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

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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-(3H)-quinazolinone (125IQ2-P), is hydrolyzed by alkaline phosphatase to a water-insoluble, radiolabeled drug, 2-(2'-hydroxyphenyl)-6-[125I]iodo-4-(3H)-quinazolinone (125IQ2-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 (∆G(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 in silico 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 125IQ2-P into alkaline phosphatase-expressing solid human tumors grown s.c. in nude rats results in efficient hydrolysis of the compound and retention of ~70% of the injected radioactivity, whereas similar injection into normal tissues (e.g., muscle) does not produce any measurable hydrolysis (~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 radioimaging of 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 γ-emitting diagnostic (e.g., 123I and 124I) or energetic electron-emitting therapeutic (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-(3'H)-quinazolinone (IQ4-P). We showed that the prodrug (IQ4-P) is a nonfluorescent, water-soluble compound that is hydrolyzed by alkaline phosphatase to the highly fluorescent, water-insoluble IQ4-OH analogue. We also assessed the biodistribution of 125I-IQ4-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 125I-IQ4-P in mice were sometimes very different. On investigation, we learned (3) that the synthesis of the ammonium 2-(2'-phosphoryloxyphenyl)-6-tributylstannyl-4-(3'H)-quinazolinone (SnQ2-P) precursor of IQ4-P produces a mixture of two compounds, SnQ2-P(I) (5 mol wt, 590) and SnQ2-P (6 mol wt, 609), whose radioiodination leads to the formation of 125I-IQ2-P(I) (7) and 125I-IQ2-P (8), respectively (Fig. 2). Further studies have shown that IQ2-P(I) (10) is not hydrolyzed by alkaline phosphatase and the injection of 125I-IQ2-P(I) (7) into normal mice leads to the localization of approximately 10% to 15% ID/g in liver and kidneys. On the other hand, IQ2-P (4) and 125I-IQ2-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). 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 ($DG_{binding}$) and inhibition constant ($K_I$) were estimated. Our results indicate that these in silico procedures are effective tools for use in the development of radio pharmaceuticals with characteristics advantageous for noninvasive, site-specific, alkaline phosphatase–mediated insolubilization of a radioactive prodrug for diagnosis ($^{123}$I/$^{124}$I-labeled) and therapy ($^{131}$I-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. The two zinc ions and one magnesium ion at the active site were

Figure 1. EMCIT approach. Con- version of water-soluble prodrug substrate to an insoluble product. AP, alkaline phosphatase; *I, $^{123}$I/$^{124}$I/$^{125}$I/$^{131}$I.
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, \( v \) (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 \( r = 1.1 \) Å, \( e = 0.25 \) 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.** Pathway for the synthesis of iodinated \((^{127}I/^{125}I)\) quinazolinone derivatives. Reagents and conditions: i, salicylaldehyde, \( p \)-toluenesulfonic acid; ii, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; iii, hexa-n-butylditin/palladium(0)/dioxane, reflux; iv, phosphoryl oxychloride/pyridine/0°C; v, 28% aqueous ammonium hydroxide; vi, DMSO/H2O; vii, NaI/Iodogen (*I: \(^{123}\)I, \(^{124}\)I, \(^{125}\)I, or \(^{131}\)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/125I]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-tributylstannyl-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 (iodogen, 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,Pd (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 by HPLC. 125IQ2,P (10 μCi, 4.6 pmol) was also incubated with 80 units PLAP per 80 μL PBS at 37°C for 5 min. The retention times of each sample on TLC and HPLC were determined and compared with those before the addition of PLAP.

Hydrolysis of 125IQ2,P (4) and 125IQ2,P (8) by Alkaline Phosphatase–Expressing Tumor Cells In vitro as Assessed by Fluorescence Microscopy, Autoradiography, and Confocal Microscopy. Cells from several tumor cell lines (BT-474, human breast ductal carcinoma; MCF7, human breast adenocarcinoma; LS174T, human colorectal adenocarcinoma; HTB-182, human squamous lung cell carcinoma; OVCAR3, human ovarian adenocarcinoma; SKOV3, human ovarian adenocarcinoma; TE671, human rhabdomyosarcoma; NE, mouse teratocarcinoma; HMEC, human mammary epithelial cells; obtained from the American Type Culture Collection, Manassas, VA) were trypsinized, suspended in medium, and seeded onto glass slides. Each cell line was seeded in duplicate and each experiment was repeated three to five times. After an overnight incubation at 37°C, the medium was removed and the cells were reincubated for up to 24 h with 4 (fluorescence microscope studies, 0.2–20 μg/200 μL) or 8 (autoradiography, 100 μCi/200 μL) in medium (pH 7.4). To ascertain the cell-mediated alkaline phosphatase specificity of 125IQ2,P (4) hydrolysis, the same medium was added to chambers containing no cells as well as to chambers containing NE cells, known to express alkaline phosphatase on the exterior surface of their plasma membrane (8), and 10 mmol/L levamisole, a specific inhibitor of alkaline phosphatase (15, 16). After incubation, the cells were repeatedly washed in PBS and then fixed in ice-cold ethanol. For fluorescence microscopy, the cells were counterstained with 4,6-diamidino-2-phenylindole (a nuclear stain) and the distribution and intensity of fluorescent crystals were observed. For autoradiography, the slides were dipped vertically into Kodak (New Haven, CT) NTB-2 emulsion melted at 41°C, withdrawn, left to drain and gel, and then stored at –20°C in a black box containing Drierite (1 day to 2 weeks). Next, the emulsion-coated slides were incubated at 16°C in Kodak developer D-19 for 3 min, fixed in Kodak Fixer for 5 min, rinsed in distilled water, and mounted in Permount. Finally, the slides were viewed under a microscope and the distribution and the frequency of grains over the entire cell monolayer were noted.

The ability of tumor cells grown in vivo as solid tumors to hydrolyze IQ2,OH in vitro was also examined. Mouse NE teratocarcinoma cells were injected i.m. into the right flank of C3H/1 mice (n = 5). When the tumors became palpable, the animals were killed and the tumors were dissected, cut into 2-mm3 fragments, and incubated with IQ2,P (4) for 1 h. The tumor fragments were then washed in PBS, pressed between two glass slides, and observed under the light fluorescence microscope.

IQ2,P is a negatively charged molecule that is not expected to be internalized by mammalian cells. Because the alkaline phosphatase–mediated hydrolysis of this prodrug leads to the formation of the fluorogenic, water-insoluble IQ2,OH derivative, NE teratocarcinoma cells (1 × 106/mL) were grown in vitro and the cell clusters that attached to the slides were washed in medium before the addition of IQ2,P (1 mg/mL). Following 37°C incubation for 2 h, the cells were washed and examined using a confocal microscope. Fifty fluorescence images (0.3 μm apart) were obtained from each cluster, and the images were reconstructed into a three-dimensional image.

In vivo Hydrolysis of 125IQ2,P (8) following Intratumoral or I.m. Injection in Rats. Nude rats (n = 5) were injected s.c. with 2 × 106 human TE671 human rhabdomyosarcoma cells (a cell line that rapidly and efficiently hydrolyzes IQ2,P to IQ2,OH in vitro). When the tumors became palpable, 8 of (10 μCi in 20 μL saline) was injected intratumorally or i.m. (thigh). Twenty-four hours later, the rats were killed, the radioactivity within the s.c. tumors, blood, injected muscle, and contralateral muscle was determined (per gram of tissue or blood) in a gamma counter, and the percentage injected dose was calculated.

Results

Bioinformatics (Data Mining)

To ascertain the potential of the data mining approaches in the identification of hydrodases, 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 (IPR001801), alkaline phosphatase (IPR001952), and 5'-nucleotidase and apyrase (IPR00679). Our searches validated alkaline phosphatase as an enzyme that is overexpressed 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 Zn$^{2+}$ and one Mg$^{2+}$) 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 Glu$^{429}$ 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, Glu$^{429}$ borders a pocket that extends from the catalytic Ser92 to the phosphoryloxy moiety. This moiety interacts through H-bond binding with the His320, His432, Arg166, and Ser92 residues in PLAP. In addition to the tightly bound phosphorylox moiety, several water molecules are located within the active site and they form an extensive hydrogen-bonding network. Moreover, the three metals, two Zn$^{2+}$ (referred as Zn1 and Zn2) and one Mg$^{2+}$, are close in space: d(Zn1 – Zn2) = 4.02 Å, d(Zn2 – Mg) = 4.76 Å, and d(Zn1 – Mg) = 7.00 Å. The 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 phosphorylox groups (18).

**Docking of IQ2-P(10) and IQ2-P (4) with PLAP.** IQ2-P(10) 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 of this quinazolinone derivative with PLAP produce only one cluster, and the position differences among 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(10) is able to form H-bonds with His135 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(10) forms only one H-bond with Arg166 and no H-bonds with His320 and His432 (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(10) is unable to form potential contacts with the catalytic amino acid residues and metal ions of PLAP. Overall, IQ2-P(10) 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(10) will be hydrolyzed by PLAP.

Unlike the absolutely rigid structure of IQ2-P(10), 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 ($\phi_{P-O-C} = 118.47^\circ$) to allow itself to drop completely into the bottom of the binding pocket and to face the metal triplet. Similar
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 His320Ne2 and His432Ne2. 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 0.2Å 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.

Chemical Studies

The pathway for synthesis of iodinated (127I/125I) 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-toluene sulfonic 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 trimethylstannylquinazolinone 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 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 radiolabeled 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 vitro as solid tumors to hydrolyze IQ2-P in vitro. Mouse
NE teratocarcinoma cells were injected i.m. into mice, and when the tumors became palpable, the tumor-containing muscles were dissected and incubated with IQ2-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 IQ2-P to IQ2-OH by tumor cells (Fig. 11)]. That the hydrolysis of IQ2-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 125IQ2-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 125IQ2-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 125IQ2-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)

Figure 8. Fluorescence microscopy of alkaline phosphatase–expressing mouse NE teratocarcinoma cells incubated (pH 7.4) in vitro with IQ2-P (4) (± 10 mmol/L levamisole), showing its hydrolysis and crystallization of IQ2-OH (2) (green) and lack of hydrolysis with alkaline phosphatase–specific inhibitor levamisole.

Figure 9. Confocal microscopy of clusters of NE teratocarcinoma cells incubated with IQ2-P (4) showing entrapment of IQ2-OH (2) (green) between cells.
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 intrinsically various phosphatases (10, 28–35), we expect the hydrolysis of radiiodinated prodrug within the extracellular spaces of such tumors and the indefinite entrapment of the


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 hydrolytic enzyme; (catalysis sites of a biologically active molecule, such as identification and characterization of the binding and can push forward dramatically drug discovery. important role in finding and optimizing lead compounds assisted molecular modeling techniques can play an

and classify tumors based on cell membrane-associated information on the structure of various human genes, including those that lead to the expression of cell surface proteins/enzymes, and the rapid development of high-throughput methods, such as microarray-based analysis of gene expression, can potentially have a major positive effect on our EMCIT approach. Newly developed, high performance, data mining techniques can be used to search systematically the entire set of expressed genes to identify and classify tumors based on cell membrane-associated hydrolytic enzyme signatures that are specifically overexpressed by cancerous cells. On the other hand, computer-assisted molecular modeling techniques can play an important role in finding and optimizing lead compounds and can push forward dramatically drug discovery. Their intrinsic advantages include (a) assistance in the identification and characterization of the binding and catalysis sites of a biologically active molecule, such as a hydrolytic enzyme; (b) identification and design of the three-dimensional chemical structures that have the required physical, chemical, and biological characteristics; and (c) prediction of the free binding energies and the H-bond interactions between the identified molecules, such as a radioiodinated prodrug that is a substrate for a hydrolytic enzyme, and their intended target.

For proof of principle, the use of advanced bioinformatics data mining and molecular modeling techniques to identify the target enzyme and to characterize the prodrug in silico has been corroborated by the synthesis of radioiodinated prodrug and in vitro identification of its activity. First, the data mining approach was used to identify hydrolases, which are (a) overexpressed on the cell surface and (b) associated with cancer cells. Through the searching of several different databases, we found certain interesting hits. Among these, alkaline phosphatase, whose hydrolytic functions have been confirmed in the literature, matched our search criteria. These data mining results verify the settings of our search criteria and methods and show that we are able to identify hydrolytic proteins/enzymes for EMCIT. After selecting alkaline phosphatase as our preliminary target, we did alkaline phosphatase structure searching through the PDB and found an X-ray crystal structure of human PLAP that has been elucidated with 1.8 Å resolution, thereby making molecular docking studies possible.

Haughland et al. (36) had reported previously a class of novel substrates derived from quinazolinones for in vitro detection of cell-associated enzyme activity, particularly that of glycosidase, phosphatase, and sulfatase, by fluorescence microscope. Using similar synthetic approaches, we have synthesized a radioiodinated derivative of quinazolinone (2). However, after careful structural characterization, we found recently3 that the synthesis of this radiohalogenated derivative produces two iodinated and phosphorylated quinazolone derivatives (prodrugs): IQ2-P(I) (10; Fig. 2) and IQ2-P (4; Fig. 2). To explore the potential bioactivity of the two prodrug isoforms, in silico molecular modeling experiments were done to identify and assess the effects of their structural differences. After retrieving the structure of human PLAP from the PDB, the two isoforms were docked into the active site of PLAP. The docking results clearly show that IQ2-P(I) is less suitable than IQ2-P as a substrate for PLAP (Table 1) because its estimated free binding energy is much higher (IQ2-P(I), −9.81 kcal/mol; IQ2-P, −12.86 kcal/mol) and there are fewer H-bonding interactions (IQ2-P(0), 4 H-bonds; IQ2-P, 7 H-bonds) mainly due to the rigidity of this derivative [i.e., IQ2-P is a better substrate because its structure fits more favorably the binding pocket of PLAP (Fig. 4)]. These expectations were substantiated in experiments showing the rapidalkaline phosphatase–mediated hydrolysis of IQ2-P and the lack of hydrolysis of IQ2-P(0) (Fig. 6). Our molecular modeling results provide us with insights into the structural relationship between alkaline phosphatase and such quinazolinone ligands and will continue to guide us in structural modification and optimization of IQ2-P derivatives.

A series of biological experiments support the in silico computational predictions. Alkaline phosphatase–dependent hydrolysis of 125I IQ2-P(0) (7) is undetectable whereas that of 125I IQ2-P (8) is highly efficient (Fig. 6). In addition, microscopy studies show that incubation of IQ2-P with various mouse and human tumor cell lines, but not with normal cells and tissues,5,5 leads to the formation and precipitation of fluorescent IQ2-OH outside the cells (Figs. 7–11), indicating

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phosphatase overexpression by tumor cell membranes and the absence of cellular internalization of IQ$_2$-P (a multitude of phosphatases reside in the intracellular environment). That the enzyme responsible for IQ$_2$-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 agents that can be efficiently hydrolyzed in vivo to highly water-insoluble, radiolabeled analogues when solid tumors, whereas minimal activity is seen in muscle tissues that are adjacent to the solid tumor (Fig. 11), and (c) functions as a substrate for alkaline phosphatasas overexpressed by tumor cells when the radiopharmaceutical is injected into cancer-bearing animals. 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 IQ$_2$-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 $^{125}$IQ$_2$-P into human tumors grown in nude rats leads to the entrapment of ~70% of the injected radioactive dose within these solid tumors, whereas minimal activity (~1%) is retained when $^{125}$IQ$_2$-P is injected into the leg muscles of these rats (Fig. 12). Previously, we had also shown that, once formed within a tissue, IQ$_2$-OH is retained for up to 48 h at the location where it is formed (2).

When a prodrug, such as IQ$_2$-P, is labeled with $^{131}$I and given to a tumor-bearing animal, the concentration of $^{131}$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 IQ$_2$-OH) should lead to the deposition of a cytotoxic dose in every tumor cell that is within the range of the emitted particles (~1 mm). Because the molecular weight of such prodrugs is low (<500), these IQ$_2$-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 $^{131}$IQ$_2$-P to $^{131}$IQ$_2$-OH alkaline phosphatase–mediated turnover, and (c) deposit a tumor-icidal dose. On the other hand, because $^{131}$IQ$_2$-P clears rapidly from blood and all normal tissues, the dose deposited by the decay of $^{131}$I within them is expected to be very low. Our findings indicate that the maximum tolerated dose for $^{131}$IQ$_2$-P is ~1 mCi for a 25-g mouse, corroborating these expectations (in humans, the maximum tolerated dose would be expected to be ~2 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.

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