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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.

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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.

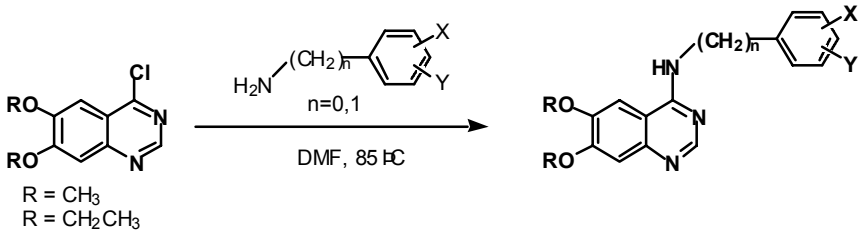

Figure 1. Synthesis of substituted benzyl- and anilino-dialkoxyquinazolines.

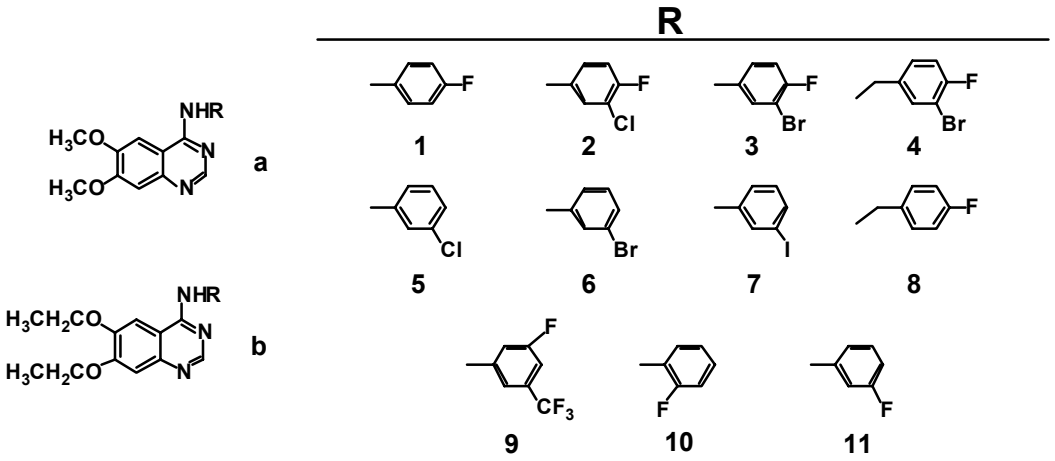

Figure 2. Dialkoxyquinazoline analogs prepared as part of this effort.

Appendix 1. cont.

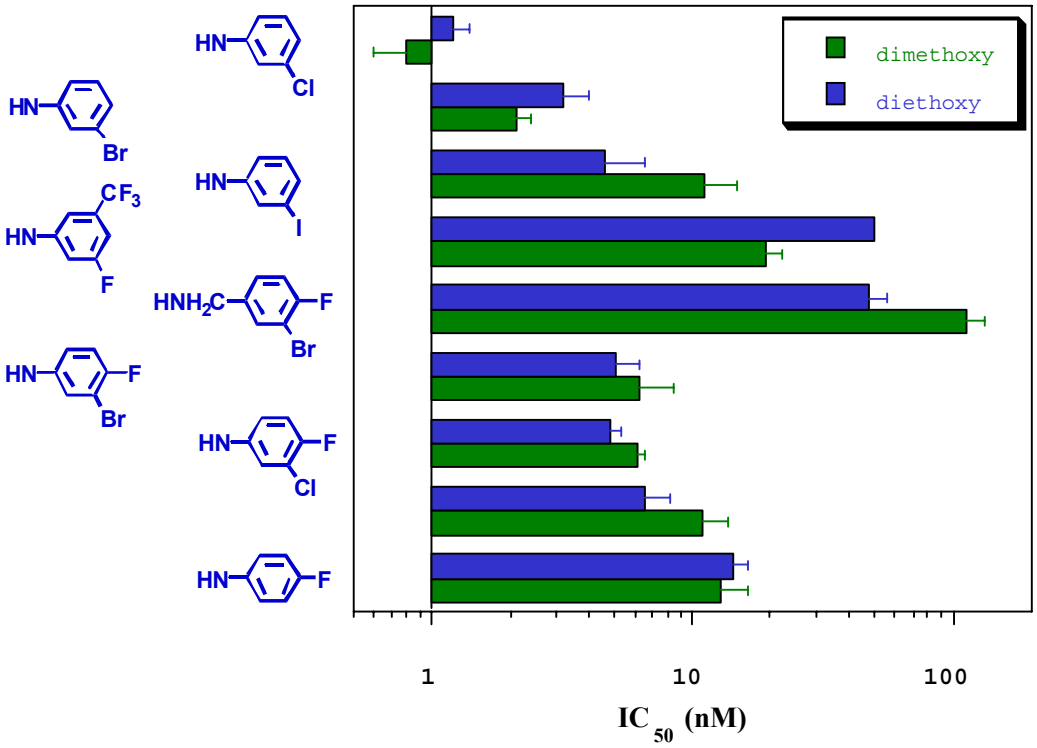

Figure 3. Inhibition of EGFR tyrosine kinase phosphorylation.

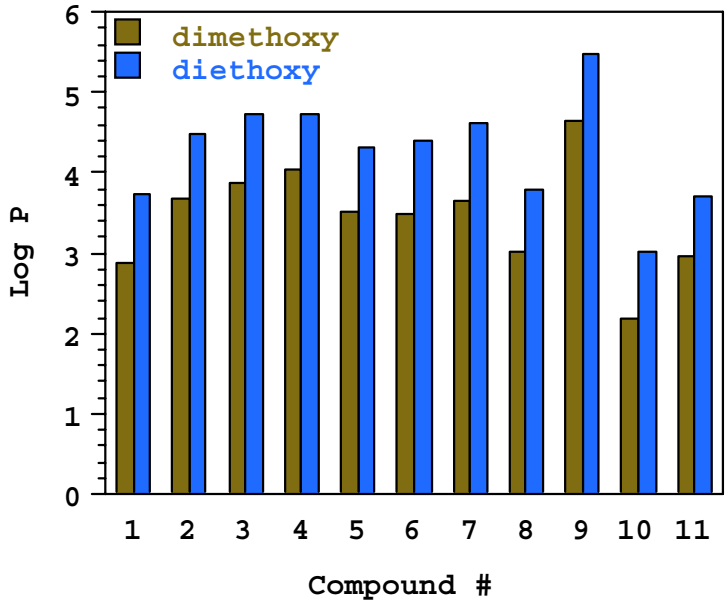

Figure 4. Log P estimation by HPLC for the quinazoline compounds

Appendix 1. cont.

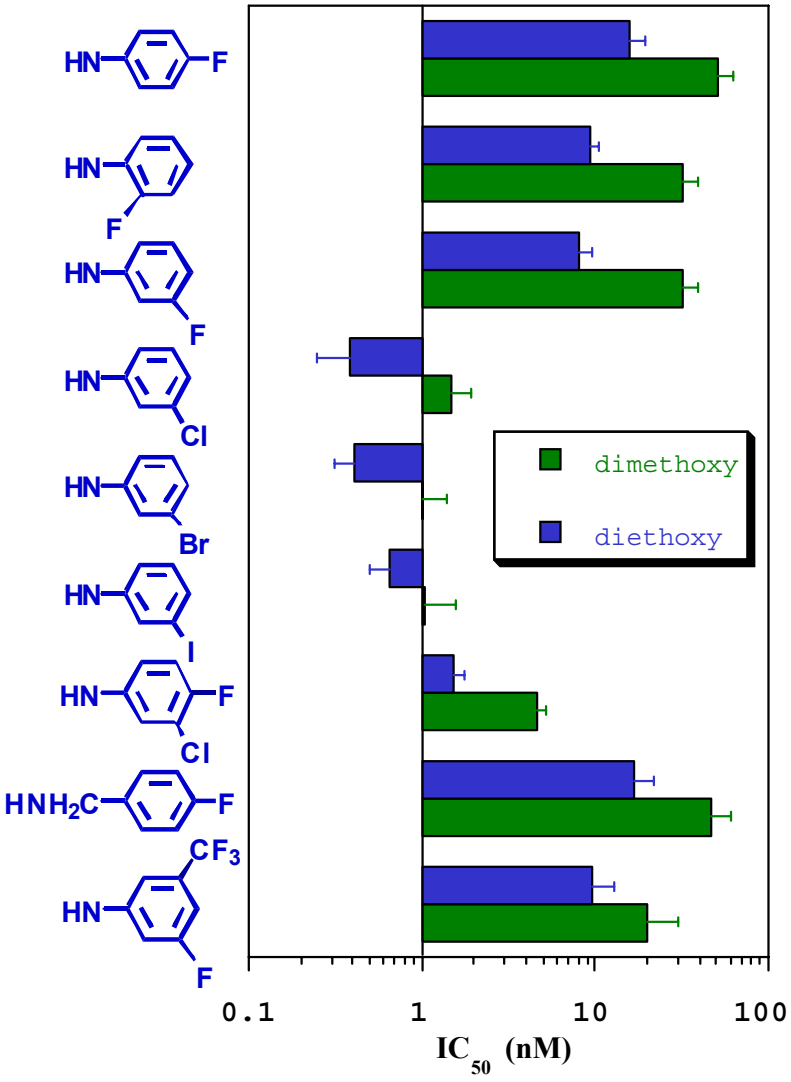

Figure 5. EGF Receptor binding affinity of the quinazoline compounds.

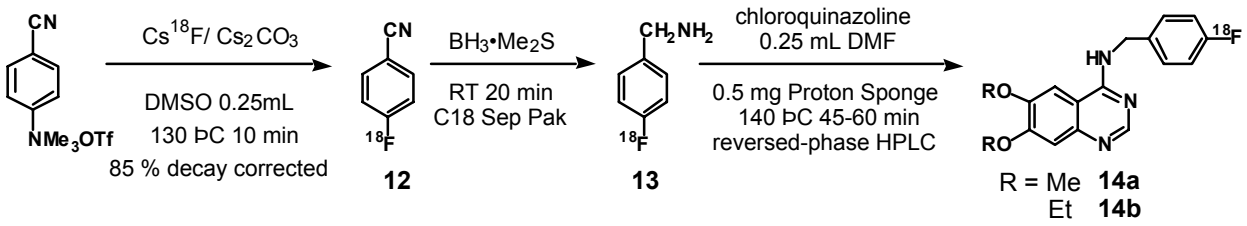

Figure 6. Synthesis of 4-(4'-[^{18}F]fluorobenzylamino)-6,7-dimethoxy- and

4-(4’-[^{18}F]fluorobenzylamino)-6,7-diethoxy- quinazoline

Appendix 1. cont.

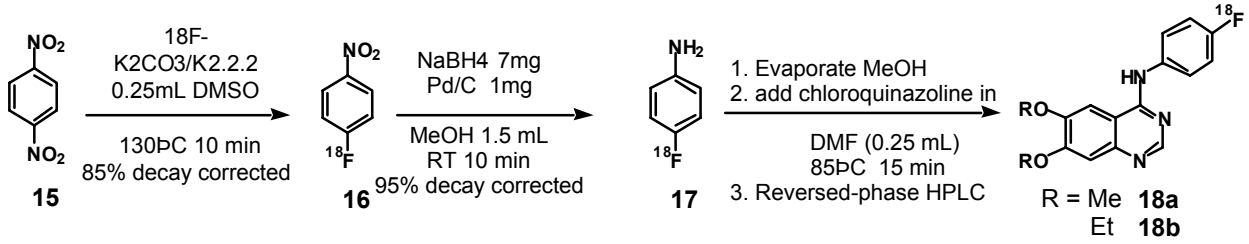

Figure 7. Synthesis of 4-(4’-[^{18}F]fluoroanalino)-6,7-dimethoxy- and 4-(4’-[^{18}F]fluoroanalino)-6,7-diethoxy- quinazoline.

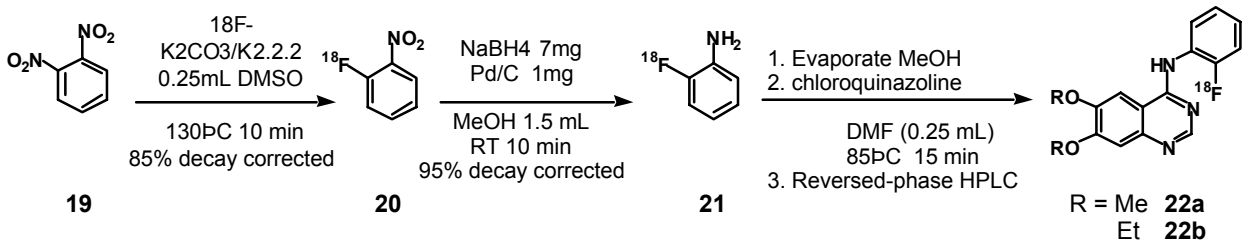

Figure 8. Synthesis of 4-(2'-[^{18}F]fluoroanalino)-6,7-dimethoxy- and 4-(2’-[^{18}F]fluoroanalino)-6,7-diethoxy- quinazoline

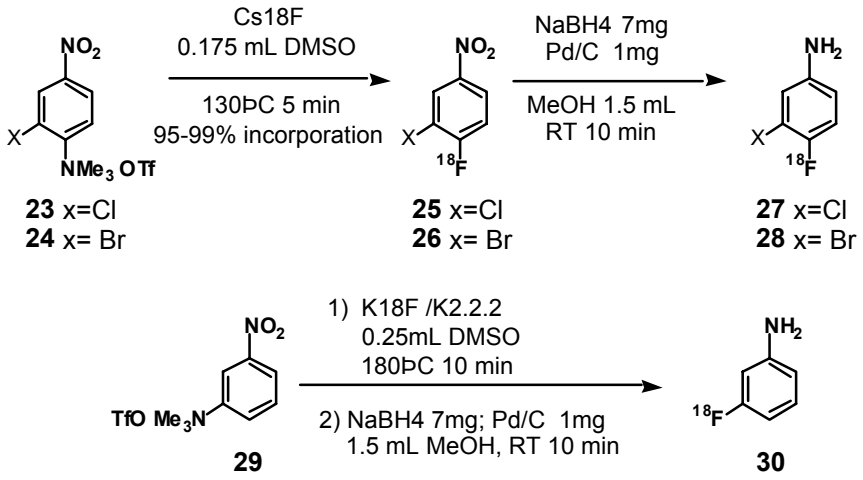

Figure 9. Synthesis of [^{18}F]fluoroanilines

Appendix 1. cont.

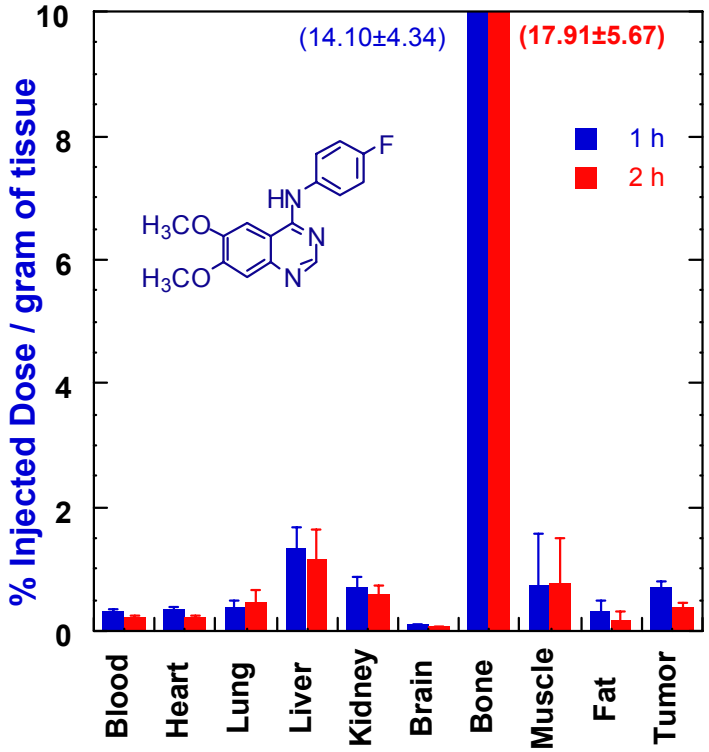

Figure 10. In vivo distribution of **18a** in MDA-468 tumor bearing mice.

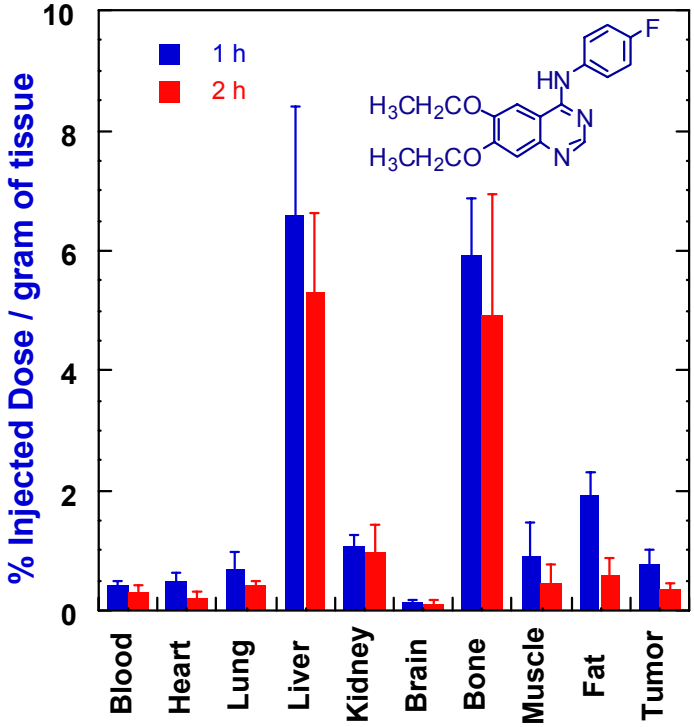

Figure 11. In vivo distribution of **18b** in MDA-468 tumor bearing mice.

Appendix 1. cont.

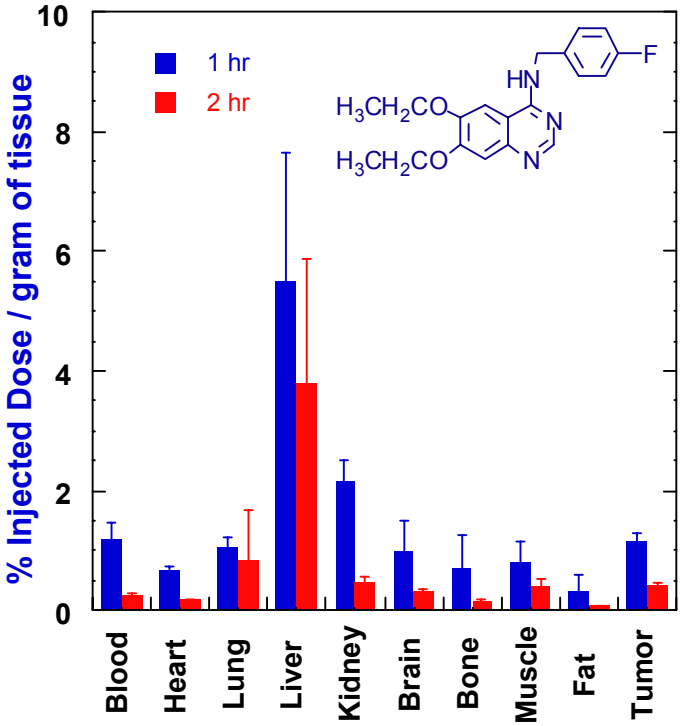

Figure 12. In vivo distribution of **14b** in MDA-468 tumor bearing mice.

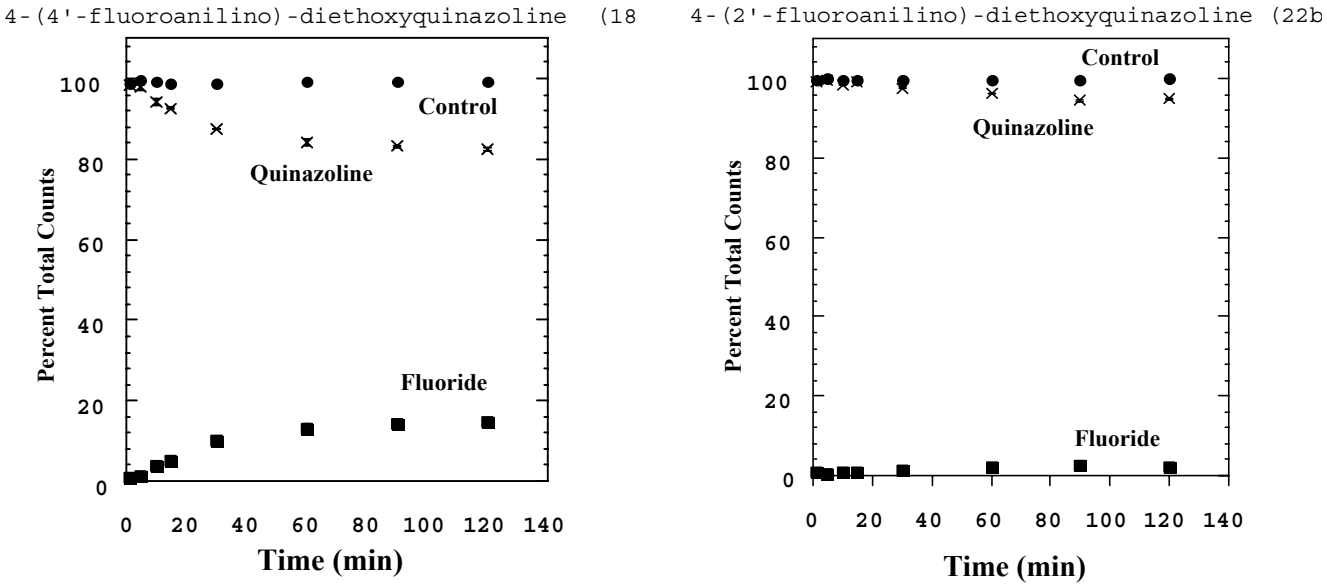

Figure 13. Hepatocyte metabolism kinetic profile of para- (**18b**) versus ortho- (**22b**) [^{18}F]fluoroanilinodiethoxyquinazoline analogs. Control contained no hepatocytes.