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Award Number: DAMD17-98-1-8064

TITLE: Development of Novel Epidermal Growth Receptor-Based Radiopharmaceuticals: Imaging Agents for Breast Cancer

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REPORT DATE: September 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2001	Final (1 Sep 98 - 31 Aug 01)

4. TITLE AND SUBTITLE
Development of Novel Epidermal Growth Receptor-Based Radiopharmaceuticals: Imaging Agents for Breast Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8064

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The goal of this research was to develop epidermal growth factor receptor (EGFR) nuclear medicine breast cancer imaging agents. Our approach was to synthesize small molecule inhibitors of the EGFR tyrosine kinase (tk) suitable for labeling with single photon or positron-emitting radioisotopes and evaluate the imaging potential of these new molecules. We have synthesized and fully characterized 22 quinazoline compounds. All compounds inhibit EGFR tk phosphorylation activity in the nanomolar range. All compounds tested exhibited specificity for the EGFR tk versus the ErbB2 and ErbB4 tyrosine kinases. A radiometric binding assay using an iodine-125 labeled quinazoline was developed to determine the affinity of the quinazolines for the EGFR tk ATP binding site. The affinities ranged from 0.4-51 nM. The octanol/water partition coefficients (Log P; lipophilicity) of the new compounds ranged from 2.2-5.5. Six compounds have been labeled with fluorine-18. Biodistribution in EGFR overxpressing tumor bearing mice demonstrated tumor uptake but highlighted delivery and metabolism issues. The 2-fluoro quinazoline was not metabolized in an in vitro hepatocyte study. From this work a breadth of agent characteristics was created establishing the foundation for future research toward the optimal EGFR imaging agent.

14. SUBJECT TERMS
Epidermal Growth Factor Receptor, Breast Cancer, Positron Emission Tomography, Quantitative Imaging, Prognostic Indicator

15. NUMBER OF PAGES
37

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Appendix 10 Synthesis of 4-(3'-[^{125}I]iodoanalino)-6,7-dialkoxyquinazolines: Radiolabeled epidermal growth factor receptor tyrosine kinase Inhibitors.

Introduction

The goal of this research was to develop nuclear medicine imaging agents, to aid in the early detection, diagnosis and treatment of breast cancer, based on the epidermal growth factor receptor (EGFR) biomarker. EGFR is a member of the ErbB family of receptor tyrosine kinases, cell surface receptors responsible for cell proliferation and differentiation. EGF receptors are overexpressed in 45% of breast tumors.[1] Tumors possessing these receptors fail to respond to conventional treatment (hormonal based chemotherapy) leading to decreased patient survivability. EGFR based therapeutic agents have been in development by many drug companies over the last 10 years.[2-4]

Our approach was to design and synthesize small molecule inhibitors of the EGFR tyrosine kinase (tk) suitable for labeling with single photon and positron-emitting radioisotopes and evaluate the imaging potential of these new molecules. Our selection of the quinazoline inhibitors was based on the known structure activity relationships from the work of Fry and Bridges at Parke-Davis in the mid 1990's.[5, 6] Several new quinazoline analogs were synthesized. The analogs were put through a battery of tests including full chemical characterization, assessment of lipophilicity, determination of their ability to inhibit EGFR tk activity and measurement of their affinity for the EGFR tk. Those compounds that possessed suitable potency and binding characteristics were radiolabeled and subjected to secondary evaluation in in vitro cell studies, in vitro metabolism assays and in vivo distribution studies in tumor bearing mice. From this study we have created a library of agent characteristics and established the foundation for future research toward the optimal EGFR imaging agent.

Body

Task 1: Months 1-24: Synthesize and characterize new epidermal growth factor tyrosine kinase inhibitors.

We have synthesized the 22 quinazoline analogs shown in Figure 2. Four analogs, **10** and **11** were synthesized during this last year. All of the analogs were synthesized from the common intermediates 4-chloro-6,7-dimethoxy- and 4-chloro-6,7-diethoxy-quinazoline by the reaction shown in Figure 1. Production of the common intermediates allowed the rapid production of new analogs. In many cases the product crystallized out of solution during the reaction. The isolated yields from this series of reactions ranged from 65-90%. All products and intermediates were purified and were chemically characterized by NMR, mass spectroscopy, melting point and elemental analysis.

Task 2: Months 3-27: Perform in vitro assays to determine tyrosine kinase affinity and inhibition. Measure the lipophilicity of the inhibitors.

Mouse BaF3 hematopoietic cells that ectopically express either EGFR, ErbB-2 or ErbB-4 were pretreated with the kinase inhibitors. Subsequently, the cells were stimulated with EGF (for cells expressing EGFR) or Neuregulin (for cells expressing ErbB-2 and ErbB-4). The cells were lysed and analyzed for receptor tyrosine phosphorylation by immunoprecipitation with anti-EGFR or anti-ErbB antibodies followed by immunoblotting with an anti-phosphotyrosine antibody. Inhibition of receptor phosphorylation for 16 of the quinazoline analogs is shown in Figure 3. Additionally, the inhibition of cellular DNA synthesis by the EGFR inhibitors was performed in human mammary tumor MCF-10A cells. The cells were incubated for 48 hours

with varying concentrations of the EGFR inhibitors. Inhibition of DNA synthesis was identified by the lack of incorporation of tritiated thymidine into the DNA. The concentration of each inhibitor that inhibits 50% incorporation of the thymidine into the DNA was determined. These assays were performed by Dr. David Riese at Purdue University.

Almost all of the quinazoline analogs are potent inhibitors of EGFR tk phosphorylation. Most of the compounds exhibit nM inhibition. The exceptions to this are the 3-bromo-4-fluorobenzyl analogs, **4a** and **4b**, and the 3-fluoro-5-trifluoromethyl compound, **9b**. The compounds were specific for the EGFR tk as demonstrated by the fact that the inhibition of ErbB2 and ErbB4 tk phosphorylation was 10-70 fold less than EGFR tk. In an attempt to find a less labor-intensive assay that would be predictive of EGFR tk inhibition, we measured the inhibition of DNA synthesis in EGF responsive MCF 10A cells. In the initial subset of the analogs it was found that there was a good correlation between the inhibition of DNA synthesis and EGFR tk phosphorylation. However, as more compounds were tested the DNA synthesis inhibition did not significantly correlate with the EGFR tk phosphorylation. Thus, the DNA synthesis assay was not a good predictor of the potency of these compounds.

As part of this effort we developed an EGFR tyrosine kinase receptor binding assay. Receptor binding of the quinazoline compounds was determined by a competitive radiometric assay using [^{125}I]-3'-iodoanailino-6,7-dimethoxy quinazoline as the radioligand.[7] Commercially available A431 human carcinoma cell membrane homogenate was the EGFR source (Receptor Biology, Beltsville, MD). By varying the concentration of the quinazoline analog, the inhibition of specific binding at the 50% level was determined. The IC_{50} values for 18 of the quinazoline compounds is shown in Figure 5.

All of the compounds tested bind to the EGFR tk with high affinity. The dimethoxy analogs of compounds **1a**, **8a**, **9a**, **10a** and **11a** (4-fluoroanilino, 4-fluorobenzyl, 3-fluoro-5-trifluoromethylanilino, 2-fluoroanilino and 3-fluoroanilino, respectively) exhibit the lowest affinities (IC_{50} >20 nM). In all cases the affinity of the diethoxy compound is higher than the corresponding dimethoxy compound. This is analogous to the reported relationship between the diethoxy and dimethoxy compounds in terms of their inhibition of EGFR phosphorylation activity (diethoxy > dimethoxy).[6] There is not a good correlation between the binding and inhibition of phosphorylation (R^2=0.36). The binding assay is a more useful as a predictor for good imaging agents and is much less cumbersome to perform.

The lipophilicity of the quinazoline analogs was determined by measuring the octanol/water partition coefficients (Log P) for each of the compounds. The HPLC method of Minick, et al.[8] was used to determine the Log P (Log [octanol/water]) experimental values. The Log P values are shown in Figure 4. The Log P values range from 2.2 for **10a** (2-fluoroanilino-dimethoxyquinazoline) to 5.49 for **9b** (3-fluoro-5-trifluoromethylanilino-diethoxyquinazoline). The data predict the relative changes in lipophilicity between any two compounds as would be expected based on the physical differences between the two molecules. In general Log P values can be used to predict the non-specific binding of the compounds to non-receptor sites. Thus, a compound with a higher Log P might be taken up and remain in non-target tissues increasing the background activity and blurring the image. The best compounds are generally in the range of 2.0-3.5.

Task 3: Months 6-36: Label receptor tracers with fluorine-18, carbon-11, iodine-123, iodine-125, bromine-76. Optimize the synthetic routes for efficient production of high yield, high specific activity tracers.

We radioiodinated two of the quinazolines with iodine-125 from the trimethyltin precursor. A detailed description of the radiochemistry including the synthesis of the precursor is given in the manuscript attached as appendix 10.

Six compounds were labeled with fluorine-18. These syntheses are shown in Figures 6-8. The route to the labeled compounds is analogous to the synthesis of the cold compounds shown in Figure 1. Either the labeled benzylamine **13** or the labeled anilines **17** and **21** were coupled to the appropriate chloroquinazoline to give the desired labeled compounds. The synthesis of the fluorobenzylamine was based on the recently published accounts [9] and the synthesis of the fluoroanilines was an adaptation of previous work by Feliu [10]. Details of the fluoroaniline work are given in Appendix 8. All of the radiolabeled quinazoline compounds were purified by HPLC and corresponded in retention time to the non-radioactive analogs. All compounds were produced in 27-30% decay corrected yield for a 155 min. synthesis and purification.

In addition to alkylating 4-chloroquinazoline with 2- and 4- fluoroaniline and 4-fluorobenzylamine, we have optimized the production of other fluoroanilines shown in Figure 9. We have labeled 3-chloro- **27** and 3-bromo-4-[^{18}F]fluoroaniline **28** by reacting cesium [^{18}F]fluoride with the respective 4-trimethylammonium triflate . We also produced 3-[^{18}F]fluoro-nitrobenzene **30** in good yield from the 3-nitrophenyl trimethylammonium triflate **29**. This labeled synthon will allow the production of 3-fluoroanilino compounds.

Task 4: Months 9-36: Evaluate uptake of labeled tracers in tumor cells possessing differing EGFR titer.

We have added [^{125}I]**7a** to whole MB-468 cells (EGFr +) in suspension and aliquots of the media and cells were removed at various times. The cells were separated from the media and each were counted. A significant portion of the activity remained in the media and no change was noted over the time course of the study.

Collaborating with Dr. Buck Rogers at the University of Alabama, the time course of uptake of [^{125}I]**7a** and [^{125}I]**7b** in whole cells that express EGFr (MB468) and those that don't express EGFr (MB453) was examined. The influence of the presence of EGF in the growth media and the introduction of blocking doses of non-radioactive **6a** was also studied. The results were mixed and inconsistent.

A single study was performed using membrane preparations from MB468 cells (EGFr+) and MB453 cells (EGFr -). [^{125}I]**7a** and [^{125}I]**7b** were added to the preparations with and without a blocking dose of **5a**. The results of this study are shown in Table 1. The EGFr positive membranes take up [^{125}I]**7a** and [^{125}I]**7b** and demonstrate receptor mediated uptake in the presence of the blocking compound. The EGFr negative membranes show no binding characteristics. The amount of radiolabel taken up in all cases in the MB453 membranes is equivalent to the background (non-specific) seen in the blocked MB468 membranes. This study became the basis for the receptor binding assay that was developed (described above).

Task 5: Months 18-36: Evaluate whole body distribution in normal and tumor-bearing nude mice.

The distribution of three of the labeled compounds was studied in mice bearing MB468 tumors, known to overexpress EGFR. The distribution of the compounds is shown in Figures 10-12.

All three compounds demonstrated some uptake in the tumor over the 2 hour study. In all cases there appeared to be washout of the label from the tumor. The blood values were low for the two fluoroanilino compounds, **18a** and **18b**. The clearance from the blood was most evident for compound **14b**. The only tumor to blood ratio that increased from 1 to 2 hours was that of the fluorobenzyl compound **14b** which indicates retention of the compound in the tumor. Tumor to muscle ratios for **18a** and **18b** were less than 1 at all time points whereas the tumor to muscle ratios of **14b** were greater than 2 to 1 at both time points. High bone uptake was seen for **18a** and **18b** indicating metabolic defluorination. This is the first time that this type of metabolism has been seen with a fluorine-18 labeled compound, however, this type of metabolic defluorination of para-fluoro anilines has been described in the literature.[11] The percent of the injected dose recovered from these animals was very low, on the order of 2-5%. This would indicate that the compound is being cleared very rapidly from the body through the liver and kidney so that it is not available to bind go to the tumor and bind to the receptors.

Using cryopresserved hepatocytes from In Vitro Technologies (Baltimore, MD) the differences between the metabolism of the 2-[^{18}F fluoroanilino, **22a**, and 4-[^{18}F]fluoroanilino, **18a**, positional analogs was investigated. The fluorine-18 labeled compounds were added to thawed hepatocytes suspended in Krebs-Hensleit buffer. Compound added to a buffer solution with no hepatocytes served as a control. At various time points, aliquots of the solution were added to an equivalent amount of methanol and centrifuged to pellet the cells and debris. The supernatant liquid was analyzed using thin layer chromatography and HPLC. Figure 13 shows the metabolism of the two compounds from the TLC data. The amount of [^{18}F]fluoride ion or baseline metabolites was significantly less for the 2-fluoro case indicating limited defluorination. This data supports the hypothesis that the 4 position in the anilines is more susceptible to defluorination.

Key Research Accomplishments

- Synthesized and fully characterized 22 quinazoline analogs including developing a new route to the diethoxy compounds.
- Most of the analogs inhibited epidermal growth factor receptor tyrosine kinase activity at pico- or nano-molar concentrations.
- Analogs tested were selective for the EGFR tk over the ErbB2 tk and the ErbB4 tk.
- Developed an EGFR tk binding assay and determined the affinity of 18 of the compounds.
- 14 out of the 18 analogs exhibited high affinity for the EGFR tk receptor.
- The lipophilicity of the new analogs ranges from 2.2-5.5.
- Successfully labeled two analogs with iodine-125.
- Successfully labeled six analogs with fluorine-18.

- Optimized the synthesis of labeled fluoroanilines and coupling to the chloroquinazolines.
- Biodistribution of radiolabeled compounds highlighted delivery and metabolism issues.
- Solved metabolism issue by synthesizing 2- fluoro compound and testing in isolated hepatocytes.

Reportable Outcomes

- Abstract presented at the 13th International Symposium on Radiopharmaceutical Chemistry entitled "Synthesis of 4-(4'-[18F]fluorobenzylamino)-6,7-diethoxyquinazoline: a positron emitting radioprobe for the epidermal growth factor receptor" *J. Lab. Comp. Radiopharm.* 42, Suppl. 1:S693-S695, 1999. – Appendix 2.
- Abstract presented at California Breast Cancer Research Symposium, September 1999 "Development of PET imaging agents for breast cancer" – Appendix 3.
- Abstract presented at 219th American Chemical Society National Meeting, March 2000 "Development of positron labeled epidermal growth factor receptor tyrosine kinase inhibitors: PET probes for breast cancer" – Appendix 4.
- Abstract presented at DoD Era of Hope Meeting, June 2000 "Development of radiolabeled epidermal growth factor receptor inhibitors. New probes for tumor imaging" – Appendix 5.
- Abstract presented at 2000 International Chemical Congress of the Pacific Basin Societies, December 2000 "Fluorine-18 labeled 4-(4'-fluoroanilino)-6,7-dialkoxy-quinazolines: synthesis and evaluation of new breast cancer receptor imaging agents." – Appendix 6.
- Abstract presented at 221st American Chemical Society National Meeting, March 2001 "Novel cancer probes: development of receptor tyrosine kinase-based imaging agents." – Appendix 7.
- Abstract presented at the 14th International Symposium on Radiopharmaceutical Chemistry entitled "Synthesis of [^{18}F]fluoroanilines: precursors to [^{18}F]fluoroanilino-quinazolines." *J. Lab. Comp. Radiopharm.* 44, Suppl. 1:S880-882, 2001.– Appendix 8.
- Abstract to be presented at California Breast Cancer Research Symposium, Fall 2001 "Epidermal growth factor imaging agents for breast cancer detection and new therapeutic drug development." – Appendix 9.
- Manuscript: Lim, JK; Negash, K; Hanrahan, SM; VanBrocklin, HF. Synthesis of 4-(3'[^{125}I]Iodo-phenylamino)-6,7-dialkoxyquinazoline: Epidermal Growth Factor Receptor Tyrosine Kinase (EGFrTK) Inhibitors. *J. Lab. Comp. Radiopharm.*, **43**:1183-1191, 2000. – Appendix 10.
- Manuscript: Lim,JK, Hom, DL, Negash, K, Ono, MY, Taylor, SE, Riese II, DJ, VanBrocklin, HF. Quinazoline-based Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors: Development of Tumor Imaging Agents. *J. Med. Chem.* in preparation.
- Funding applied for based on this work: NIH RO1 Proposal CA94253 "Targeted Molecular Probes for Tumor Imaging".

- Personnel funded through this effort: Henry, F. VanBrocklin, Ph.D. (PI), Scott Taylor, Ph.D, James P. O'Neil, Ph.D, Stephen Hanrahan,

Conclusions

Over the three years of this project sufficient data has been produced to identify some key patterns in the characteristics of these potential imaging agents. While the compounds we have developed demonstrate appropriate affinity and specificity for the EGFR tk, the compounds do not possess the desired distribution characteristics. The compounds studied may have been too lipophilic to cross the cell membrane and as was seen in the case of the 4-fluoroanilino compounds, **18a** and **18b**, metabolic defluorination led to high bone uptake. The 2-fluoroanilino compounds that were just synthesized and characterized in this last year possess more desirable lipophilic and metabolic characteristics but the binding affinity is 10-30 nM.

The biodistribution data of the fluoro compounds corroborates the results from the clinical trials of the 3-bromoanilino-dimethoxyquinazoline EGFR inhibitor, **6a**. The compound cleared rapidly from the body minimizing its potential therapeutic efficacy. In addition to the diagnostic potential of these quinazoline analogs, the information that was gathered about the distribution properties of these molecules may be very important for the future development of tyrosine kinase inhibitors as cancer therapeutics not only for the EGFR but also for ErbB2 and VEGF receptors.

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Appendix 1.

Table 1. Receptor mediated binding of the EGFr tk inhibitors

	Percent of Radiolabel bound to membranes			
Radiolabel	MB468 (EGFr+) Membranes		MB453 (EGFr-) Membranes	
	(-)**6a**[x]	(+)**6a**[y]	(-)**6a**[x]	(+)**6a**[y]
[^{125}I]**7a**	72%	26%	28%	29%
[^{125}I]**7b**	65%	33%	32%	37%

[x] No blocking dose added to the membranes.

[y] Receptor blocking dose added to the membranes.

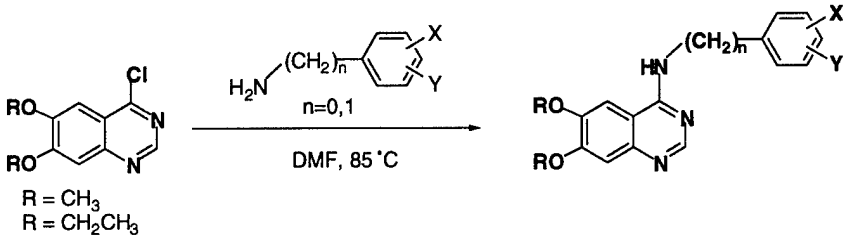

Figure 1. Synthesis of substituted benzyl- and anilino-dialkoxyquinazolines.

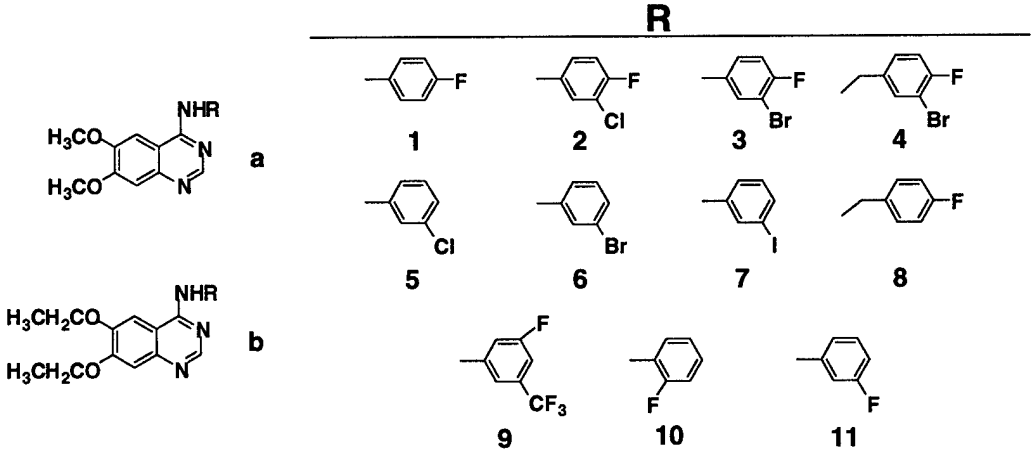

Figure 2. Dialkoxyquinazoline analogs prepared as part of this effort.

Appendix 1. cont.

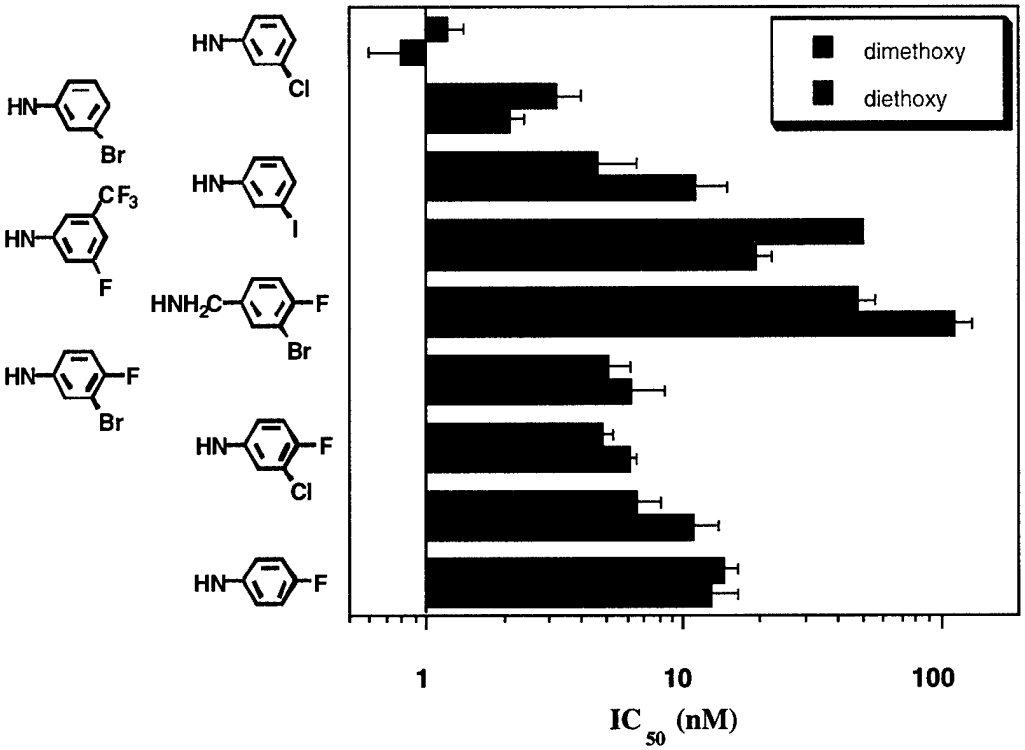

Figure 3. Inhibition of EGFR tyrosine kinase phosphorylation.

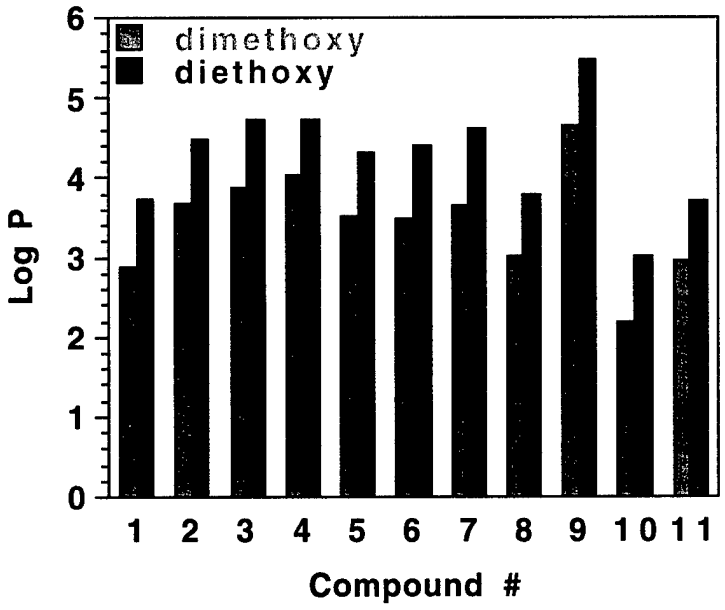

Figure 4. Log P estimation by HPLC for the quinazoline compounds

Appendix 1. cont.

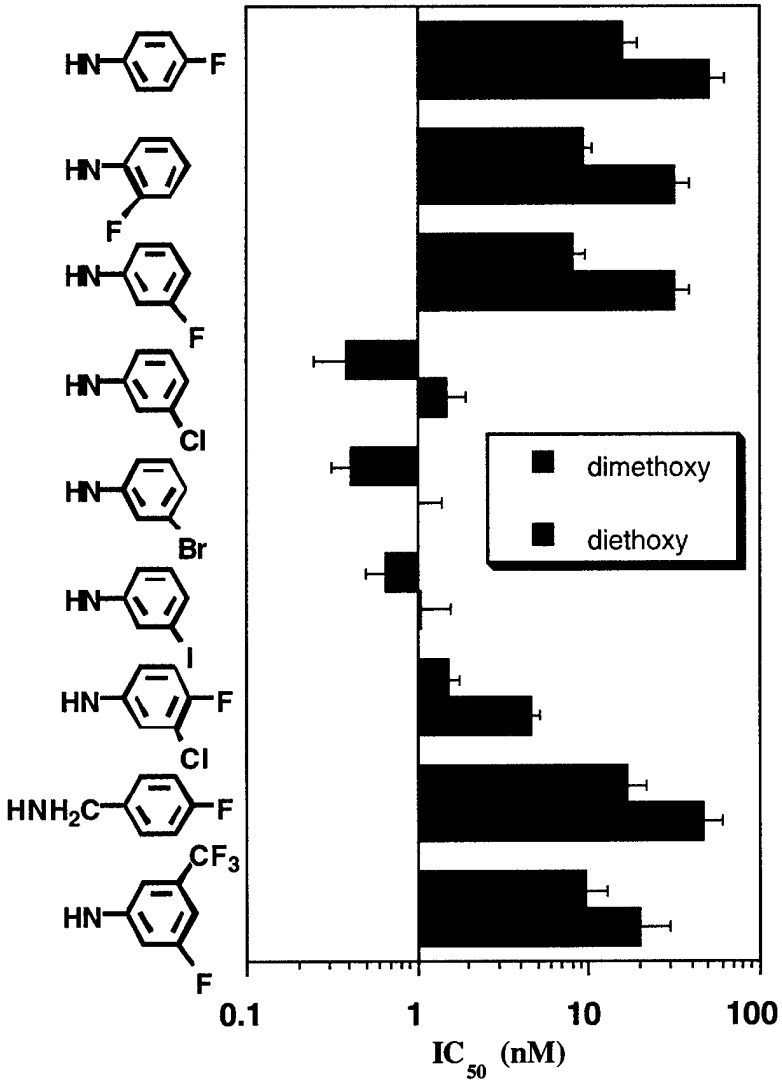

Figure 5. EGF Receptor binding affinity of the quinazoline compounds.

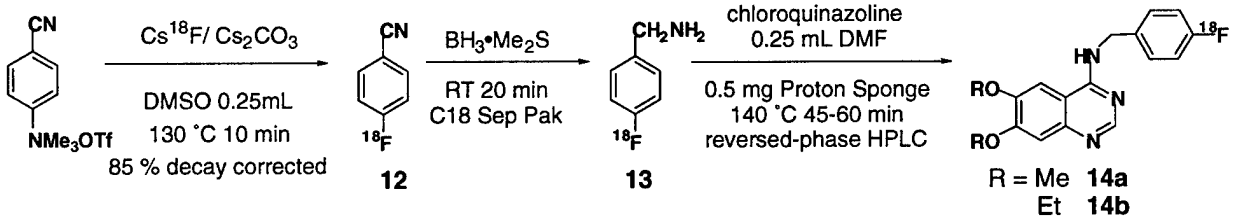

Figure 6. Synthesis of 4-(4'-[^{18}F]fluorobenzylamino)-6,7-dimethoxy- and 4-(4'-[^{18}F]fluorobenzylamino)-6,7-diethoxy- quinazoline

Appendix 1. cont.

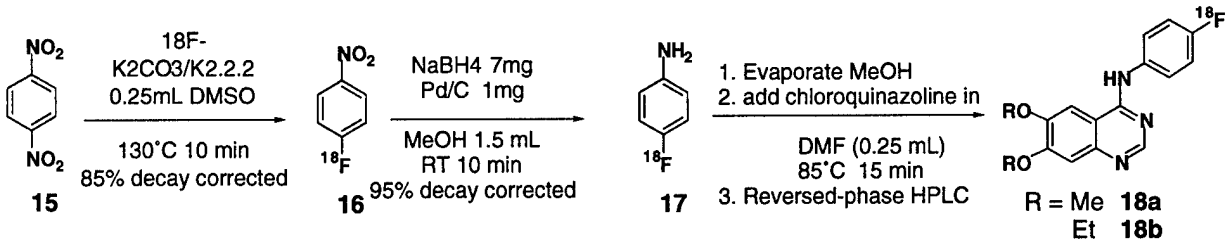

Figure 7. Synthesis of 4-(4'-[^{18}F]fluoroanalino)-6,7-dimethoxy- and 4-(4'-[^{18}F]fluoroanalino)-6,7-diethoxy- quinazoline.

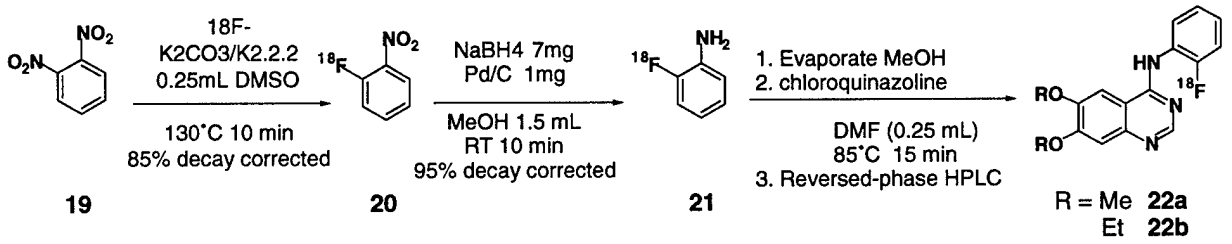

Figure 8. Synthesis of 4-(2'-[^{18}F]fluoroanalino)-6,7-dimethoxy- and 4-(2'-[^{18}F]fluoroanalino)-6,7-diethoxy- quinazoline

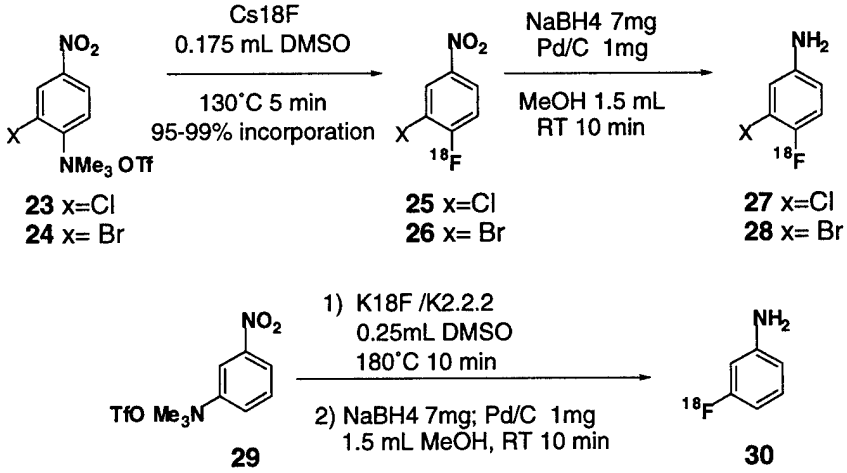

Figure 9. Synthesis of [^{18}F]fluoroanilines

Appendix 1. cont.

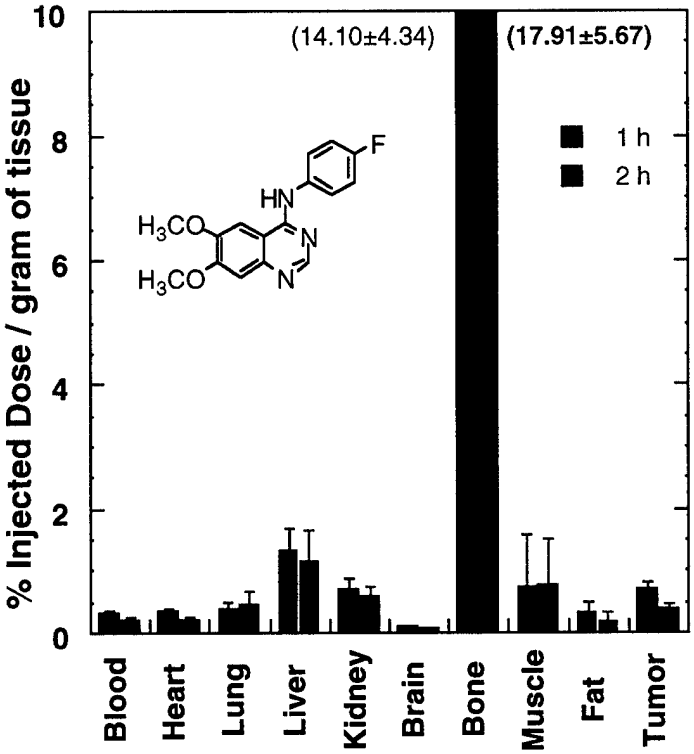

Figure 10. In vivo distribution of **18a** in MDA-468 tumor bearing mice.

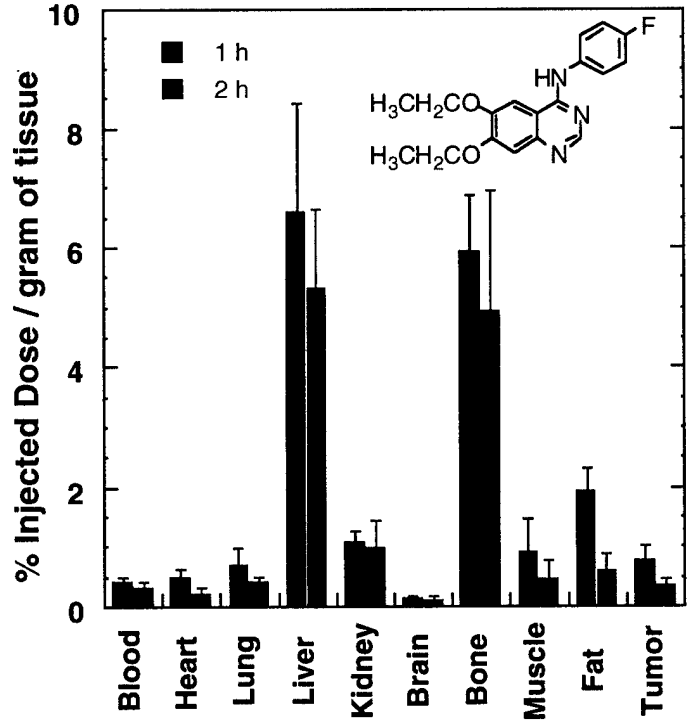

Figure 11. In vivo distribution of **18b** in MDA-468 tumor bearing mice.

Appendix 1. cont.

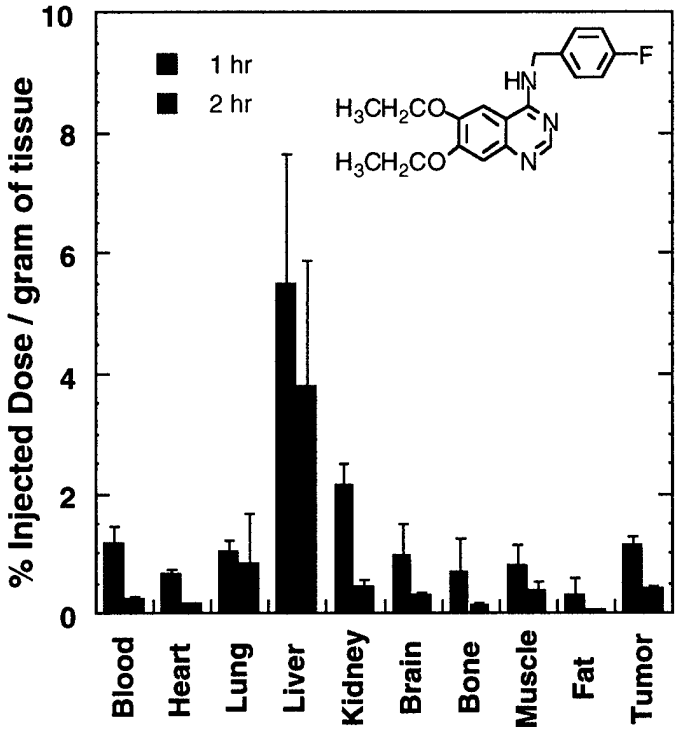

Figure 12. In vivo distribution of **14b** in MDA-468 tumor bearing mice.

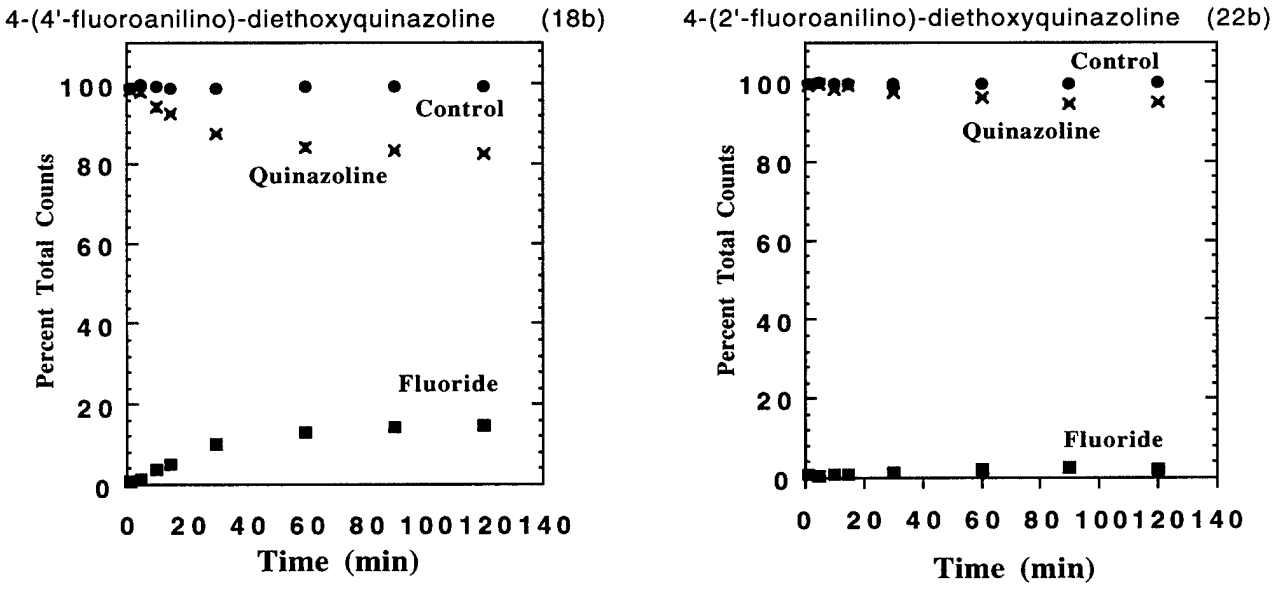

Figure 13. Hepatocyte metabolism kinetic profile of para- (**18b**) versus ortho- (**22b**) [^{18}F]fluoroanilinodiethoxyquinazoline analogs. Control contained no hepatocytes.

Appendix 2.

SYNTHESIS OF 4-(4′-[^{18}F]FLUOROBENZYLAMINO)-6-7-DIETHOXYQUINAZXOLINE: A POSITRON EMITTING RADIOPROBE FOR THE EPIDERMAL GROWTH FACTOR RECEPTOR

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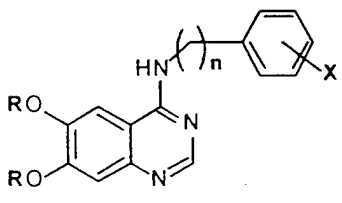
Key Words: epidermal growth factor receptor, tyrosine kinase, inhibitor, quinazoline, fluorine-18, PD15305

The oncogenic epidermal growth factor receptor (EGFr) is a transmembrane protein with an extracellular binding domain and an intracellular tyrosine kinase region. Binding of EGF to the receptor initiates the phosphorylation of tyrosine by the tyrosine kinase, propagating a signal that ultimately stimulates cell growth and proliferation.(1) EGFrs are upregulated in several different tumors including lung cancer, endometrial cancer and breast cancer.(2) Our interest lies in developing EGFr-based imaging agents for breast cancer, where EGFr is upregulated in 45% of the tumors and correlated with poor prognosis.(3,4)

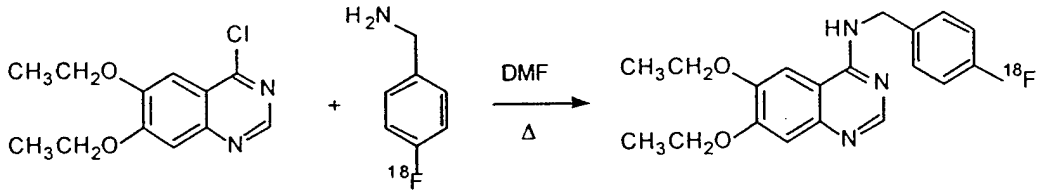
Inhibitors of the receptor tyrosine kinase are target drug candidates for anticancer therapy. A class of EGFr tyrosine kinase inhibitors, 4-anilinoquinazolines, has been shown to be particularly potent and selective.(5) One analog, 4-(3′-bromoanilino)-6,7-dimethoxyquinazoline (**1**, PD153035), inhibits EGFr activity with an IC_{50} value of 25 pM (free receptor assay) and has progressed to phase I clinical trials.(6) The radioiodinated 3'-iodo analog of **1** has shown receptor mediated uptake in the human tumor cell line MDA-468.(7) Two positron emitting labeled quinazoline analogs, [^{18}F]fluoroethoxy (8) and [^{11}C]methoxy (8-10), have been reported. Preliminary biodistribution studies have shown differential uptake of the carbon-11 compound in implanted tumors versus healthy tissue, demonstrating potential as a tumor imaging agent.(9)



COMPD	R	n	X	IC_{50} (nM)
1	Me	0	3'-Br	2.38 ± 1.27
2	Me	1	4'-F	14.02 ± 5.18
3	Et	0	3'-Br	5.91 ± 3.41
4	Et	1	4'-F	7.57 ± 4.64

We have synthesized a series of fluorine and iodine quinazoline compounds suitable for radiolabeling and assessed their ability to inhibit EGFr tyrosine kinase activity.(11) Two of the fluorine compounds, **2** and **4**, are shown above along with their respective IC_{50} values from an *in vitro* assay using whole cells. Compounds **1** and **3** are shown for comparison. There is a loss of inhibition in the transition from 3′-bromoanilino to the 4′-fluorobenzyl while an increase in inhibition from the methoxy to ethoxy is seen within the fluorine series. Compound **4** also demonstrates better inhibition than the 3′-iodo compounds (data not shown).

We previously reported the preparation of 4-(4'-[^{18}F]fluorobenzylamino)-6,7-dimethoxyquinazoline, **[^{18}F]2**, by coupling [^{18}F]fluorobenzylamine with 6,7-dimethoxychloroquinazoline.(11) [^{18}F]fluorobenzylamine was prepared by reduction of the corresponding benzonitrile precursor with lithium aluminum hydride. This method necessitated a substantial synthetic work-up to purify the intermediate [^{18}F]fluorobenzylamine. We have since adapted a recent literature procedure (12) whereby the nitrile moiety of *p*-[^{18}F]fluorobenzonitrile was reduced with borane-dimethylsulfide resulting in high radiochemical purities (>95%, determined by thin-layer radiochromatography) in a much shorter time and with a simplified workup. Our only deviation from the reported reaction was the use of Cs[^{18}F] as the nucleophilic fluoride ion in the reaction with 4-*N,N,N*-trimethylammonium-benzonitrile triflate (0.5 mg). The [^{18}F]fluorobenzylamine was purified on a C_{18} Sep Pak® cartridge and eluted with DMF. The first 0.5 mL of DMF from the Sep Pak® was discarded.



5 **6** **[^{18}F]4**

The preparation of 4-(4'-[^{18}F]fluorobenzylamino)-6,7-diethoxyquinazoline **[^{18}F]4** is shown in the scheme above. The [^{18}F]fluorobenzylamine **6**/ DMF solution was added to 4-chloro-6,7-diethoxyquinazoline **5** (0.5 mg) and proton sponge (0.5 mg) followed by heating at 140 °C for 45-60 minutes. The solution was applied to a C_{18} Sep Pak® cartridge and eluted with methanol. The methanolic solution was diluted with water and purified by reversed phase HPLC (Whatman M9/50 ODS3 semi-preparative column 65:35 methanol/water, pH 7.40, 6 mL/min). The desired **[^{18}F]4** eluted at 14 minutes. The total synthesis time was approximately 2.5 hours from the end of bombardment. While the radiochemical purity was >99%, the overall yield was low.

We have successfully synthesized 4-(4'-[^{18}F]-fluorobenzylamino)-6,7-diethoxyquinazoline and are continuing to improve the radioligand synthesis for further evaluation of this EGFr ligand as a potential tumor imaging agent.

Acknowledgments

The authors wish to thank Scott Taylor, Steve Hanrahan, Michelle Huesman, Andrew Gibbs, Alan Glabe and Refaat Sheleby for their assistance. This work was supported by the Director, Office of Science, Office of Biological and Environmental Research, Medical Science Division of the U.S. Department of Energy under contract No. DE-AC03-76SF00098 and by the Army Medical Research and Materiel Command, U.S. Department of Defense under grant No. DAMD17-98-1-8064 (HFV). Support for Dr. Lim is provided by NIH training grant No. NCI T32CA66527.

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Appendix 3.

Abstract for the California Breast Cancer Research Symposium

September 17-18, 1999 Los Angeles, CA.

Development of PET Imaging Agents for Breast Cancer

Henry F. VanBrocklin, John K. Lim. Center for Functional Imaging, Lawrence Berkeley National Laboratory, Berkeley CA.

Abstract:

The aim of this project is to develop new radioactively labeled pharmaceuticals (radiopharmaceutical) for the early detection and diagnosis (staging) of breast cancer using nuclear medicine imaging methods such as positron emission tomography (PET). The nuclear medicine procedure involves the injection of a radiopharmaceutical that is attracted to and preferentially accumulates in the breast tumor. The patient is then placed in an imaging device called a scanner to show the physician where the radiopharmaceutical has gone. In this case we are looking for accumulation in tumors that have a special receptor called the epidermal growth factor receptor. Only 45% of breast cancers have epidermal growth factor receptors, however, tumors with these receptors are harder to treat and do not respond to conventional hormonal treatment. Therefore, it is important that the earlier these tumors are detected the sooner any treatment can begin. Nuclear Medicine imaging agents can provide information about the tumor faster than biopsy, is less invasive than a biopsy and is about as harmful as an X-ray.

We have produced several new chemical compounds that have demonstrated an ability to bind to the epidermal growth factor receptor. We will report our latest efforts on the development of the chemistry that will be used to introduce the radioactive label into the these compounds.

Nuclear medicine techniques have a positive impact on the reduction of human and economic costs of breast cancer. These techniques are non-invasive and can provide localization information as well as some clinical evaluation of the type of disease in a single procedure, thus reducing the need for separate detection and biopsy procedures. This has both human and economic benefits by providing reliable, fast detection and diagnostic information. This in turn can have a positive impact on survivability by fostering rapid treatment response.

Appendix 4.

Poster presentation at the 219th American Chemical Society National Meeting, Anaheim, CA, March 2000

DEVELOPMENT OF POSITRON LABELED EPIDERMAL GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITORS: PET PROBES FOR BREAST CANCER. ***Henry F. VanBrocklin*** [1], *Andrew R. Gibbs* [1], *Stephen M. Hanrahan* [1], *Darren L. Hom* [1], *John, K. Lim* [1], *David J. Riese II* [2]. (1) Center for Functional Imaging, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, (2) Department of Medicinal Chemistry and Molecular Pharmacology Purdue University, West Lafayette, IN 47907.

Epidermal growth factor receptor (EGFr) expression is upregulated in 45% of breast cancers. Over expression of these receptors has been correlated with poor patient prognosis as well as poor response to hormonal therapy. Inhibitors of the EGF receptor tyrosine kinase (tk) are target drug candidates for antitumor therapy. Several classes of compounds, including 4-analinoquinazolines, have been shown to inhibit EGFr tk phosphorylation, the first step in the signal transduction pathway. We have synthesized several analogs of 4-(3′-bromoanilino)-6,7-dimethoxyquinazoline (PD153035), a compound which has demonstrated potent inhibition of EGFr tk activity, suitable for labeling with fluorine-18 or carbon-11. Screening assays have demonstrated the potent inhibition of EGFr tk phosphorylation as well as the cytotoxicity of these new ligands. We will describe the synthesis, the inhibition and cytotoxicty results along with the preliminary efforts towards the synthesis of the radiolabeled probes.

Appendix 5.

DOD "Era of Hope" Abstract June 2000.

DEVELOPMENT OF RADIOLABELED EPIDERMAL GROWTH FACTOR RECEPTOR INHIBITORS. NEW PROBES FOR TUMOR IMAGING.

Henry VanBrocklin[1], David Riese II[2], John Lim[1], Andrew Gibbs[1], Stephen Hanrahan[1], Darren Hom[1], Michele Ono[1], and Scott Taylor[1]

[1]Center for Functional Imaging, Lawrence Berkeley National Laboratory, Berkeley, CA 94720 and [2]Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907

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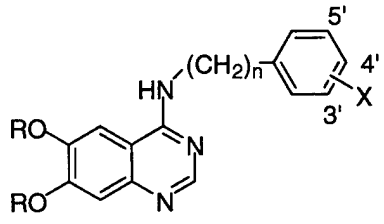
The oncogenic transmembrane epidermal growth factor receptors (EGFr) are upregulated in a variety of human neoplasms including lung, endometrial and breast tumors. The present investigation explores the development of small molecule EGFr-based imaging agents for breast cancer, where receptor upregulation correlates with poor therapeutic response and prognosis. We have synthesized ten quinazoline analogs suitable for radiolabeling, based on the known structure activity relationships of compounds developed at Parke-Davis as therapeutic agents. We have evaluated their ability to inhibit EGFr tyrosine kinase phosphorylation, using EGFr ectopically expressed in BaF3 hematopoetic cells, as well as their ability to inhibit DNA synthesis in an EGF dependent cell line, MCF-10A cells (see table below). All of the compounds tested inhibit the tyrosine kinase phosphorylation. We have identified a significant correlation between the inhibition of EGFr phosphorylation and inhibition of DNA synthesis ($p<0.005$). The DNA synthesis assay is less cumbersome, thus more attractive for high throughput screening of new inhibitors and may be more indicative of therapeutic potency. We have labeled compounds **3** and **8** with iodine-125 and demonstrated receptor-mediated uptake using MB468 cell membranes. Compounds **5** and **10** have been labeled with positron emitting fluorine-18. Initial evaluation in tumor bearing mice showed slight retention in the tumor 2 hours post injection. Based on these initial results, labeled compounds from this series of EGFr inhibitors show potential as imaging agents to detect EGF receptors in breast tumors.



analog #	n	R	X	EGFr Phos. (nM)	DNA Inhibition (nM)
1	0	Me	3'-Cl	Not Detd	Not Detd
2	0	Me	3'-Br	2.2 ± 0.4	226 ± 32
3	0	Me	3'-I	11.1 ± 3.7	585 ± 108
4	0	Me	3'-F,5'-CF_3	19.1 ± 2.9	1634 ± 100
5	1	Me	4'-F	10.9 ± 6.8	489 ± 87
6	0	Et	3'-Cl	Not Detd	Not Detd
7	0	Et	3'-Br	3.3 ± 0.9	74 ± 18
8	0	Et	3'-I	4.6 ± 2.0	159 ± 28
9	0	Et	3'-F,5'-CF_3	>50	>3000
10	1	Et	4'-F	6.6 ± 1.6	211 ± 29

The U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8064 supported this work.

Appendix 6.

Oral Presentation at 2000 International Chemical Congress of the Pacific Basin Societies. Honolulu, HI, December 2000

FLUORINE-18 LABELED 4-(4'-FLUOROANILINO)-6,7-DIALKOXY-QUINAZOLINES: SYNTHESIS AND EVALUATION OF NEW BREAST CANCER RECEPTOR IMAGING AGENTS

Henry F. VanBrocklin (1), David J. Riese, II (2), Andrew R. Gibbs (1), John K. Lim (1).
(1) Center for Functional Imaging, Lawrence Berkeley National Laboratory, 1 Ccylotron Rd. Berkeley CA, 94720, (2) Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907.

Epidermal growth factor receptors (EGFr) are over expressed in several human neoplasms including lung, endometrial and breast tumors. We have developed a series of aminoquinazoline EGFr inhibitors based on the structure activity relationships of compounds under investigation as anticancer agents. We have synthesized anilino- and benzylamino analogs, containing fluorine and iodine, suitable for labeling with positron and single photon isotopes. All of the compounds tested inhibit EGFr tyrosine kinase phosphorylation. [18F]Fluoro-for-nitro exchange on 1,4-dinitrobenzene followed by reduction of the nitro group afforded 4-[18F]fluoroaniline which was subsequently coupled to either 6,7-dimethoxy- or 6,7-diethoxy-4 chloroquinazoline to yield the title compounds. The total synthesis time was 2 hours and decay corrected yields for both compounds were high (36-40%). Initial evaluation in MDA-468 tumor bearing mice exhibited uptake in the tumor and clearance organs. These results demonstrate the potential of EGFr-based agents for tumor imaging.

Appendix 7.

Oral Presentation at the 221st American Chemical Society National Meeting, Anaheim, CA, March 2001

NOVEL CANCER PROBES: DEVELOPMENT OF RECEPTOR TYROSINE KINASE-BASED IMAGING AGENTS

H.F. VanBrocklin, M.Y. Ono, D.L.Hom, S.E. Taylor, S.M. Hanrahan, D.J. Riese, III
Center for Functional Imaging, Lawrence Berkeley National Laboratory, Berkeley, CA.
Department of Medicinal Chemistry and Molecular Pharmacology, Purdue Univ., West Lafayette, IN.

Receptor tyrosine kinases (RTKs) (e.g. epidermal receptor growth factor family – EGFR, ErbB2, ErbB3, ErbB4; and VEGF, etc.) play an important role in the proliferation of many cancers. The inhibition of RTKs has been targeted for new antineoplastic therapeutics. The objective of this research is to screen potential candidate compounds as imaging agents to measure the density of RTKs in tumors. Potent small molecule inhibitors have been identified for the EGFR system. We have produced over 20 quinazoline analogs, EGFR phosphorylation inhibitors, suitable for labeling with positron emitting isotopes (i.e. carbon-11 or fluorine-18), and assessed a variety of in vitro and in vivo characteristics. The affinity, enzyme inhibition, distribution and metabolism of these analogs will be presented. The paradigm developed for the EGFR imaging agents may be applicable to image other RTKs.

SYNTHESIS OF [18F]FLUOROANILINES: PRECURSORS TO [18F]FLUOROANILINOQUINAZOLINES

Henry F.VanBrocklin, James P. O'Neil, Darren L. Hom, Andrew R. Gibbs

Center for Functional Imaging, Lawrence Berkeley National Laboratory, Berkeley, California, 94720

Keywords: fluorine-18, fluoronitrobenzene, fluoroaniline, EGFR

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases. These receptors play an important role in cell proliferation and transformation of cellular morphology (1). EGFR overexpression has been noted in a number of human neoplastic lesions including breast, ovary, lung and prostate cancers (2). EGFR has been an attractive target for therapeutic drug discovery programs (3-5) and recently, a target for radiolabeled probes (6-11).

As part of our ongoing program to develop EGFR imaging agents, we have developed a general method for the synthesis of [^{18}F]fluoroaniline synthons. Conditions for the subsequent coupling reaction with chloroquinazoline derivatives were also investigated, providing fluoroanilinoquinazoline analogs of interest.

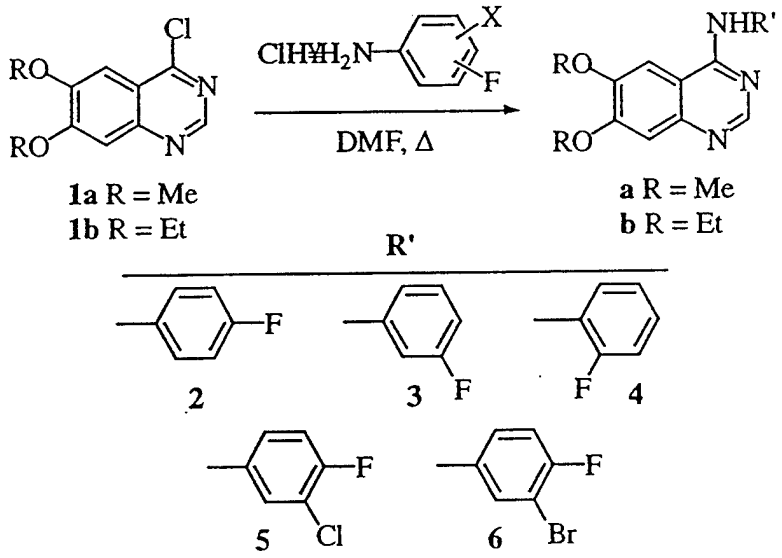


Figure 1. Synthesis of non-radioactive fluoroanilinoquinazoline analogs

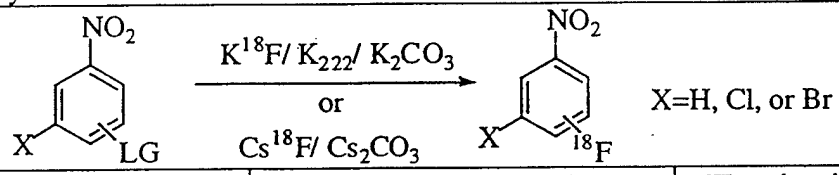
The synthetic scheme for the non-radioactive fluoroanilino-dialkoxyquinazolines is shown in Figure 1. All of the fluoroanilines were commercially available with the exception of 3-bromo-4-fluoroaniline. This was produced in 2 steps from 1-bromo-2-fluorobenzene. The chloroquinazolines were synthesized according to literature procedures (12). The coupling reactions proceeded cleanly in DMF to give the desired quinazolines in 84-91% yield.

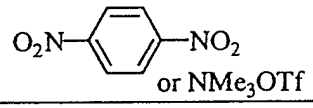
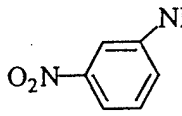
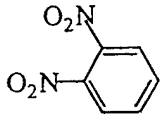
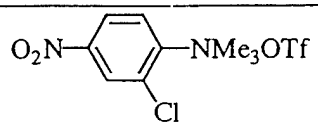
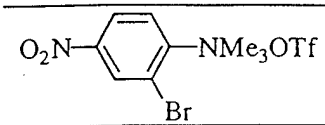
The first step in the radioactive synthesis of the quinazoline compounds is shown in Table 1. The precursors to the corresponding [^{18}F]fluoronitrobenzenes were either recrystallized (commercially available dinitrobenzenes) or were produced (trimethylammonium triflates) according to the literature procedure (13). The bromo analog was produced in 2 steps from 4-nitro-N-methylaniline. The nucleophilic [^{18}F]fluoride displacement reactions were carried out in the presence of either potassium carbonate/ kryptofix or cesium carbonate in DMSO using 1 mg of the precursor. All of the reactions gave the expected yields for [^{18}F]fluorine incorporation.

The conversion of the [^{18}F]fluoronitrobenzenes to the [^{18}F]fluoroanilines and subsequent coupling to the chloroquinazolines is shown in Figure 2. The [^{18}F]fluoronitrobenzenes are removed from the DMSO by C18 Sep Pak. The Sep Pak is eluted with 1.5 mL of methanol into a vial containing the reductants. This procedure, first reported by Feliu (14), is quantitative and complete in ten minutes at room temperature. The reaction, quenched with 0.2 mL of conc. HCl, is filtered and concentrated to dryness by heating under a stream of nitrogen or in vacuo. Up to 23% of the activity is volatilized during the solvent removal process.

A DMF solution (0.25 mL) of the desired dialkoxychloroquinazoline (2.2 mg) was added to the dry aniline•HCl and heated at 85 °C. The coupling reaction was

Table 1. Synthesis of fluoronitrobenzenes



Precursor	Fluorination Conditions	Fluorination Yield (decay corrected)
	0.5 mg K_2CO_3/ 5 mg K_{222} 130°C 10 min	76-85%
	0.5 mg K_2CO_3/ 5 mg K_{222} 130°C 10 min	38%
	0.5 mg K_2CO_3/ 5 mg K_{222} 130°C 10 min	75-90%
	$Cs^{18}F$ / 4.8 mmol Cs_2CO_3 130°C 5 min	33-80%
	$Cs^{18}F$ / 4.8 mmol Cs_2CO_3 130°C 5 min	75-80%

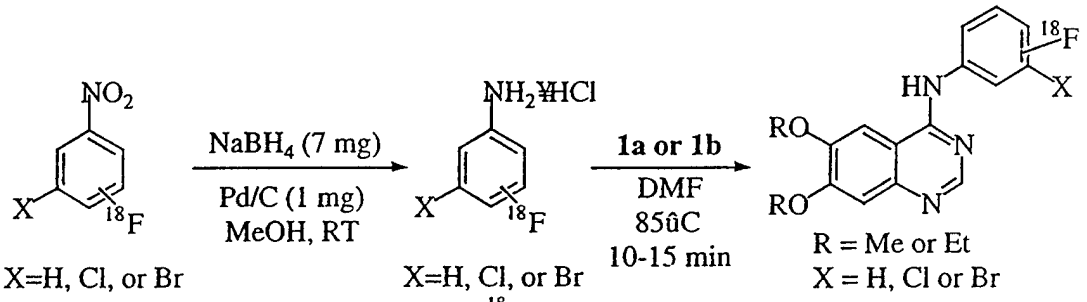


Figure 2. Synthesis of the [^{18}F]fluoroanilines and corresponding [^{18}F]fluoroanilino-dialkoxyquinazolines

Complete, as evidenced by HPLC, in 10-15 min. HPLC purification of the basified reaction (0.1 mL of 10N NaOH) gave the desired [^{18}F]fluoroanilino-dialkoxyquinazoline in ~155 min with decay corrected yields of 27-30%. Our unoptimized yields are lower than that reported by Snyder(9) for the fluorochloro-dimethoxy quinazoline, however, the 3'-[^{18}F]fluoroanilino analog was produced in 11% decay corrected yield compared to 1% reported by Mishani (7). This reduction and coupling strategy represents a general method that may be useful for the production of a variety of fluoroanilino radiopharmaceuticals.

This work was supported by the US Department of the Army grant # DAMD17-98-8064, the National Institutes of Health grant # CA79823 and the UC Breast Cancer Research Program grant # 4IB-0059.

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Appendix 9.

Abstract Submitted for the California Breast Cancer Research Symposium

Fall 2001 Oakland, CA.

Epidermal Growth Factor Imaging Agents for Breast Cancer Detection and New Therapeutic Drug Development

Henry F. VanBrocklin, Michele Y. Ono, D.L. Hom, S.E. Taylor, S.M. Hanrahan, Fred T. Chin, David J. Riese, III*

Department of Functional Imaging, Lawrence Berkeley National Laboratory, Berkeley, CA.
*Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN.

The overall goal of this project is to develop new radioactively labeled pharmaceuticals (radiopharmaceuticals) for the detection of epidermal growth factor receptors in breast cancer. These radiopharmaceuticals, when injected into a subject with breast cancer, would accumulate in the tumor based on the concentration of the epidermal growth factor receptors present in the tumor. By measuring the amount of radiopharmaceutical in the tumor using non-invasive positron emission tomography (PET) imaging, the amount of receptors in the tumor can be determined. Up to 45% of breast tumors have epidermal growth factor receptors and these tumors do not respond well to conventional treatment. Drug companies have been attempting to develop therapeutic agents to treat tumors containing epidermal growth factor receptors for several years.

We have produced over 30 new chemical compounds, based on therapeutic agents developed by Parke-Davis, suitable for radiolabeling with a positron-emitting isotope. We have evaluated their ability to recognize the epidermal growth factor receptor. All of the compounds recognize the receptor. In addition, we have developed the chemical steps to attach fluorine-18, a positron emitting isotope, to the new compounds. We have tested some of these radiopharmaceuticals in breast cancer models. We are continuing to make improvements on the imaging characteristics of these new radiopharmaceuticals.

Nuclear medicine techniques using the radiopharmaceuticals can provide valuable diagnostic information about the location and the biochemical nature of the tumor in a single study. These radiopharmaceuticals may also help in the development of new drugs to treat tumors with elevated epidermal growth factor receptors by monitoring the receptor content in tumors following a given treatment.

Journal of Labelled Compounds and Radiopharmaceuticals
J. Labelled Cpd. Radiopharm. **43**, 1183-1191 (2000)

SYNTHESIS OF 4-(3'-[^{125}I]IODOANILINO)-6,7-DIALKOXYQUINAZOLINES: RADIOLABELED EPIDERMAL GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITORS

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SUMMARY

The preparation of two radioiodinated analogs of the epidermal growth factor receptor tyrosine kinase (EGFrTK) inhibitor PD153035 (4-(3'-bromoanilino)-6,7-dimethoxyquinazoline) are reported herein. The two analogs, 4-(3'-[^{125}I]iodoanilino)-6,7-dimethoxyquinazoline and 4-(3'-[^{125}I]iodoanilino)-6,7-diethoxyquinazoline, were synthesized via iododestannylation of the corresponding 4-(3'-trimethylstannylanilino)-6,7-dialkoxyquinazolines to form the desired I-125 labeled products in good yield, high radiochemical purity (>99%) and high specific activity.

KEY WORDS: epidermal growth factor receptor, iodine-125, PD153035, quinazoline, tyrosine kinase inhibitor.

INTRODUCTION

Protein tyrosine kinases (PTKs) regulate cell division, growth and differentiation. Activation of the PTKs is one of the first steps in the signal cascade that initiates these cellular processes. The epidermal growth factor receptor (EGFr) is a member of a family of PTK-linked receptors where the tyrosine kinase domain is an integral part of the receptor. The EGFr is a 170 kD transmembrane protein possessing an extracellular ligand binding domain and an intracellular tyrosine kinase domain. The binding of EGF to the ligand domain of two adjacent receptors promotes a

Received 27 October 1999
Revised 29 November 1999
Accepted 17 July 2000

conformational change that brings the two receptors together. The dimerization activates the tyrosine kinase towards phosphorylation of tyrosine residues on the adjacent receptor (interphosphorylation) as well as phosphorylation of other enzymes, thus propagating the signal throughout the cell (1-3).

The aberrant expression and activation of growth factor receptors in normal cells has been implicated in the promotion and proliferation of malignant growths (4). EGFr overexpression has been noted in a number of human neoplastic lesions (5) including lung cancer, endometrial carcinoma and breast cancer (6). Therapeutic response and patient survival has been negatively correlated with EGFr upregulation (7, 8). Small molecules capable of selective inhibition of the EGFr activation have been the target of intense research over the last several years in an effort to develop a therapeutic antitumor drug (9-11). Based on the concentration of receptors in the tumors, growth factor receptors have been sited as potential targets for imaging as well as for radiotherapeutic agents (12).

Several classes of compounds are being investigated as tyrosine kinase inhibitors (11). One class, 4-anilinoquinazolines, has been shown to be particularly potent and selective ATP site inhibitors (13). The most potent member of this class, 4-(3'-bromoanilino)-6,7-dimethoxyquinazoline (PD153035, **1**), inhibits EGFr phosphorylation with a K_i of 5 pM and has demonstrated selectivity with only mM inhibition of other growth factor receptors (14).

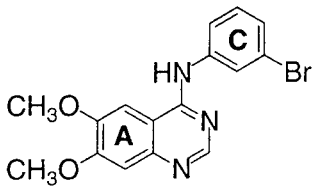

Figure 1. 4-(3'-bromoanilino)-6,7-dimethoxyquinazoline, PD153035, **1**.

Several investigators have initiated programs to develop imaging agents based on the small molecule EGFr inhibitors. A number of radiolabeled analogs of PD153035 have been reported in prefatory communications. The compounds incorporate labeled substituents on the A or C rings of the anilino- or benzylamino- quinazoline (Figure 1). The C ring substituted analogs include 4-(3'-[^{125}I]iodoanilino)- (15), 4-(3'-[^{18}F]fluoro-5'-trifluoromethylanilino)- (16), 4-(3',4'-dichloro-6'-[^{18}F]fluoro-anilino)- (16), 4-(4'-[^{18}F]fluorobenzylamino)-dimethoxyquinazolines (17) and 4-(4'-[^{18}F]fluorobenzylamino)-diethoxyquinazoline (18). The 7-[^{18}F]fluoroethoxy- (19) and the 6- or 7-[^{11}C]methoxy- (19, 20) constitute the A ring labeled analogs. Preliminary in vitro studies with the 3'-[^{125}I]iodo analog demonstrated receptor mediated uptake in cells containing high EGFr titer (15). A more recent study of the ^{11}C-methoxy derivative demonstrated some uptake in human neuroblastoma xenographs in mice (21, 22). While neither of these studies were unequivocal, the evidence suggests that further studies towards the development of in vivo imaging agents for EGFr expression in tumors is warranted.

As part of an ongoing effort to develop positron-emitting EGFr imaging agents in our laboratory, we sought to produce a labeled compound for use in radiometric binding studies. To this end we report herein the detailed synthesis of two iodine-125 labeled analogs of PD153035.

RESULTS AND DISCUSSION

The two iodinated analogs presented were chosen for labeling based on known structure activity relationships derived from the inhibition of EGFr tyrosine kinase activity by numerous analogs of PD153035 produced by Parke-Davis (23). The investigators found that replacing the bromine on PD153035 with iodine increased the IC_{50} value 35 times, albeit the iodo compound still retained subnanomolar inhibition of the EGFr tyrosine kinase (IC_{50} = 0.89 nM). Replacement of the 6 and 7 methoxy moieties with 6 and 7 ethoxy groups decreased the IC_{50} of PD153035 nearly 5 fold, 0.025 nM to 0.006 nM. Based on these data we prepared 3'-iodoanalino-6,7-diethoxyquinazoline and labeled it with ^{125}I in addition to the corresponding 6,7-dimethoxy analog.

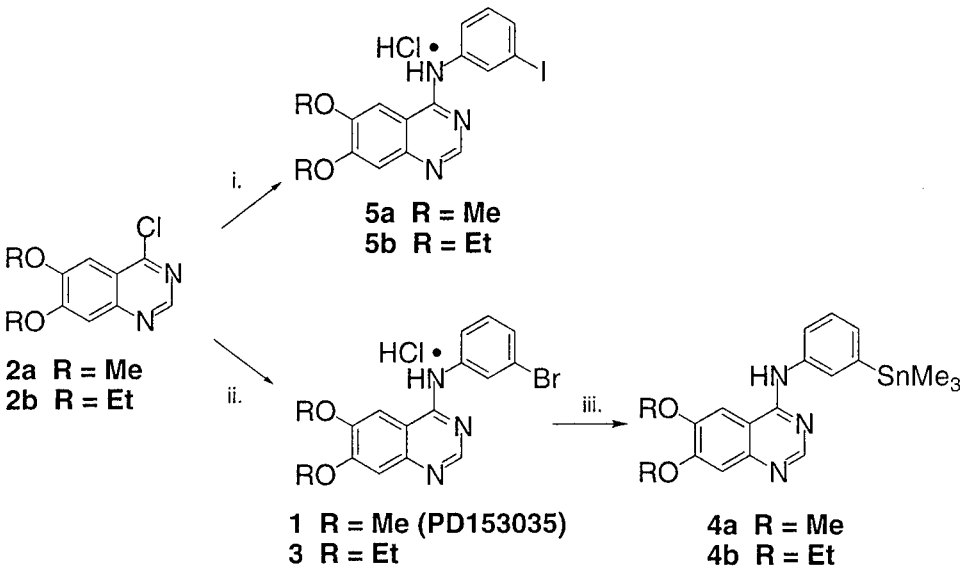

Figure 2. Synthetic routes for the iodoanilino- and trimethylstannylanilino-dialkoxyquinazolines. (i.) 3-iodoaniline, DMF, Δ; (ii.) 3-bromoaniline, DMF, Δ; (iii.) $(SnMe_3)_2$, $Pd(Ph_3)_4$, dioxane.

The synthetic route for the preparation of the analinoquinazolines is outlined in Figure 2. The common intermediates, 4-chloro-6,7-dimethoxy- **2a** and 4-chloro-6,7-diethoxyquinazoline **2b**, were used to prepare the nonradioactive iodine compounds and the trialkylstannyl precursors for labeling. The procedure outlined by Bridges *et al.* (23) was followed for the synthesis of **2a** starting from the

commercially available dimethoxyanthranilic acid. The corresponding diethoxyanthranilic acid was not commercially available; however, it was produced in one step by the saponification of the available 2-amino-4,5-diethoxy-methyl benzoate (24). The synthesis of **2b** proceeded analogously to that of **2a**.

The bromo- and iodo-anilino analogs, **1**, **3**, **5a** and **5b**, were produced by heating the appropriate bromo- or iodo-aniline with the respective chloroquinazoline, **2a** or **2b**, in anhydrous DMF. This reaction proceeded very cleanly and gave high yields (84-91%) of the haloanilino compounds. This compares favorably to the reactions reported by Bridges *et al.* (23) where they used isopropanol as the solvent. The bromoanilino compounds, **1** and **3**, were converted into the corresponding trimethylstannyl derivatives by reacting with hexamethylditin in the presence of a catalytic amount of tetrakis(triphenylphosphine)palladium(0) in 55-60% yield.

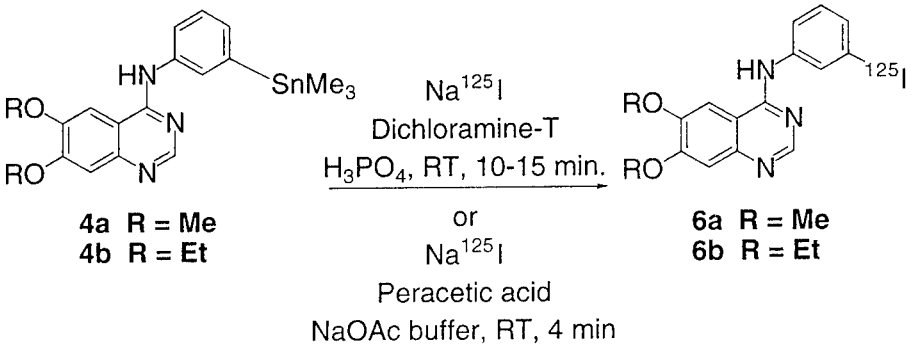

Figure 3. Radiosynthesis of 3'-[^{125}I]iodoanilino-quinazoline analogs of **6a** and **6b**.

The conversion of the stannylated compounds to the desired ^{125}I labeled quinazolines by two different methods is shown in Figure 3. The iododestannylation reaction proceeded rapidly, less than 15 minutes using dichloramine-T and less than 5 minutes using peracetic acid. The original radioidinations were carried out following an old Berkeley dichloramine-T procedure. Recently, we converted to the peracetic acid method. Purification by solid phase extraction (reversed-phase, C18) and HPLC gave either **6a** or **6b** in 40-65% radiochemical yield. The yields for the peracetic acid reactions were about 50% higher than the dichloramine-T reactions. The radiochemical purity of **6a** and **6b** was greater than 98% confirmed by normal phase TLC and coinjection with cold standard on reversed phase analytical HPLC (multiple solvent systems). Using either method, only one major radioactive product was identified on the HPLC; no other mono- or di-substituted species were formed in the reaction.

The specific activities of **6a** and **6b** ranged from 466-1900 Ci/mmol and the values were found to be independent of the method used to produce the labeled products. An HPLC chromatogram (Figure 4) of the crude **6a** dichloramine-T reaction mixture shows the separation between the radioiodinated material and the non-radioactive 3'-chloroanilinoquniazoline mass peak. The chloroanilinoquinazoline

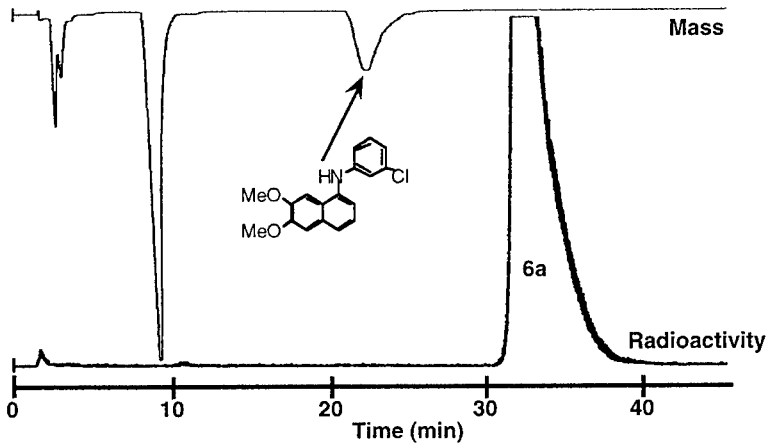

Figure 4. HPLC chromatogram of **6a** produced by the dichloramine-T method. (conditions: analytical C18, 50:50 MeOH:H_2O, pH 7.4, flow 2 mL/min)

byproduct is not present in the peracetic acid produced radiotracer as expected. Comparing the two iodination methods, the peracetic acid reaction is more favorable for this chemistry.

EXPERIMENTAL

All chemicals were purchased from Aldrich Chemical Co. and were used without further purification. The 2-amino-4,5-diethoxy-methyl benzoate was purchased from Aldrich Specialty Chemicals. Chloroquinazolines **2a** and **2b** were prepared according to literature procedures (23,24). NMR spectra were obtained on a Bruker VBAMX400 400 MHz spectrometer. Elemental analyses were performed by the Microanalytical Laboratory at the College of Chemistry, University of California, Berkeley. Melting points were taken on a Mel-Temp™ apparatus and are reported uncorrected. Mass spectra were obtained on a Perkin Elmer SCIEX spectrometer at the SynPep Corporation, Dublin, CA, USA. Purification of the radioiodinated compounds was carried out by HPLC (column and conditions noted below in the synthesis section) with an in-line Linear™ UV-106 spectrophotometer to detect mass and a NaI detector and Ortec NIM components to measure the radioactivity.

4-(3'-bromoanilino)-6,7-dimethoxyquinazoline (1).

The preparation of **1** followed the method described below for **3**. Yield 87%. ^{1}H NMR spectra were identical to those reported in the literature (23).

4-(3'-bromoanilino)-6,7-diethoxyquinazoline (3).

A clear, pale yellow anhydrous DMF solution (3 mL) of 4-chloro-6,7-diethoxyquinazoline **2b** (0.1 g, 0.396 mmol) was combined with 3-bromoaniline

(64.6 μL, 0.593 mmol) to form a clear, pale pink solution. Within 15 minutes of heating the reaction flask at 80° C under argon, precipitation of a white solid was observed. The heterogeneous solution was heated for an additional 45 minutes, then cooled to room temperature for 15 minutes. The solid was filtered and washed with ethyl acetate (20 mL) to give the hydrochloride salt of **3** as a bright white, pulpy solid. Yield 0.15 g (89%). m.p. 260° C. ^{1}H NMR ($CDCl_3$): δ 8.63 (*s*, 1H, Ar*H*), 7.88 (*s*, 1H, Ar*H*), 7.87 (*s*, 1H, Ar*H*), 7.59, (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.38 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.31 (*t*, 1H, ArH, J = 8.0 Hz), 4.18 (*m*, 4H, OCH_2CH_3), 1.42 (*m*, 3H, OCH_2CH_3). APCI mass spec. 388.1, 390.1 [M+1]. Elemental analysis $C_{18}H_{19}BrClN_3O_2$ calcd. C 50.90, H 4.51, N 9.89; found C 49.32, H 4.66, N 9.44.

4-(3'-trimethylstannylanilino)-6,7-dimethoxyquinazoline (4a).

The preparation of **4a** starting from 40 mg (0.11 mmol) of the free base of **1** was carried out in a manner analogous to **4b**. Yield 30 mg (61%). ^{1}H NMR ($CDCl_3$): δ 8.64 (*s*, 1H, Ar*H*), 7.73 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.62 (*s*, 1H, Ar*H*), 7.40 (*t*, 1H, Ar*H*, J = 8.0 Hz), 7.28 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.26 (*s*, 1H, Ar*H*), 7.02 (*s*, 1H, Ar*H*), 4.02 (*s*, 6H, OCH_3), 0.30 (*s*, 9H, $Sn(CH_3)_3$). APCI mass spec. 446.1, 444.0, 442.1 [M+1]. Elemental analysis $C_{19}H_{23}N_3O_2Sn$ calcd. C 51.38, H 5.22, N 9.46; found C 51.59, H 5.47 N 9.12.

4-(3'-trimethylstannylanilino)-6,7-diethoxyquinazoline (4b).

The hydrochloride salt of **3** was first converted to the free base by partitioning the solid between ethyl acetate (3 mL) and 1*N* sodium hydroxide (2 mL). After thoroughly shaking the two layers, the ethyl acetate layer was separated and the aqueous base solution was extracted with additional ethyl acetate (2 x 3 mL). The ethyl acetate fractions were pooled, dried over magnesium sulfate, filtered, and solvent removed in vacuo to give an oil, then were recrystallized from diethyl ether to give white crystals (yield 60 mg, 66%). A toluene solution (5 mL) containing the free base **3** (75 mg, 0.493 mmol), hexamethylditin (48 μL, 0.592 mmol) and a catalytic amount of tetrakis(triphenylphosphine)palladium(0) (22.3 mg, 10 mol%) was heated at 105° C under argon for 16 hours, resulting in an intense, dark black solution. The palladium catalyst was removed by eluting through a short silica pad to give a clear yellow solution. The solution was concentrated in vacuo, then purified by radial chromatography (2 mm silica plate thickness; eluted first with hexanes, then gradually adjusted to 30% ethyl acetate/hexane, and finally to 100% ethyl acetate). Purity was confirmed by a single spot in TLC (silica, 75% ethyl acetate/hexane, R_f 0.55). Yield 50 mg (56%). ^{1}H NMR ($CDCl_3$): δ 8.62 (*s*, 1H, Ar*H*), 7.75 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.61 (*s*, 1H, Ar*H*), 7.39 (*t*, 1H, Ar*H*, J = 8.0 Hz), 7.27 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.23 (*s*, 1H, Ar*H*), 7.02 (*s*, 1H, Ar*H*), 4.22 (*m*, 4H, OCH_2CH_3), 1.55 (*m*, 6H, OCH_2CH_3), 0.30 (*s*, 9H, $Sn(CH_3)_3$). APCI mass spec. 474, 472.1, 470 [M+1]. Elemental Analysis $C_{21}H_{27}N_3O_2Sn$ calcd. C 53.42, H 5.76, N 8.90; found C 53.76, H 6.06, N 8.54.

4-(3'-iodoanilino)-6,7-dimethoxyquinazoline (5a).

The preparation of **5a** followed the method described above for **3**. The chloroquinazoline **2a** (0.28 g, 1.22 mmol) was reacted with 1.5 equiv. of iodoaniline to yield 0.5 g (92%) of **5a**. m.p. 252-253° C. ^{1}H NMR (DMSO): δ 7.91 (*s*, 1H, Ar*H*), 7.31 (*s*, 1H, Ar*H*), 7.16 (*s*, 1H, Ar*H*), 6.80 (*d*, 1H, Ar*H*, J = 8.0 Hz), 6.70 (*d*, 1H, Ar*H*, J = 8.0 Hz), 6.36 (*s*, 1H, Ar*H*), 6.32 (*t*, 1H, Ar*H*, J = 8.0 Hz), 3.05 (*s*, 3H, OCH_3), 3.03 (*s*, 3H, OCH_3). APCI mass spec. 408.0 [M+1]. Elemental Analysis $C_{16}H_{15}ClIN_3O_2$ calcd. C 43.31, H 3.41, N 9.47; found C 43.66, H 3.45, N 9.51.

4-(3'-iodoanilino)-6,7-diethoxyquinazoline (5b).

The preparation of **5b** followed the method described above for **5a**. The chloroquinazoline **2b** (0.12 g, 0.46 mmol) was reacted with 1.5 equiv. of iodoaniline to yield 0.19 g (88%) of **5b**. m.p. 265-266° C. ^{1}H NMR (DMSO): δ 8.86 (*s*, 1H, Ar*H*), 8.20 (*s*, 1H, Ar*H*), 8.10 (*s*, 1H, Ar*H*), 7.75 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.67 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.30 (*s*, 1H, Ar*H*), 4.27 (*m*, 4H, OCH_2CH$_3$), 1.44 (*m*, 6H, OCH$_2$CH_3). APCI mass spec. 436.0 [M+1]. Elemental analysis $C_{18}H_{19}ClIN_3O_2$ calcd. C 45.83, H 4.06, N 8.91; found C 46.11, H 3.45, N 8.85.

4-(3'-[^{125}I]iodoanilino)-6,7-dimethoxyquinazoline (6a) or 4-(3'-[^{125}I]iodoanilinio)-6,7-diethoxyquinazoline (6b).

Dichloramine-T Method: A 4 mL vial equipped with a Teflon cap and a stir bar was charged with the 3'-trimethylstannylanilino-6,7-dialkoxyquinazoline precursor **4a** (~0.5 mg, 1.1 μmol) or **4b** (~0.5 mg, 1.1 μmol) in acetonitrile (200 μL). To the vial was added a solution of Na^{125}I (~1 mCi) followed by the addition of 85% phosphoric acid (50 μL) and dichloramine-T (20 μL of a 2 mg/mL solution in acetonitrile). After reacting at room temperature for 10-15 minutes, the mixture was quenched with 10% $Na_2S_2O_5$ (50 μL). The quenched solution was diluted with 5-10 mL of deionized H_2O, then eluted through an activated C18 Sep-Pak® trapping >95% of the measured activity. The cartridge was then eluted with 0.2 mL of methanol, then an additional 1.6 mL of methanol. The second elution fraction containing the bulk of the activity was slowly evaporated under a gentle stream of argon. The residue was dissolved in 2 mL of a buffered methanol/water solution (phosphoric acid/triethylamine) and chromatographed by HPLC (Waters μBondapak™ C18 column, 3.9 x 300 mm, 50% MeOH/water final pH 7.40, flow = 2 mL/min, retention time **6a**: 25-34.5 min or **6b**: 53-70 min.). The product was concentrated by trapping on an activated C18 Sep-Pak®. **6a** (0.5 mCi, 53% yield) or **6b** (0.45 mCi, 45% yield) was collected in 1 mL of methanol eluent.

Peracetic Acid Method: A 4 mL vial equipped with a Teflon cap and a stir bar was charged with the 3'-trimethylstannylanilino-6,7-dialkoxyquinazoline precursor **4a** (~0.5 mg, 1.1 μmol) or **4b** (~0.5 mg, 1.1 μmol) in acetonitrile (200 μL). To this vial was added sodium acetate buffer (0.6M, pH 4.5, 100 μL) and Na^{125}I followed by peracetic acid (30 μL, Aldrich). The reaction was stirred for 4 min. at room

temperature and then quenched with 10% $Na_2S_2O_5$ (100 μL). The solution was made basic with saturated $NaHCO_3$ and transferred to a syringe. The reaction vial was rinsed with acetonitrile (100 μL) and added to the syringe along with 8 mL of water. The solution was passed through an activated C18 Sep-Pak® and the trapped radioiodinated product was eluted with 3 mL of MeOH and concentrated for HPLC purification (as noted above). The yield of **6a** and **6b** using this method was 60-65%.

Specific Activity Measurement: The purified labeled products, **6a** and **6b**, were concentrated and analyzed by reversed phase analytical HPLC (Phenomenex Bondclone™ 10 C18 column, 3.9 x 300 mm, 50-80% MeOH/water final pH 7.40, flow = 2 mL/min). The HPLC derived specific activity was calculated based on the activity injected and the mass/UV response measured relative to a standard curve.

CONCLUSION

We have successfully labeled two EGFr tyrosine kinase inhibitors with iodine-125 using two different synthetic methods. The peracetic acid method produced slightly higher yields of cleaner product in a one half to one third the time and is the preferred method for producing these compounds in the future. The compounds were produced in sufficiently high yield, high radiochemical purity and high specific activity. Both these compounds demonstrated receptor mediated affinity for the EGFr tyrosine kinase binding site in initial binding studies. One of these compounds may find utility in the radiometric binding assay we are developing for the measurement of the receptor binding affinity of novel EGFr inhibitors.

ACKNOWLEDGMENTS

This work was supported in part by the Director, Office of Science, Office of Biological and Environmental Research, Medical Science Division of the U.S. Department of Energy under contract no. DE-AC03-76SF00098 and by the Army Medical Research and Materiel Command, U.S. Department of Defense under grant no. DAMD17-98-1-8064 (HFV). Support for Dr. Lim was provided by NIH training grant no. NCI T32CA66527.

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