 10.44 (1H, s), 9.92 (1H, s), 8.84 (1H,s), 8.59 (1H, s) 8.18 (1H, m), 7.83 (3H, m), 7.32 (2H, m), 6.83 (1H, dt, *J1* = 30.7, *J2* = 11.5 Hz), 6.36 (1H, d, *J* = 30.7 Hz), 3.12 (2H, d, *J* = 11.5 Hz), 2.19 (6H, s).

*2.1.3. 4-Dimethylamino-but-2-enoic acid [4-(3-iodo-phenylamino)-quinazolin-6-yl]-amide (***5c***)*

This compound was prepared similarly to compound 5a (ML04) described above.

*2.1.4. 6-Nitro-4-(3-iodo-phenylamino)quinazoline (***4c***)*

MS (*m/z*) 392.2 (MH^+); ^{1}H NMR (DMSO-d_6) δ 10.56 (1H, s), 9.67 (1H, d, *J* = 3.2 Hz), 8.78 (1H, s), 8.58 (1H, dd, *J1* = 12 Hz, *J2* = 3 Hz), 8.27 (1H, bs), 7.96 (2H, m), 7.54 (1H, bd, *J* = 11 Hz), 7.22(1H, t, *J* = 11 Hz).

2.1.5. 6-Amino-4-(3-iodo-phenylamino)quinazoline

This reduction step was performed at 0°C.

MS (*m/z*) 363 (MH^+); ^{1}H NMR (DMSO-d_6) δ 9.365 (1H, s), 8.347 (1H, s), 8.32(1H, t, *J* = 2.4 Hz), 7.91 (1H, dd, *J1* = 10.4 Hz, *J2* = 2.4 Hz), 7.52 (1H, d, *J* = 11.6 Hz), 7.39 (1H, bd, *J* = 9.6 Hz), 7.32 (1H, d, *J* = 2.8 Hz), 7.23 (1H, dd, *J1* = 11.6 Hz, *J2* = 2.8 Hz), 7.13 (1H, t, *J* = 10.4 Hz), 5.95 (2H. bs).

*2.1.6. (2E)-4-bromo/chloro-N-{4-[(3-iodo-phenyl)amino]-quinazolin-6-yl}-2-butenamide (***6c***)*

MS (*m/z*) 465.5 $(MH+)^+$(Cl derivative), 509.2 $(M+)^+$(Br derivative), ^{1}H 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, bdt, *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 ratio 1:1): C, 43.27; H, 2.82; N, 11.21. Found: C, 43.60; H, 2.81; N, 11.12.

*2.1.7. 4-Dimethylamino-but-2-enoic acid [4-(3-iodo-phenylamino)-quinazolin-6-yl]-amide (***5c***)*

MS (*m/z*) 474 (MH+); ^{1}H 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, 48.91; H, 4.36; N, 13.59.

*2.1.8. 4-Methylamino-but-2-enoic acid [4-(phenylamino)-quinazolin-6-yl]-amides (***7a–c***)*

Methylamine (2 mol/L in THF, 1 mL) was added to a solution of (*2E*)-4-bromo-N-[4-(phenylamino)-quinazolin-6-yl]-2-butenamides 6a–c (10 mg) in dry DMSO (70 μL), at 0°C. After a 15-minute reaction, 0.015 mol/L NaOH (10 mL) were added. The solution was passed through 2 × C-18 cartridges (Waters Sep-Pak Plus, Massachusetts, USA), pre-activated with 10 mL EtOH and 20 mL of sterile water), and the cartridges were dried under nitrogen stream. The product was eluted with 4 mL of THF, and dried with Na_2SO_4. Filtration with a 0.45-μm filter and THF evaporation under reduced pressure gave the crude products 7a–c, which were used for the radiolabeling without any further purification.

7a: MS (*m/z*) 419.2 (M+); 7b: MS (*m/z*) 413 (MH+); 7c: MS (*m/z*) 460 (MH+).

2.2. Radiochemistry

2.2.1. Radiolabeling of compounds **5a–c**

Carbon-11 MeI was prepared according to well documented procedures [18]. Briefly, [^{11}C]CO2 (37 GBq, 1000 mCi) was trapped at −160°C. The temperature of the cooling trap was increased to −50°C, and the activity was transferred by a stream of argon (40 mL/min) into reactor-1 containing 300 μL of 0.25N $LiAlH_4$ in THF at −50°C. After 90 seconds the solvent was removed under reduced pressure. In this manner, more than 80% of the activity was recovered. The reactor temperature was increased to 160°C, HI was added, and [^{11}C]MeI was distilled (argon flow of 15 mL/min.) through a NaOH column to the second reactor, containing 8 mg of the appropriate 4-mono-methylamino-but-2-enoic acid [4-(phenylamino)-quinazolin-6-yl]-amide precursor in a mixture of 0.3 mL anhydrous THF, 0.1 mL CH_3CN and 0.2 mL DMSO at −20°C. At the end of the 2-minute distillation step, an average of 550 mCi (*n* = 30) was trapped in the second reactor. The reactor was sealed, and heated to 100°C for 5 minutes. At the end of the 5-minute reaction, THF and CH_3CN were removed under flow of argon at 80°C. The mixture was cooled to 40°C, 0.6

mL HPLC solvent (2% THF, 60% CH_3CN, 38% 0.1 mol/L ammonium formate) were added, and the crude product [average of 370 mCi ($n = 30$)] was automatically injected to the HPLC [Bischoff Nucleosil 100-7-C18 reverse phase preparative column (7 μm, 250 $\times$ 16 mm), flow rate of 13 mL/min]. The labeled products were collected (Rt. 18 minutes 5a (ML04), 17 minutes 5b and 18.5 minutes 5c) in a flask containing 60 μL of 1 mol/L NaOH in 85 mL of water. The solution was passed through a C-18 cartridge (Waters Sep-Pak Plus, preactivated with 10 mL EtOH and 20 mL of sterile water), and the cartridge was washed with 4 mL of sterile water. The products were eluted with 0.75 mL of EtOH, followed by 4.25 mL of saline, and collected into the product vial after a total radiosynthesis time of 50 minutes. Identifications of the products and of chemical and radiochemical purities were determined by reverse-phase HPLC C18 analytical column (47% CH_3CN, 53% 0.1 mol/L ammonium formate, flow rate of 1.4 mL/min). The average ($n = 30$) final doses were approximately 0.74 GBq (20 mCi, 10% decay corrected radiochemical yield); specific activity was 2.1-3.3 Ci/μmol.

2.3. Biology

2.3.1. 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), and placed over ice before the addition of 3.25 mL cold, freshly prepared lysis buffer (50 mmol/L N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer (HEPES), pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 1 mmol/L 4-(2-aminoethyl-)benzenesulfonylfluoride hydrochloride (AEBSF), 1 μg/mL aprotinin, 300 μg/mL benzamidine, 10 μg/mL leupeptin, and 10 μg/mL soy-trypsin inhibitor) for 10 minutes. The cells were scraped from the plates with a rubber policeman and content was homogenized with a dounce homogenizer and centrifuged (Sorval centrifuge, rotor 5, 10,000 rpm, 10 minutes, 4°C). The supernatant, which contained the EGFR, was collected and frozen at −70°C in aliquots.

2.3.2. ELISA screen

EGFR autophosphorylation IC_{50} values were obtained by means of an ELISA screen. All of 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 ($\times$4) and 200 μL TBST buffer (10 mmol/L Tris-HCl, pH 7.4 (TBS), 0.2% Tween 20, 170 mmol/L NaCl) ($\times$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. After removing unbound mAb108 the plate was washed, and PBS, containing 5% milk (1% fat) was added to block the unbound sites in the plate for 30 minutes. One 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 minutes, different concentrations of each inhibitor were added 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 minutes and without washing the plate, ATP-$MnCl_2$ solution was added to each well for exactly 5 minutes, the final concentration being 5 μM ATP, 5 mmol/L $MnCl_2$.

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

For the colorimetric detection of phosphotyrosine content in EGFR, commercial TAGO antirabbit peroxidase-conjugated antibody diluted 1:10,000 in blocking TBST was added to the wells and allowed to react for 45 minutes. 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]. After 5-10 minutes the plate was read on Dynaytec MR 5000 ELISA reader at 405 nm. Analysis of the data was performed with GraphPad Prism, version 2.01 (GraphPad Software, Inc., California, USA). For each compound, two independent assays, at least, with similar results, were performed. Each assay was carried out in duplicate.

2.3.3. Irreversibility test protocol

A431 cells (5×10^5) were grown in six-well plates (35 mm diameter, Nalge Nunc) for 24 hours to ~90% confluence, and then incubated in serum-free media for 18 hours. Duplicate sets of cells were treated with varying concentrations of designated compounds to be tested as irreversible inhibitors for 1 hour. Addition of the inhibitor vehicle made the final solution 0.05% DMSO and 0.1% EtOH. After removal of inhibitor from the medium, PBS wash ($\times$2) and addition of serum-free media to the wells one set of cells was stimulated with 20 ng/mL EGF for 5 minutes at 37°C, and then washed with PBS. Cell extracts were made by adding 0.4 mL boiling Laemmli buffer (10% glycerol, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 62.5 mmol/L Tris, pH 6.8), containing 0.001% bromophenol blue, to the cells, scraping them with a rubber policeman and heating to 100°C for 10 minutes. The other set of cells was incubated for 2 hours, washed with PBS, incubated another 2 hours, washed again, incubated an additional 4 hours and then stimulated with EGF. Cell lysates were prepared similar to the first set of cells. This assay was

performed three times, with similar results, for compound 5a and once for compounds 5b and 5c.

2.3.4. Western blot analysis

A quantity of 30 μg of protein lysates was separated by SDS-PAGE (8% acrylamide) and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in blocking TBST for 30 minutes and incubated overnight with PY20 antiphosphotyrosine antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:2,000 in blocking TBST. The membrane was washed thoroughly with TBST, and incubated for 1 hour with a horseradish peroxidase–conjugated antimouse 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 detection reagent. Quantification of each band was performed using Adobe Photoshop 5.0 ME and National Institutes of Health NIH-image 1.61/ppc programs.

3. Results and discussion

3.1. Chemistry

To achieve a higher biological stability, we chose to label the recently reported and more chemically stable irreversible inhibitors [15] that also possess a better water solubility. These compounds demonstrated *in vivo* efficacy in human xenograft animal models, indicating that improved bioavailability could be related to enhancement of antitumor activity. In these new irreversible EGFR inhibitors, the acryl-amide group at the 6-position of the quinazoline ring was replaced with a 4-dimethylamino-crotonyl amide. In this group, the β carbon of the amide is partially positively charged and thus sufficiently reactive to the nucleophilic attack, performed by the cysteine moiety at the receptor-binding site. The increased chemical stability of these new labeled inhibitors may be explained by the higher energy gap between the HOMO LUMO electronic orbitals of the β carbon center relative to the β carbon center in the acryloyl group. This enhanced chemical stability may lead to higher biological stability and therefore improved bioavailability. Thus, if potency is not dramatically affected and remains in the nanomolar range, the new labeled inhibitors will remain at the receptor binding site long enough to allow covalent binding. The synthesis of compounds 5a–c is outlined in Fig. 2. 6-Nitroquinazolone was reacted with $SOCl_2$ to produce the 4-chloro-6-nitroquinazoline (3) with 80% chemical yield and 97% purity. Compound 3 was used as is in the next step. Reaction of 4-chloro-6-nitroquinazoline (3) was performed with three different aniline derivatives. Each of them contained elements that will enable future radiolabeling with fluorine-18, bromine or iodine isotopes. The coupling of the aniline derivatives and the 4-chloro-6-nitroquinazoline was performed in i-PrOH at reflux, and the products were obtained as a bright yellow precipitate.

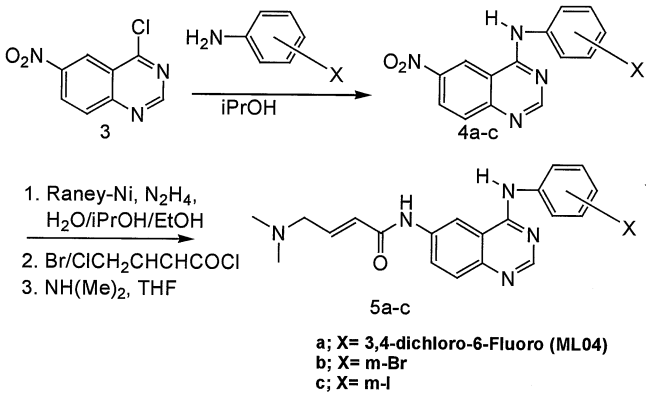

Fig. 2. Synthesis of irreversible epidermal growth factor receptor inhibitors.

In the next step, the nitro group at the 6-position of the quinazoline ring was reduced to amine. It should be noted that an undesired reduction at the anilino moiety of the halogen position was observed only with the iodo derivative. Therefore, the reduction step of this derivative was performed at 0°C. Even at this temperature we observed the formation of 10% of the reduction side-product that was separated from the desired product by silica chromatography. Reaction with bromo/chlorocrotonyl chloride [15] gave the (*2E*)-4-bromo/chloro-N-{4-[(phenyl)amino]-quinazolin-6-yl}-2-butenamides (6a–c) with 66% yield. The crude materials were purified by flash chromatography on silica gel column. Compounds 6a–c were used as precursors for either preparing the standard final compounds 5a–c (Fig. 2) or preparing the starting materials 7a–c for the radiolabeling with C-11 MeI (Fig. 3). For the preparation of the standard compounds, compounds 6a–c were reacted with dimethylamine at 80°C for 15 minutes. The standard compounds 5a–c were purified by flash chromatography with silica gel column and obtained with 73% yield. For synthesis of the precursor for the radiolabeling, the chloro/bromo-crotonylmide derivatives 6a–c were reacted with

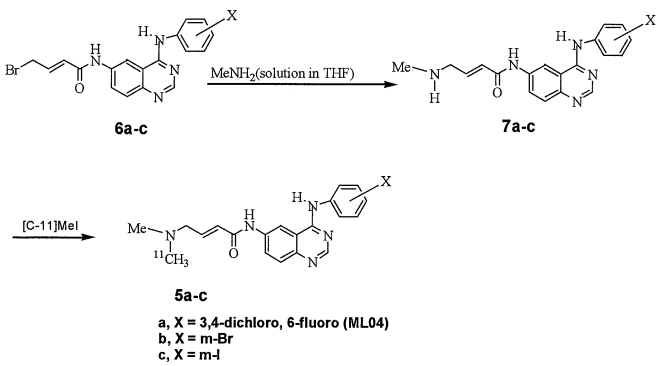

Fig. 3. Carbon-11 labeling of irreversible epidermal growth factor receptor inhibitors.

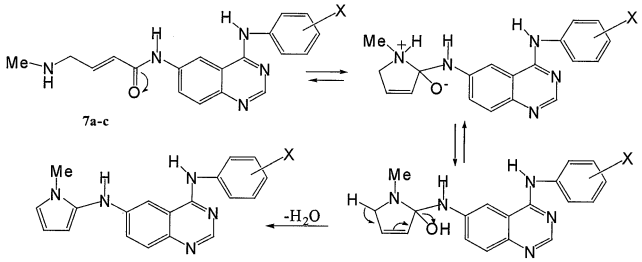

Fig. 4. Hypothesized mode of decomposition of 7a–c precursors.

monomethylamine in THF at 0°C for 10-15 minutes. Higher reaction temperatures caused the formation of several side-products. It should be noted that the crude mixture of this reaction could not be purified by silica gel nor by alumina gel column and was used as is for the radiosynthesis. When kept as a solution at room temperature or when loaded on column for purification, decomposition was observed by NMR, MS and HPLC. Therefore, at the end of the work-up, the solvent was quickly removed under reduced pressure without heating, and the crude product was kept at −20°C before use. We hypothesized that one possible mode of decomposition could have occurred through an intramolecular nucleophilic attack of the secondary amine on the carbonyl group to form a five-member ring (Fig. 4). Hydrogen migration followed by water elimination could lead to the stable aromatic methyl-pyrole group. The decomposition mixture contains several inseparable side-products. This hypothesis was supported by the MS analysis of the crude mixture, which showed the mass of the pyrole-derivative side products. It should also be noted that the chlorocrotonylamide derivatives were stable throughout the reaction with either mono-methylamine or dimethylamine.

3.2. In vitro screening

The nonlabeled compounds were evaluated in a cell-free system by means of ELISA to determine their EGFR autophosphorylation IC_{50} values. The assays were performed with A431 cell lysates (as described in the Methods and materials section), and the IC_{50} values of the three compounds are summarized in Table 1. The three compounds present similar results. Moreover, their IC_{50} values, in the low nanomolar range, indicate that the inhibitory potency of these new irreversible compounds towards the EGFR is on par with the parent compound, ML03. These data support the potential use of this family of compounds as PET bioprobes.

To assess the irreversible effect of the compounds on the EGFR autophosphorylation, the inhibitors were incubated with intact A431 cells for 1 hour (as described in the Methods and materials section), and the degree of EGFR phosphorylation was measured either immediately after or 8 hours after removal of the inhibitor from the medium.

One acceptable criterion for determining an irreversible effect of a compound is by measuring the degree of phosphorylation of the EGFR 8 hours after removal of the inhibitor from the medium. According to this assay, if an inhibition of 80% or more is achieved after 8 hours then the compound is considered to be irreversible, whereas an inhibition of 20-80% sets the compound as “partially irreversible” [19]. The results in Table 1 indicate that the three compounds possess similar potencies with regard to their inhibitory effect upon EGFR phosphorylation. The inhibition of phosphorylation was retained 8 hours after removal of the compounds from the media, reflecting the irreversible effect of these inhibitors, most likely due to covalent bonding to the EGFR.

3.3. Radiochemistry

We initially considered labeling these new inhibitors with fluorine-18 on the anilino moiety. However, labeling these compounds with fluorine-18 would have involved a six-step radiosynthesis, with the introduction of the labeled isotopes at the beginning of the procedure. Both the long synthesis time and the difficulty associated with automation of the procedure disfavored labeling with fluorine-18. Labeling with carbon-11, on the other hand, would have enabled the desired introduction of the radioisotope into the molecule at the last step of the synthesis. The strategic radiolabeling approach is based on the C-11 methylation

Table 1
IC_{50} values in the ELISA screen with A431 cell lysates and IC_{80} values for inhibition of epidermal growth factor receptor (EGFR) autophosphorylation in intact A431 cells immediately after and 8 hours post removal of the compound from the medium

Structure	A431 Lysate	Intact A431 Cells	
	IC_{50} app	IC_{80}* Range (immediately after removal of inhibitor)	IC_{80}* range (8 h after removal of inhibitor)
ML01	0.208 nmol/L	3–5 nmol/L	—
ML03	0.037 nmol/L	6.7–20 nmol/L	6.7–20 nmol/L
5a (ML04)	0.06 ± 0.04 nmol/L	4–10 nmol/L	10–50 nmol/L
5b	0.46 ± 0.12 nmol/L	4–10 nmol/L	~50 nmol/L
5c	0.09 ± 0.01 nmol/L	10–20 nmol/L	10–20 nmol/L

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

reaction of the mono-methylamine group of the amide attached to the 6-position of the quinazoline ring (Fig. 3). This carbon-11 radiolabeling reaction used a commercial module (Nuclear Interface, Munster, Germany). The well known C-11 methyl-iodide labeling reagent was prepared according to documented procedures [18]. The C-11 methyl-iodide was distilled out to the second reactor, which contained the precursor in a mixture of THF, DMSO, and acetonitrile at −20°C. The C-11 methylation reaction usually occurs at high temperatures; however, because of the instability of the precursor, the high temperature would have caused decomposition and the labeled product would not have been obtained. Therefore, upon investigating the relationship between temperature and yield, we found out that the optimal temperature for this reaction was 100°C. After a 5-minute reaction and THF evaporation, HPLC solvent was added and the crude mixture was purified by a built-in HPLC to yield the final products. The desired fraction was collected into the solid-phase extraction flask. To dilute the acetonitrile in the mobile phase, the flask was preloaded with 60 μL of 1 mol/L NaOH in 85 mL of water. Basic conditions were provided to obtain 5a–c as a free base. Finally, the products were separated by a C18 cartridge and were eluted with ethanol and saline. The automated procedure described enabled us to reliably and reproducibly ($n > 30$) obtain 5a–c in good yields (0.55-0.74 GBq, 15-20 mCi). The highest final product activity dosage achieved was 37 mCi, which is sufficient for human studies. HPLC analysis of the products solution revealed high radiochemical (>99%) and chemical purities. The high specific radioactivity achieved in the automated radiosynthesis (100 GBq/μmol, 2.7 Ci/μmol, EOB) is an important factor for the developments of PET biomarkers in general, as well as for 5a–c, designed to target *in vivo* low capacity receptor systems, in particular.

In conclusion, based on our previous results with both reversible and irreversible labeled EGFR inhibitors, we synthesized a new generation of carbon-11–labeled PET biomarkers. An automated radiosynthesis for the preparation of these radiolabeled bioprobes was developed, leading to the production of these labeled compounds with good yields, high specific activities and high radiochemical purities. These new labeled tracers have the potential of a higher biological stability, and a lower metabolic rate, which hopefully will lead to higher specific uptake in EGFR-positive tumors. The potency of these labeled tracers toward the EGFR was found to be high, and their inhibitory effect was demonstrated to be long lasting. Altogether, these data reflect the good potential of these compounds to serve as biomarkers for molecular imaging of EGFR-positive tumors.

Acknowledgments

Support was provided by TK Signal, Ltd., Nazaret, Israel, and by Rotem Industries Ltd., Beer Sheva, Israel.

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