In silico design, synthesis, and biological evaluation of radioiodinated quinazolinone derivatives for alkaline phosphatase–mediated cancer diagnosis and therapy


1Department of Radiology, Harvard Medical School, Boston, Massachusetts and 2Bauer Center for Genomics Research, Harvard University, Cambridge, Massachusetts

Abstract

As part of the development of enzyme-mediated cancer imaging and therapy, a novel technology to entrap water-insoluble radioactive molecules within solid tumors, we show that a water-soluble, radioactive quinazolinone prodrug, ammonium 2-(2'-phosphoryloxyphenyl)-6-[125I]iodo-4-(3'H)-quinazolinone ([125I]IQ2-P), is hydrolyzed by alkaline phosphatase to a water-insoluble, radiolabeled drug, 2-(2'-hydroxyphenyl)-6-[125I]iodo-4-(3'H)-quinazolinone ([125I]IQ2-OH). Biodistribution data suggest the existence of two isoforms of the prodrug (IQ2-P(I) and IQ2-P), and this has been confirmed by their synthesis and characterization. Structural differences of the two isoforms have been examined using in silico molecular modeling techniques and docking methods to describe the interaction/binding between the isoforms and human placental alkaline phosphatase (PLAP), a tumor cell, membrane-associated, hydrolytic enzyme whose structure is known by X-ray crystallographic determination. Docking data show that IQ2-P, but not IQ2-P(I), fits the active binding site of PLAP favorably and interacts with the catalytic amino acid Ser92, which plays an important role in the hydrolytic process. The binding free energies (\(\Delta G_{\text{binding}}\)) of the isoforms to PLAP predict that IQ2-P will be the better substrate for PLAP. The in vitro incubation of the isoforms with PLAP leads to the rapid hydrolysis of IQ2-P only and confirms the \(\Delta G_{\text{binding}}\) expectations. Fluorescence microscopy shows that in vitro incubation of IQ2-P with mouse and human tumor cells causes the extracellular, alkaline phosphatase–mediated hydrolysis of the molecule and precipitation of fluorescent crystals of IQ2-OH. No hydrolysis is seen in the presence of normal mouse and human cells. Furthermore, the intratumoral injection of [125I]IQ2-P into alkaline phosphatase–expressing solid human tumors grown s.c. in nude rats results in efficient hydrolysis of the compound and retention of \(\sim 70\%\) of the injected radioactivity, whereas similar injection into normal tissues (e.g., muscle) does not produce any measurable hydrolysis (\(\sim 1\%\)) or retention of radioactivity at the injected site. These studies support the enzyme-mediated cancer imaging and therapy technology and show the potential of such quinazolinone derivatives in the in vivo radio-detection \(\sim 123\text{I}/124\text{I}\) and therapy \(\sim 131\text{I}\) of solid tumors.

Introduction

Our laboratory is developing a novel technology [enzyme-mediated cancer imaging and therapy (EMCIT)] that aims to concentrate radioactive molecules within solid tumors (Fig. 1; refs. 1, 2). In one of its embodiments, a radioactive prodrug is hydrolyzed to its water-insoluble form by an enzyme that is specifically overexpressed on the exterior surfaces of tumor cell plasma membranes. On enzymatic hydrolysis, the water-soluble molecule loses its prosthetic group and the resulting compound is water-insoluble and precipitates. The precipitated molecules are concentrated and permanently trapped within the extracellular spaces of targeted solid tumors. The substrates can be radiolabeled with \(\gamma\)-emitting diagnostic (e.g., \(\text{123I}\)) and energetic electron-emitting therapeutic (\(\text{131I}\)) radionuclides. Once precipitated and trapped within the tumor, the prolonged residence time of the drug should permit the noninvasive detection and therapy of tumors.

Well-curated, web-based, protein databases provide annotation that can be methodically mined to derive subsets of well-studied proteins with defined characteristics. Because EMCIT technology depends on the overexpression of an extracellular hydrolytic enzyme, we carried out systematic and thorough searches [UniProt (Universal Protein Resource), SRS (Sequence Retrieval System), and InterPro (database of protein families, domains, and functional sites)] for enzymes that (a) have hydrolytic activity, (b) are either expressed extracellularly or attached to the plasma membrane by glycosylphosphatidylinositol anchors, and (c) are known/thought to be
active in cancer. Using this approach, alkaline phosphatase, a hydrolytic enzyme overexpressed on the plasma membranes of many types of tumor cells (3–11), was identified.

In 2002, we reported (2) the synthesis and characterization of ammonium 2-(2-¶-phosphoryloxyphenyl)-6-iodo-4-(3H)-quinazolinone (IQ₂_P), 125IQ₂_P, and 2-(2-¶-hydroxyphenyl)-6-iodo-4-(3H)-quinazolinone (IQ₂_OH). We showed that the prodrug (IQ₂_P) is a nonfluorescent, water-soluble compound that is hydrolyzed by alkaline phosphatase to the highly fluorescent, water-insoluble IQ₂_OH analogue. We also assessed the biodistribution of 125IQ₂_P in normal mice, observing that, 24 h after i.v. injection, most tissues have approximately 0.5% to 2% injected dose per gram (% ID/g), whereas higher activity (~5–7% ID/g) occurs in normal liver and kidneys.

Recently, on reevaluating biodistribution data from repeated studies, we observed that the pharmacokinetics of 125IQ₂_P in mice were sometimes very different. On investigation, we learned (3) that the synthesis of the ammonium 2-(2-¶-phosphoryloxyphenyl)-6-tributylstannyl-4-(3H)-quinazolinone (SnIQ₂_P) precursor of IQ₂_P produces a mixture of two compounds, SnIQ₂_P(I) (5; molecular weight, 590) and SnIQ₂_P (6; molecular weight, 609), whose radioiodination leads to the formation of 125IQ₂_P(I) (7) and 125IQ₂_P (8), respectively (Fig. 2). Further studies have shown that IQ₂_P(I) (10) is not hydrolyzed by alkaline phosphatase and the injection of 125IQ₂_P(I) (7) into normal mice leads to the localization of approximately 10% to 15% ID/g in liver and kidneys. (3) On the other hand, IQ₂_P (4) and 125IQ₂_P (8) are readily hydrolyzed by alkaline phosphatase and their pharmacokinetics in normal mice following i.v. injection show minimal retention in all normal tissues (0.01–0.4% ID/g at 24 h after injection). (3) These findings indicate that the compound has minimal affinity to normal cells and is not significantly hydrolyzed by them.

To explore the enzyme–substrate structural activity of the two prodrug isoforms, in silico molecular modeling studies have been done. This is possible because the X-ray crystal structure of placental alkaline phosphatase (PLAP) has been elucidated with 1.8 Å resolution (12). Structures of the isoforms were thus built and docked onto PLAP, the prodrug–PLAP complex was minimized, and the binding free energy (ΔGbinding) and inhibition constant (Kᵢ) were estimated. Our results indicate that these in silico procedures are effective tools for use in the development of radiopharmaceuticals with characteristics advantageous for noninvasive, site-specific, alkaline phosphatase–mediated insolubilization of a radioactive prodrug for diagnosis (123I/124I-labeled) and therapy (131I-labeled) of solid tumors.

Materials and Methods

Bioinformatics (Data Mining)

Three protein databases (i.e., UniProt, SRS, and InterPro) were thoroughly mined for enzymes having the following two characteristics: (a) they are either expressed on the cell surface or attached to the membrane by glycosylphosphatidylinositol anchors (a variety of proteins use glycosylphosphatidylinositol to link them to the cell surface) and (b) they are, or are suspected of being, involved in cancer. The Protein Data Bank (PDB) further identified whether the three-dimensional structure of the protein or the catalytic part of the enzyme has been resolved by crystallography or nuclear magnetic resonance.

Computational Methodology

Data Set of PLAP and Ligands. Three-dimensional coordinates of the PLAP structure were obtained from the PDB (code 1EW2; ref. 12). The PLAP dimer structure was produced according to the symmetry of atom coordination. The necessary repairing of missing atoms of residues was done in DeepView Swiss-PdbViewer. (4) The two zinc ions and one magnesium ion at the active site were

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retained, whereas the heteroatoms, including cofactors and phosphate, were removed. All bound water molecules, except the five involved in interactions between phosphate and PLAP (WAT 1, WAT 71, WAT 110, WAT 315, and WAT 421), were discarded. Polar hydrogens were added and the united partial charges for PLAP atoms were taken from the Amber force field in the Insight II package (Accelrys, Inc., San Diego, CA). The atomic solvation parameters were assigned to PLAP using the AutoDock module Addsol. All the residues and charges were visually inspected to insure consistency and reasonableness of assignment. All histidines were allocated as singly protonated. Consequently, two zinc-coordinated histidines were protonated on H6, whereas other histidines were protonated on He. The amino acid side chains of arginine, lysine, aspartate, and glutamate residues were treated as ionized. CAChe molecular modeling software (Fujitsu America, Inc., Beaverton, OR) was used to build the IQ2_{p(I)} (10) and IQ2_{P} (4) ligand structures (Fig. 2). To explore the possible low-energy conformations, the ligands were energy minimized by applying the PM5 method (MOPAC module in CAChe). The partial atomic charges were calculated, and all partial charges were further modified using the AutoDockTools package so that the charges of the nonpolar hydrogen atoms were assigned to the atom to which the hydrogen was attached. Both ligands were used in the charged form (i.e., the phosphates were deprotonated and all their torsion angles were defined so that they could be explored during molecular docking).

Docking Protocol of PLAP and Ligands. The AutoDock 3.0 program (13) was employed for automated molecular docking. The active site of PLAP was defined using the AutoGrid module in AutoDock. The grid site was constrained to a 24.75 Å cubic space centered on the original phosphate in the crystal structure. This selected grid box included the entire binding site of PLAP and provided sufficient space for ligand translational and rotational walk. The default variables of the AutoDock 3.0 program were used, except that the maximum number of energy evaluations was set to 1.5 \times 10^6. For each of the 50 independent runs done, a maximum of 2.7 \times 10^4 genetic algorithm operations was generated on a single population of 50 individuals. Operator weights for crossover, mutation, and elitism were default variables, 0.80, 0.02, and 1, respectively. Metal ions were modeled in AutoDock by Amber force field potentials. However, for zinc variables (zinc radius and well depth), we selected \( r = 1.1 \text{ Å}, \quad \epsilon = 0.25 \text{ kcal/mol}, \) and a charge of +2.0 e (14).

Binding Affinity Prediction. Based on the traditional molecular force field model of interaction energy, a score function at the level of binding free energy was derived and adopted in the AutoDock 3.0 version (13). We applied this scoring approach and calculated the total binding free energy (\( \Delta G \)) and inhibition constant (\( K_i \)) values for quinazolinone derivative–PLAP complexes according to the algorithm in the AutoDock 3.0 program. From the simulated models, the docked complex of quinazolinone derivative–PLAP with the lowest interaction energy and most reasonable geometric qualities was selected. Further

![Figure 2](image-url)  
**Figure 2.** Pathway for the synthesis of iodinated \( ^{127/125}\text{I} \) quinazolinone derivatives. Reagents and conditions: i, salicylaldehyde, p-toluene sulfonic acid; ii, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; iii, hexa-n-butyltin/palladium(0)/dioxane, reflux; iv, phosphorus oxychloride/pyridine/0°C; v, 28% aqueous ammonium hydroxide; vi, DMSO/H2O; vii, Na*I/Iodogen (\( ^{1}\text{I, }^{123}\text{I, }^{124}\text{I, }^{125}\text{I, or }^{131}\text{I} \)); viii, alkaline phosphatase.
energy minimization and geometric optimization were done on the selected complex until there were no conflicts among the ligand, the metal ion, the water molecules, and PLAP. The obtained complex was used for structure analysis and interpretation of the potential bioactivities of the ligands.

**Chemical Studies**

**Synthesis of $^{[127I]}$Iodinated Quinazolinone Derivatives.** Reagents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO) and used without further purification. Carrier-free sodium $^{[125I]}$iodide was obtained from GE Healthcare Corp. (Piscataway, NJ). Nuclear magnetic resonance spectra were recorded on a Varian XL-200 spectrometer. Analytic TLC was carried out on Sigma-Aldrich silica gel plastic sheets. Column chromatography was used for routine purification of reaction products. The column output was monitored with TLC. High-performance liquid chromatography (HPLC) analyses were done on a Waters 515 binary system equipped with a Waters 2487 UV detector and a γ-ray detector from IN/US Systems, Inc. (Tampa, FL). HPLC solvents consisted of aqueous 0.05 mol/L phosphate buffer (pH 2.5; solvent A) and methanol (solvent B). For radiochemical analyses, a Zorbax SB-C18 reversed-phase column (9.4 mm × 25 cm; Agilent Technologies, Santa Clara, CA) was used with 90% A and 10% B followed by a linear gradient to 100% B at 6 min and switched back at 13 min to 90% A and 10% B for a further 10 min (flow rate of 3 mL/min). Electron spray mass spectra were recorded on a Micromass Platform LCT
mass analyzer. Compounds 1 to 4 (Fig. 2) were synthesized according to previously published procedures (2). The synthetic details of compounds 5 to 8 will appear elsewhere.3

Radiolabeled (125I) Ammonium 2-(2'-Phosphoryloxyphenyl)-6-Iodo-4-(3H)-Quinazolinone (125IQ2,P) (8). Phosphate buffer (10 μL, 0.01 mol/L, pH 7.4) and ammonium 2-(2'-phosphoryloxyphenyl)-6-butyrylthiannel-4-(3H)-quinazolinone (6, 1 μL of 5 μg/μL DMSO solution) were placed in a reaction vial coated with 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (lodogen, 10 μg). Na125I (37–74 MBq in 2–4 μL 0.1 mol/L sodium hydroxide) was added. After vortex mixing at ambient temperature for 2 min, the reaction solution was analyzed by HPLC (Waters) on a reversed-phase Zorbax SB-C18 column using a linear gradient from 10% 0.05 mol/L disodium hydrogen phosphate (pH 2) to 100% methanol at a flow rate of 3 mL/min for 6 min, with UV absorption at 280 nm (Waters 486 detector) and γ-ray detection (γ-ray, IN/US Systems) to analyze the eluates. All products were collected in pure methanol, which was then removed by purging with argon, and each residue was dissolved in water.

Biological Studies

Alkaline Phosphatase–Dependent Conversion of Prodrugs 125IQ2,P (7) and 125IQ2,P (8) to Drug 125IQ2,OH (9). To assess alkaline phosphatase–dependent hydrolysis of 8 to 9 (Fig. 3), the phosphorylated derivative 125IQ2,P (10 μCi, 4.6 pmol) was incubated at 37°C for 5 min with bovine intestinal mucosa alkaline phosphatase [20 units/20 μL 0.01 mol/L PBS (pH 7.4) containing 50 mmol/L NaCl, 10 mmol/L MgCl2, and 0.1 mmol/L ZnCl2] and the formation of 9 was determined (per gram of tissue or blood) in a gamma counter (γ-ray detection). The activity was also determined for 8 in the identification of hydrolases, we primarily carried

To ascertain the potential of the data mining approaches in the identification of hydrolases, we primarily carried
out searches of the UniProt and SRS databases. We hypothesized that if our approaches were valid, the search would identify alkaline phosphatase, a hydrolytic enzyme that is overexpressed on the plasma membranes of many tumor cells (3–11). The search yielded a total of 1,349 matches of 120,961 entries and included a multitude of enzymes involved in the hydrolysis of various bonds (e.g., phosphomonoester and glycosidic). To narrow our focus to a few well-characterized protein families, we repeated these searches on the InterPro database (version 2.0) of protein signatures, considered the gold standard for the analysis of protein families (17), and this yielded 60 hits for enzymes at the cell surface. To restrict our scope further, we also searched InterPro for enzymes/enzyme families with glycosylphosphatidylinositol anchors, and this resulted in three hits: renal dipeptidase (IPR000180), alkaline phosphatase (IPR001952), and 5'-nucleotidase and apyrase (IPR006179). Our searches validated alkaline phosphatase as an enzyme that is overexpressed on the cell surface and involved in cancer. In addition, the PDB search afforded a total of 40 matches of available structure models of alkaline phosphatase or alkaline phosphatase substrate/alkaline phosphatase inhibitor complexes, including a high-resolution structure of the human PLAP isoform determined at 1.8 Å resolution (PDB code 1EW2; ref. 12). We then used this structure for molecular docking studies.

**Computational Studies**

**Active Site of PLAP.** The active site of PLAP involves Asp91, Ser92, Gly93, the metal triplet (two Zn2+ and one Mg2+) and its substrate (phosphate), as well as Arg166 and other amino acids in the immediate vicinity (Fig. 4A). The structure suggests that there is a hydrophobic pocket in the active site and that the phosphate–PLAP interaction involves Glu429 and Arg166. The participation of these two amino acids allows precise description of this pocket, which probably stabilizes the hydrophobic moiety of the substrate. Located at the entrance of the cleft that leads to the active site, Glu429 borders a pocket that extends from the catalytic Ser92 to the phosphoryloxy moiety. This moiety interacts through H-bond binding with the His317 and His153, two amino acid residues (especially catalytic Ser92) and the metal triplet (especially Zn1) are the core of the active site, which has been shown to participate in PLAP-mediated hydrolysis of phosphoryloxy groups (18).

**Docking of IQ2-P(I) (10) and IQ2-P (4) with PLAP.** IQ2-P(I) is a fused phosphorus heterocyclic compound, composed basically of four rings, causing the whole structure to be quite rigid and bulky. All 50 docking poses are within 0.02 Å root mean square deviation, indicating a single binding mode. The lowest energy pose is shown in Fig. 4B. The oxygen atom of the carbonyl group in the quinazolinone moiety of IQ2-P(I) (10) is able to form H-bonds with His317 and His153, two amino acid residues positioned at the upper boundary of the binding pocket. However, unlike the pose of the phosphate group in the PLAP crystal structure, the phosphorus group of IQ2-P(I) (10) forms only one H-bond with Arg166 and no H-bonds with His317 and His153 (Fig. 4B). In addition, because the steric four-ring structure cannot induce the phosphorus group to contact the bottom of the binding pocket, the phosphorus atom remains at a distance from the oxygen atom in the side chain of Ser92 [d(P–O) = 4.35 Å] and from Zn1 [d(P–Zn1) = 3.16 Å], showing that the phosphorus group of IQ2-P(I) (10) is unable to form potential contacts with the catalytic amino acid residues and metal ions of PLAP. Overall, IQ2-P(I) is therefore positioned in the upper cleft of the binding pocket of the enzyme, and its steric rigid-ring structure makes the phosphorus group less flexible in approaching the core of the active site, suggesting a very low likelihood that IQ2-P(I) will be hydrolyzed by PLAP.

Unlike the absolutely rigid structure of IQ2-P(I), IQ2-P (4) is a relatively flexible molecule in which two rings, the quinazolinone moiety and the benzene ring, are joined via a single rotatable C-C bond. The phosphorus group is also quite free because it is connected with the benzene ring through a single P-O-C bond. In this case, all 50 docking poses are clustered within 0.5 Å root mean square deviation, indicating a strong consensus for a single binding mode. The lowest energy pose is shown in Fig. 4C. Along the active site, the quinazolinone moiety packs in the upper cleft of the binding pocket and the iodine atom poses against the pocket (Fig. 4D). To be positioned in the middle of the binding pocket, the benzene ring adopts noncoplanar conformation with the quinazolinone ring and the dihedral angle between them is 33.22°. The phosphorus group of IQ2-P twists slightly (τ_P-O-C = 118.47°) to allow itself to drop completely into the bottom of the binding pocket and to face the metal triplet. Similar

<table>
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<th>Ligand</th>
<th>Structure</th>
<th>ΔG* (kcal/mol)</th>
<th>Kᵢ (T = 298.15 K)</th>
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<td>3.77 × 10⁻¹⁰</td>
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* Binding free energy.
† Inhibition constant.
to the binding pose of phosphate in the PLAP crystal structure, the phosphorus group of IQ2-P forms several H-bonds with amino acid residues in the active site. In addition to forming a salt bridge with the guanidino group of Arg166, the phosphorus group of IQ2-P is also capable of contacting His320Nε2 and His432Nε2. Most important, the phosphorus group of IQ2-P has hydrogen bonding with the catalytic amino acid Ser92. Since, the distance between the phosphorus atom and the oxygen atom in the side chain of Ser92 is 2.69 Å, IQ2-P can easily interact with Ser92 and is expected to be phosphorylated, whereas for IQ2-P(I) the distance is 2 Å greater. The interaction of the phosphorus group of IQ2-P with water networks has also been noted, particularly with water 315, which favors the binding of IQ2-P with PLAP. After IQ2-P(I) (10) and IQ2-P (4) were docked into PLAP, the free binding energy (ΔGbinding) and inhibition constant (Ki) of their lowest energy poses with PLAP were calculated (Table 1). The values indicate that there is ~3 kcal/mol differential of free binding energy between IQ2-P(I) and IQ2-P with PLAP. As these data show that the binding affinity of IQ2-P is ~170 times stronger than that of IQ2-P(I), the former molecule is more likely to bind with PLAP and be dephosphorylated.

Chemical Studies

The pathway for synthesis of iodinated (125I/127I) quinazolinone derivatives (Fig. 2) is based in part on a published method (2). Initially, iodoanthranilamide (1) was conjugated with 2-hydroxy-5-methylbenzaldehyde using p-toluenesulfonic acid as catalyst and oxidized with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone to give 3 (2). The yield of each step in the synthetic procedure was quite high, and the separation processes for products were not difficult. However, it recently became apparent to us that, along the reaction pathway, the phosphorylation of tin precursor 3 generates two products (5 and 6) with totally different 31P nuclear magnetic resonance spectra and that, after an overnight incubation in DMSO, 5 is converted completely to 6. The carrier-free radioiodination of this trimethylstannyquinazolinone derivative is conducted by iododestannylation using Na125I and Iodogen. HPLC analysis indicates that the radiochemical yield and purity of 125I-labeled 8 are ≥98% and that its retention time (9.05 min) matches that of authenticated 127I-labeled 4.

Biological Studies

Alkaline Phosphatase–Dependent Conversion of Prodrugs IQ2-P (4), 125IQ2-P(I) (7), and 125IQ2-P (8) to Drugs IQ2-OH (2) and 125IQ2-OH (9). When nonradiolabeled prodrug 127IQ2-P (4), a nonfluorescent, water-soluble compound, is incubated with bovine intestinal mucosa alkaline phosphatase, the resulting product of hydrolysis, analyzed by HPLC (UV visualization) and TLC (autoradiography detection), has a retention time matching that of the drug IQ2-OH (2, Fig. 5). The PLAP-mediated hydrolysis of 4 is similarly highly efficient; complete

Figure 5. Alkaline phosphatase–mediated hydrolysis of 125IQ2-P (8) to 125IQ2-OH (9). Left, TLC; right, HPLC.

Figure 6. HPLC profiles of IQ2-P (7)/IQ2-P (8) mixture before (A) and after (B) 5-min incubation with PLAP showing the absence of IQ2-P(I) hydrolysis.
dephosphorylation occurs within a few minutes. The data (Fig. 6) also show that the incubation of 7 with PLAP does not lead to the hydrolysis of this analogue (thus the acronym of inactive quinazolinone IQ2-P(I)).

**Hydrolysis of 127IQ2-P (4) and 125IQ2-P (8) by Alkaline Phosphatase–Expressing Tumor Cells In vitro as Assessed by Fluorescence Microscopy, Autoradiography, and Confocal Microscopy.** The in vitro incubation of mouse NE teratocarcinoma cells, known to overexpress alkaline phosphatase extracellularly on their plasma membranes (8), with 127IQ2-P (4) leads to the formation of bright fluorescent crystals, many of which are strongly and irreversibly bound to the exterior cell-surface (Fig. 7). Because (a) IQ2-P is nonfluorescent and water-soluble, (b) IQ2-OH is highly fluorescent and water-insoluble, and (c) no fluorescent precipitates are formed in the absence of cells, the fluorescence observed is a direct result of the alkaline phosphatase–mediated hydrolysis of IQ2-P (4) and the formation of IQ2-OH (2). To confirm that the radiiodinated prodrug 125IQ2-P (8) is also dephosphorylated by alkaline phosphatase–expressing cells and forms the tumor cell–bound drug 125IQ2-OH (9), the tumor cells were incubated with a mixture of 125IQ2-P (8) and 127IQ2-P (4) and the hydrolysis was simultaneously determined by fluorescence microscopy and autoradiography. Our findings show that ~90% of NE teratocarcinoma cells are “covered” with fluorescence (Fig. 7, columns 1 and 2, row 2). Similarly, the presence of intense black grains (125IQ2-OH) is observed on autoradiography (Fig. 7, column 3). To ascertain further that the tumor cell–mediated hydrolysis of IQ2-P occurs via alkaline phosphatase, NE cells were incubated with the prodrug in the presence or absence of levamisole, a drug that is known to inhibit specifically alkaline phosphatase (15). The results show the absence of any fluorescence in the presence of levamisole (Fig. 8), clearly supporting the view that the dephosphorylation of IQ2-P is in fact mediated through alkaline phosphatase. There is no dose dependency when concentrations of 127IQ2-P between 1 and 100 μg/mL are used.

To address whether the observed crystals are trapped within the interstitial space of tumor cell clusters, NE cells were incubated with IQ2-P, washed, and examined with a confocal microscope. The results (Fig. 9) clearly show the presence of fluorescence only between the NE cells, thus indicating that the precipitated IQ2-OH molecules are trapped extracellularly within the interstitial spaces of these “solid tumor–like” cell clusters.

The hydrolysis of IQ2-P (4) by various human tumor cell types was also examined. Such in vitro incubations of viable cells lead to the formation of large (~5–25 μm) fluorescent crystals (Fig. 10A–G). On the other hand, the incubation of normal human mammary epithelial cells (HMEC) with IQ2-P does not show hydrolysis of the compound and appearance of fluorescent crystals (Fig. 10H). Similar findings (minimal fluorescence or its absence) are also obtained following a 24-h incubation of normal mouse tissues (kidneys, liver, and spleen) with IQ2-P.3

We also examined the capacity of NE tumor cells grown in vivo as solid tumors to hydrolyze IQ2-P in vitro. Mouse

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**Figure 7.** Hydrolysis of 125IQ2-P (8)/127IQ2-P (4) (nonfluorescent) to 125IQ2-OH (9)/127IQ2-OH (2) (fluorescent) after in vitro incubation (pH 7.4) of prodrug with alkaline phosphatase–expressing NE teratocarcinoma cells grown in vitro. Columns 1 and 2, light/fluorescence microscopy; column 3, autoradiography.
NE teratocarcinoma cells were injected i.m. into mice, and when the tumors became palpable, the tumor-containing muscles were dissected and incubated with IQ$_2$-P (4), washed, and observed under the light/fluorescence microscope. In these studies, the presence of intense fluorescence is seen throughout the solid tumor regions [i.e., hydrolysis of IQ$_2$-P to IQ$_2$-OH by tumor cells (Fig. 11)]. That the hydrolysis of IQ$_2$-P occurs essentially with alkaline phosphatase–expressing tumor cells and is minimally initiated by normal cells is shown by the absence of fluorescence within normal leg muscle cells (Fig. 11).

In vivo Hydrolysis of $^{125}$IQ$_2$-P (8) following Intratumoral or I.m. Injection in Rats. When rats bearing palpable s.c. human TE671 rhabdomyosarcoma tumors were injected intratumorally or i.m. (thigh) with $^{125}$IQ$_2$-P and killed 24 h later and their tissue and blood radioactivity was assessed, the data show that 68 ± 29% of the injected dose is present per gram of the injected TE671 tumors (Fig. 12) and only 1.0 ± 0.9% is found per gram of injected muscle (i.e., ~70-fold difference). Because $^{125}$IQ$_2$-P is rapidly cleared from the blood and body following i.v. injection (0.01–0.4% ID/g remains in all normal tissues at 24 h), we believe that the ~30% of the injected dose that is released into circulation from within the injected tumor will be cleared with similar pharmacokinetics. The current results, which also indicate that the radioactive content of the blood and contralateral muscle is very low (0.09 ± 0.03%/g and <0.005 ± 0.007%/g, respectively), show that very high tumor-to-blood (~800) and tumor-to-muscle (~13,500) ratios can be achieved on the in vivo hydrolysis of this quinazolinone derivative.

Discussion
Currently, the majority of clinical anticancer drugs are systemic antiproliferative agents that preferentially kill dividing cells, primarily by attacking their DNA at the synthesis and replication levels. Such cytotoxins have many advantages, especially the ability to kill large numbers of tumor cells with constant proportion kinetics (19). However, these drugs are not fully selective for cancer cells, and their therapeutic efficacy is limited by the damage they also cause to proliferating normal cells, such as those in the bone marrow and gut epithelium.

One strategy for providing substantial increases in the clinical efficacy of such drugs is the use of relatively nontoxic prodrug forms that can be selectively activated in tumor tissue (20). Prodrugs are usually defined as agents that are transformed after administration, by metabolic or spontaneous chemical breakdown, to form pharmacologically active species. During the past 2 decades, several prodrug-based therapy approaches have been reported, among which two have shown their credibility [i.e., (a)]
antibody-directed, enzyme–prodrug therapy and (b) gene-directed, enzyme–prodrug therapy. In antibody-directed, enzyme–prodrug therapy, a noninternalizing antitumor antibody–enzyme conjugate is injected before the administration of a low-molecular-weight therapeutic molecule (prodrug). The enzyme is selected for its ability to convert the relatively noncytotoxic prodrug into a highly cytotoxic drug. The major advantages provided by antibody-directed, enzyme–prodrug therapy are (a) low normal tissue toxicity (prodrug is mildly toxic), (b) specific conversion of prodrug in tumors, (c) high prodrug-to-drug turnover, and (d) a bystander effect. However, at a minimum, antibody-directed, enzyme–prodrug therapy has two major limitations: (a) to be therapeutically effective, the cytotoxic agent must be internalized by each cell within the tumor mass (21–23) and (b) the toxic agents formed have relatively long biological half-lives (24) and have been shown to escape from within the tumor mass, thereby decreasing tumor therapeutic efficacy and increasing nonspecific normal tissue toxicity (21). The alternative concept of gene-directed, enzyme–prodrug therapy (25–27) uses a variety of vector systems, including liposomes, replicating and nonreplicating viruses, and anaerobic bacteria, to deliver to and express in tumor cells the gene coding for an exogenous enzyme. This approach expands the class of available enzymes to include those that require endogenous cofactors but loses one aspect of prodrug selectivity because the prodrugs must be able to enter cells freely.

In this article, we describe a novel approach, named EMCIT, which we believe can fulfill many of the basic requirements for the effective imaging and therapy of solid tumors. The approach takes advantage of the prodrug methodology without its limitations. EMCIT is a method for the site-specific, in vivo conversion of a water-soluble, negatively charged, radioactive molecule to a water-insoluble, radioactive molecule by an enzyme overexpressed on the surface of a solid tumor (Fig. 1). Because many tumors (e.g., ovarian, breast, prostate, lung, and teratocarcinoma) express innately various phosphatases (10, 28–35), we expect the hydrolysis of radioiodinated prodrug within the extracellular spaces of such tumors and the indefinite entrapment of the radioactive molecule within the tumor mass.


Figure 11. Hydrolysis of IQ2-P (4) (nonfluorescent) to IQ2-OH (2) (fluorescent) after 1-h in vitro incubation (pH 7.4) of 4 with alkaline phosphatase–expressing NE tumor (T) growing within leg musculature (M) of mouse. Light/fluorescence microscopy.
A series of biological experiments support the in silico computational predictions. Alkaline phosphatase–dependent hydrolysis of $^{125}$IQ$_2$-P(0) (7) is undetectable whereas that of $^{125}$IQ$_2$-P (8) is highly efficient (Fig. 6). In addition, microscopy studies show that incubation of IQ$_2$-P with various mouse and human tumor cell lines, but not with normal cells and tissues,\textsuperscript{5,5} leads to the formation and precipitation of fluorescent IQ$_2$-OH outside the cells (Figs. 7–11), indicating

phosphatase overexpression by tumor cell membranes and the absence of cellular internalization of IQ2-P (a multitude of phosphatases reside in the intracellular environment). That the enzyme responsible for IQ2-P hydrolysis is alkaline phosphatase is further corroborated by the absence of dephosphorylation in the presence of the specific alkaline phosphatase inhibitor levamisole (Fig. 8).

As mentioned above, the major purpose of our studies is the development of water-soluble, radiolabeled, imaging and therapeutic agents that can be efficiently hydrolyzed in vivo to highly water-insoluble, radiolabeled analogues that are entrapped indefinitely within the interstitial spaces of tumors. It was therefore essential to ascertain that IQ2-P \( a \) is specifically hydrolyzed by tumor cells growing in vivo, \( b \) is minimally dephosphorylated by normal mammalian tissues, and \( c \) functions as a substrate for the enzyme responsible for IQ2-P hydrolysis. Our studies confirm these three expectations as they show that \( a \) the compound is rapidly hydrolyzed to its fluorescent and water-insoluble analogue when solid tumors grown in vivo are incubated with IQ2-P (Fig. 11), \( b \) minimal hydrolysis is seen in muscle tissues that are adjacent to the solid tumor (Fig. 11), and \( c \) intratumoral injection of \( 125^\text{I} \)IQ2-P into human tumors grown in nude rats leads to the entrapment of \( \sim 70\% \) of the injected radioactive dose within these solid tumors, whereas minimal activity \( (\sim 1\%) \) is retained when \( 125^\text{I} \)IQ2-P is injected into the leg muscles of these rats (Fig. 12). Previously, we had also shown that, once formed within a tissue, IQ2-OH is retained for up to 48 h at the location where it is formed (2).

When a prodrug, such as IQ2-P, is labeled with \( ^{131}\text{I} \) and given to a tumor-bearing animal, the concentration of \( ^{131}\text{I} \) in blood and normal tissues will be too low to kill cells. However, the efficient entrapment and retention of this energetic \( \beta \)-particle emitter within solid tumors (following its extracellular enzyme-mediated hydrolysis and precipitation to \( 1^{131} \text{IQ2-OH} \)) should lead to the deposition of a cytotoxic dose in every tumor cell that is within the range of the emitted particles \((\sim 1 \text{ mm})\). Because the molecular weight of such prodrugs is low \((<500)\), these \( 1^{131} \text{IQ2-P} \) molecules are expected to diffuse easily throughout normal and malignant tissues. Consequently, they are likely to \( a \) distribute rather uniformly throughout solid tumor masses before (and after) their hydrolysis, \( b \) concentrate within tumors due to the high \( 1^{131} \text{IQ2-P} \) to \( 1^{131} \text{IQ2-OH} \) alkaline phosphatase-mediated turnover, and \( c \) deposit a tumor-cellular dose. On the other hand, because \( 1^{131} \text{IQ2-P} \) clears rapidly from blood and all normal tissues, \( 3 \) the dose deposited by the decay of \( 1^{131} \text{I} \) within them is expected to be very low. Our findings indicate that the maximum tolerated dose for \( 1^{131} \text{IQ2-P} \) is \( \sim 1 \text{ mCi} \) for a 25-g mouse, \( 6 \) corroborating these expectations (in humans, the maximum tolerated dose would be expected to be \( \sim 2 \text{ Ci} \)).

In conclusion, we have described an approach that takes advantage of \( a \) the wealth of information available on expressed gene products in cancerous cells; \( b \) the ability to effectively and methodically mine various databases for specific cancer signatures, such as hydrolytic enzymes specifically overexpressed on the plasma membrane of tumor cells; \( c \) advances in computer-assisted molecular modeling techniques that can help in identification of the active sites of a biological macromolecule, such as a hydrolytic enzyme, and in prediction of the free binding energies and the H-bond interactions between a ligand and its respective target; and \( d \) a novel technology (EMCIT) that we recently developed, which enables the specific and irreversible entrapment of radioactive molecules within the extracellular space of solid tumors (Fig. 1). We believe that the EMCIT method will satisfy most requirements for an ideal radioimaging and radiotherapeutic agent \( [i.e., \( a \) rapid and efficient concentration by the tumor, \( b \) retention by the tumor, \( c \) short residence time within normal tissues, and \( d \) high tumor-to-normal tissue uptake ratios] \). In addition, the EMCIT technology will eventually proceed to the identification of new noninvasive signatures of human solid tumors based on their cellular molecular profiles, which could provide a strategy for intervention and prevention. The search for additional target proteins, the optimization of quinazolinone derivatives, and the establishment of in vivo animal radioimaging and radiotherapy models are ongoing.

References

Unpublished results.


In silico design, synthesis, and biological evaluation of radioiodinated quinazolinone derivatives for alkaline phosphatase–mediated cancer diagnosis and therapy


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