Computational Modeling and Experimental Evaluation of a Novel Prodrug for Targeting the Extracellular Space of Prostate Tumors

Pavel Pospisil, Ketai Wang, Ayman F. Al Aowad, Lakshmanan K. Iyer, S. James Adelstein, and Amin I. Kassis

Abstract

We are developing a noninvasive approach for targeting imaging and therapeutic radionuclides to prostate cancer. Our method, Enzyme-Mediated Cancer Imaging and Therapy (EMCIT), aims to use enzyme-dependent, site-specific, in vivo precipitation of a radioactive molecule within the extracellular space of solid tumors. Advanced methods for data mining of the literature, protein databases, and knowledge bases (IT.Omics LSGraph and Ingenuity Systems) identified prostatic acid phosphatase (PAP) as an enzyme overexpressed in prostate cancer and secreted in the extracellular space. Using AutoDock 5.0 software, the prodrug ammonium 2-(2'-phosphoryloxyphenyl)-6-ido-4-(3'H)-quinazolinone (IQ2,p) was docked in silico into the X-ray structure of PAP. The data indicate that IQ2,p docked into the PAP active site with a calculated inhibition constant (Ki) more favorable than that of the PAP inhibitor α-benzylaminobenzylphosphonic acid. When 125IQ2,p, the radioiodinated form of the water-soluble prodrug, was incubated with PAP, rapid hydrolysis of the compound was observed as exemplified by formation of the water-insoluble 2-(2'-hydroxyphenyl)-6-[127I]iodo-4-(3'H)-quinazolinone (IQ2,OH). Similarly, the incubation of IQ2,p with human LNCaP, PC-3, and 22Rv1 prostate tumor cells resulted in the formation of large fluorescent IQ2,OH crystals. No hydrolysis was seen in the presence of normal human cells. Autoradiography of tumor cells incubated with 125IQ2,p showed accumulation of radioactive grains (125IQ2,OH) around the cells. We anticipate that the EMCIT approach will enable the active in vivo entrapment of radioimaging and radiotherapeutic compounds within the extracellular spaces of primary prostate tumors and their metastases. [Cancer Res 2007; 67(5):2197–205]

Introduction

Prostate cancer is the most frequently diagnosed malignancy in men (33%) and the second leading cause of cancer death in the United States (10%). The American Cancer Society estimates that 234,300 new cases of invasive prostate cancer will be identified in the United States this year. A substantial proportion of these people will develop metastatic disease at some point, and ~30,000 will die. Routine diagnosis is based on prostate-specific antigen determination in blood or discovery of a palpable mass within the prostate. Despite current therapies, the outcome is often lymph node spread, bone metastases, and death.

The development of technologies that enable noninvasive determination of this disease and therapeutic intervention at an early stage is clearly to be desired. Such technologies would be designed to detect prostate cancer, move meaningful intervention to an earlier point in its progression, prevent the development of metastatic disease, and minimize patient inconvenience and incapacitation. Toward these objectives, we present a novel approach, Enzyme-Mediated Cancer Imaging and Therapy (EMCIT; refs. 1–5), which potentially enables the active entrapment of a radioisotopically labeled compound within the extracellular spaces of primary prostate tumors and their metastases (Fig. 1). This noninvasive technique is based on the rapid uptake of radioactive molecules and their enzyme-dependent, site-specific, in vivo precipitation within solid tumors (in contrast to minimal uptake in normal tissues). The compound can be labeled with an isotope having decay characteristics suitable for positron emission tomography (PET) or single-photon emission computed tomography (SPECT) imaging (e.g., 123I and 124I) or for therapy (e.g., 131I).

The prototype for this approach was first developed for alkaline phosphatase (ALP; EC 3.1.3.1), a hydroxylase with monophosphoesteric activity that is overexpressed on the plasma membranes of many tumor cell types (1, 2, 6, 7). A suitable substrate, the prodrug ammonium 2-(2'-phosphoryloxyphenyl)-6-ido-4-(3'H)-quinazolinone (IQ2,p) and its radioiodinated analogue (125IQ2,p) were synthesized, purified, and characterized (1, 5), and their ALP-mediated hydrolysis to the water-insoluble, fluorescent 2-(2'-hydroxyphenyl)-6-ido-4-(3'H)-quinazolinone derivatives (125IQ2,OH) was shown (1, 3, 5). These studies also indicated that (a) IQ2,p is a highly water-soluble molecule (mg/mL) that is stable in human serum and readily dephosphorylated by ALP to the water-insoluble IQ2,OH derivative; (b) the in vitro incubation of 125IQ2,p/125IQ2,OH derivatives with several ALP-expressing human and mouse tumor cell lines results in the efficient and rapid formation of the corresponding water-insoluble derivatives 125IQ2,OH and 125IQ2,OH; and (c) the intratumoral injection of 125IQ2,p into ALP-expressing solid human tumors grown in rats leads to the efficient hydrolysis of the compound and the retention of ~70% of the injected radioactive, whereas similar injection into normal tissues (e.g., muscle) leads to little measurable hydrolysis (~1%) and lack of retention of radioactivity at injected sites. In addition, we observed that the pharmacokinetic properties of IQ2,p in mice were not consistent (5). Subsequently, we recognized that the

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3 http://www.cancer.org
synthesis of its stannylated quinazolinone precursor (SnQ$_2$-P) produces a mixture of two compounds, SnQ$_2$-P and SnQ$_2$-P(I) ($\sim$1:1 ratio), whose radioiodination leads to the formation of $^{125}$IQ$_2$-P and its cyclic isoform $^{125}$IQ$_2$-P(0) respectively. Both iodinated molecules have been docked onto ALP, and the data indicate that the calculated binding energies for $^{125}$IQ$_2$-P are more favorable than for its cyclic isoform (3). Furthermore, it has been shown that pure $^{125}$IQ$_2$-P can be prepared following an overnight incubation of the tin precursor mixture in DMSO and that, on its i.v. injection into mice, there is minimal retention (<0.4% injected dose per gram) of radioactivity in all normal tissues (5).

The aim of our current investigation is to identify a hydrolase analogous to ALP that would be suitable for the EMCIT of prostate cancer because ALP is not known to be expressed in prostate tumors. To find such a target candidate, we recently developed an in silico "combined data-mining approach" that is based on several resources (literature text, structured databases, and knowledge bases), the retrieval capabilities of data-mining tools to extract proteins from these resources, the filtering of entities by gene ontologies, and the enlargement of the list of potential target candidates by functionally related proteins (2). One of the targets on the list obtained by this combined data-mining approach is human prostatic acid phosphatase (PAP). This hydrolase was selected because it is a phosphatase that is expressed and secreted exclusively by the prostate and overexpressed and abundantly secreted by prostate cancer cells (8–10). Consequently, serum levels of PAP are frequently elevated in patients and are used as a marker for the disease (11–15). Although PAP has never fully achieved the status of an indispensable frontline test among urologists, lymph node metastases and/or extensions (consequent to a breach of the prostate capsule) are invariably accompanied by a rise in serum PAP (15). Moreover, it is well established that the intense and selective PAP expression seen in human prostate tumor cells correlates with serum levels of the enzyme (16).

To investigate whether the EMCIT concept that brought promising results with ALP could be applied to PAP-expressing prostate tumors, the three-dimensional structure of PAP was used with the advanced docking algorithm of AutoDock 3.0 for the automated computational docking of the potential ligand into the active site of the target. The simulated binding of both IQ$_2$-P and IQ$_2$-P(I) was achieved, and binding free energy ($\Delta G$, a measure of binding strength), intermolecular interactions, and binding affinity in terms of the inhibition constant ($K_I$) were obtained. We also incubated the prodrugs $^{127}$IQ$_2$-P and $^{125}$IQ$_2$-P with purified PAP to experimentally ascertain their hydrolysis and, thereby, confirm the docking results and, subsequently, with several human prostate tumor cell lines and normal human cell types to evaluate the dephosphorylation and precipitation of the drug ($^{125}$IQ$_2$-OH) as a product of hydrolysis.

**Materials and Methods**

**Data mining.** As we were interested in phosphatases that are localized in the extracellular space or on the cell surface of prostate cancer cells, databases were thoroughly searched for enzymes with the following characteristics: (a) citation in the literature in relation to prostatic tissues, (b) expression in the extracellular space or attachment to the plasma membrane by glycosylphosphatidylinositol or the extracellular domain of a transmembrane protein, and (c) possession of phosphatase activity. The data mining was based on our recently developed strategy (2) using a combination of the advanced pathway analysis software applications.

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![Figure 1. Scheme of EMCIT technology and data-mining strategy for identifying extracellular phosphatase. Conversion of water-soluble prodrug $^{*}$IQ$_2$-P (black star) to water-insoluble drug $^{*}$IQ$_2$-OH (gray star) is catalyzed by phosphatase (half-moon shape) overexpressed by cancer cells. Data mining identified extracellular PAP related to human prostate cancer. Arrow points to active site where in silico docking of IQ$_2$-P was directed.](image-url)

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LSGraph version 1.5 (Life Science Graph,\(^4\) IT.Omics, Lille, France) and Ingenuity Pathways Analysis (IPA) version 2.0 (Ingenuity Systems,\(^5\) Mountain View, CA; Fig. 1). Both LSGraph and IPA are knowledge-based applications that can identify the biological mechanisms, pathways, and functions most relevant to a data set of genes or proteins of interest. The following sequence of steps specific to the prostate case was used in this analysis. (\(a\)) LSGraph queried PubMed abstracts that contain evidences of protein-protein relationships and are related to the keyword ''prostate'' or ''prostatic.'' (\(b\)) The cellular localization of proteins was defined by the Gene Ontology terms ''extracellular region'' (GO ID: 0005576) and ''membrane'' (GO ID: 0016020), and the list of proteins containing these annotation terms was created. (\(c\)) This list of proteins was exported from LSGraph and submitted to the functional analysis of IPA. The entities were filtered down to the set known to be involved in prostate cancer by selecting the IPA subnetworks in which the disease cancer occurs as the most significant annotation. The final set of filtered entities was exported and stored in a Microsoft Excel spreadsheet. Extracellular phosphatases were analyzed using the original scientific literature. A phosphatase with properties suitable for EMCIT and with known three-dimensional structure was selected.

**Molecular docking.** The target identified by data mining was PAP. Three-dimensional coordinates of the crystallized structure of PAP with \(\alpha\)-benzylaminobenzylphosphonic acid (BABPA) were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB; PDB ID: 1ND5).\(^6\) To test the accuracy of the docking in reproducing the X-ray complex, docking of BABPA was first directed into monomer A (chain A) of PAP using the AutoDock software version 3.0 (Scripps Research Institute, La Jolla, CA). The program was run on a Silicon Graphics Octane workstation. We then docked IQ2-P as well as IQ2-P(I) (Fig. 2), a cyclic analogue that was discovered during the synthesis of IQ2-P (see Introduction and Results and Discussion) and that had been shown to be a very poor substrate for ALP (3).

Three-dimensional conformers of all three derivatives were prepared using Chem3D of ChemOffice Ultra\(^7\) and minimized into the optimal conformation with its module MM2 [molecular mechanics method to final root-mean-square (RMS) gradient of 0.100 \(\AA\) in vacuum]. The AutoDock Tool was applied to prepare ligands in docking format and to visualize the results. Gasteiger atomic charges were assigned and the flexibility of the molecule was determined using the AutoDock module AutoTors. All torsion angles were defined so that they could be explored during the docking process. Nonpolar hydrogens, including their partial charges, were merged to parent atoms. The atomic solvation variables were assigned by the AutoDock module Addsol. All three ligands were used in the charged form (i.e., phosphonic group in BABPA, phosphatidic group in IQ2-P and

<table>
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<th>BABPA</th>
<th>IQ2-P</th>
<th>IQ2-P(I)</th>
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<tbody>
<tr>
<td>(\Delta G \text{ [kcal/mol]})</td>
<td>-11.90</td>
<td>-13.39 (-12.86)*</td>
<td>-10.82 (-9.81)*</td>
</tr>
<tr>
<td>(K_a \text{ [at } T = 298.15 \text{ K}])</td>
<td>1.89E-9</td>
<td>1.52E-10 (3.77E-10)*</td>
<td>1.18E-8 (6.45E-8)*</td>
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<tr>
<td>(IC_{50} \text{ [units/L]})</td>
<td>0.0036±0.0003 (0.023±0.008)^*</td>
<td>0.0036±0.0003 (0.023±0.008)^*</td>
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*corresponding value for ALP (6).

\(^1\)corresponding value for ALP (8).

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\(^4\) http://www.it-omics.com

\(^5\) http://www.ingenuity.com/products/pathways_analysis.html

\(^6\) http://www.rcsb.org

\(^7\) http://www.chemoffice.com

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**Figure 2.** Docking positions of BABPA, IQ2-P, and IQ2-P(I) in active site of PAP. Energetically most favorable docked positions of BABPA inhibitor (A), IQ2-P (B), and IQ2-P(I) (C) are depicted with C atoms in yellow. Original crystallographic position of BABPA in (A) is depicted with C atoms in violet. Hydrophobic residues (orange) are within van der Waals distance to each compound. Positively charged arginines (green) and histidines (cyan) interact via H-bonds with phosphonic group of BABPA (A), phosphatidic group of IQ2-P (B), and phosphoramidic group of IQ2-P(I) (green dotted lines). In case of IQ2-P, His\(^{17}\) is ideally positioned for nucleophilic attack on phosphorus atom and Asp\(^{258}\) is close to assist in catalytic process.
phosphoramidic group in IQ2-P(I) (Fig. 2). For docking studies, these groups were deprotonated to simulate the dianionic (IQ2-P) or monoanionic (IQ2-P(I)) intermediate state (17).

The following protocol was used for docking PAP and the ligands. The active site of PAP was defined as amino acid residues 7 Å from the bound ligand. Atomic interaction energy grids were calculated with the AutoDock module AutoGrid 3.0 for atom probes corresponding to each atom type in the ligand. The probes were calculated at every 0.35-Å grid position of a grid box (box size in x, y, z = 45.3, 69, 59.3, respectively) centered at the xyz coordinates of the original phosphorus atom of BABPA in the PAP-BABPA complex. The grid box included the entire active site and provided sufficient space for ligand translational and rotational movement. All histidines were allocated as singly protonated, and arginine, lysine, aspartate, and glutamate residues were treated as ionized. For the docking process itself, the Lamarckian genetic algorithm implemented in AutoDock 3.0 was used to search the optimal ligand conformation and orientation within the PAP active site while keeping the residues of the protein rigid. In general, the default variables of AutoDock were used.

Docked compounds were clustered into groups with RMS deviation (RMSD) of docked versus X-ray atom positions <1.0 Å. Ten runs were executed and the largest cluster with the most favorable free binding energy was chosen for analysis; the best docking poses of BABPA, IQ2-B and IQ2-P(I) are shown in Fig. 2. Binding free energy (ΔG) and binding constants (K_i) were calculated and estimated within the AutoDock scoring function (Fig. 2). The obtained complex was used for interpretation of the potential bioactivities of the ligands.

Synthesis and PAP-dependent conversion of prodrugs 125IQ2-P and 125IQ2-P(I) to drug 125IQ2-0H in solution. The ammonium 2-(2'-phosphoryl(oxy)phenyl)-6-[125I/127I]iodo-(4'SF)-quinazolinone analogues 125IQ2-P(127IQ2-P) and 125IQ2-P(125IQ2-P) were synthesized as reported by Wang et al. (5). Briefly, a mixture of SnQ2-P-SnQ2-P(I) was synthesized and immediately radioiodinated in the presence of iodogen and Na125I, and the mixture of 125IQ2-P and 125IQ2-P(I) produced (2 μL, 0.1 μCi/μL) was incubated with 1 unit PAP per μL PBS (0.01 mol/L, pH 7.4) containing 5 mmol/L NaCl, 10 mmol/L MgCl2, and 0.1 mmol/L ZnCl2 at 37°C for 5 min. The retention time of each incubation sample on high-performance liquid chromatography [HPLC; reverse-phase Zorbax SB-C 18 column, 9.4 × 250 mm (Agilent Technologies), with UV absorption (Waters 486) and γ-ray (γ-ram, IN/US Systems) detectors used to analyze eluates] was determined and compared with that of the sample before the addition of PAP.

It is well known that the prostatic secretory protein PAP is often elevated in the sera of patients with prostate cancer (11–15). To determine the concentration of PAP required to hydrolyze 125IQ2-P to 125IQ2-OH, the SnQ2-P-SnQ2-P(I) mixture was incubated for >24 h in DMSO before its radioiodination, as this procedure has been shown to lead to the quantitative conversion of SnQ2-P to SnQ2-P and the production of 125IQ2-P after radioiodination (5). This 100% pure radioiodinated quinazolinone derivative (2 μL, 0.1 μCi/μL) was then incubated with various concentrations of PAP (between 0.001 and 1 unit/μL) for 5 min, and the formation of 125IQ2-OH was assessed by HPLC. After integrating the peak areas of the compounds, the ratios of conversion of 125IQ2-P to 125IQ2-OH were determined. A plot of PAP concentration (abscissa) versus formation of 125IQ2-OH (ordinate) furnished a sigmoidal line from which the IC50 value and the minimum concentration of PAP necessary for any hydrolysis were calculated.

In vitro hydrolysis of 125IQ2-P/125IQ2-P(I) by human prostate tumor cells and normal mammalian cells assessed by fluorescence microscopy and autoradiography. The following cell lines were used: LNCaP [American Type Culture Collection (ATCC)], a cell line isolated from a lymph node lymph node biopsy of a patient with confirmed diagnosis of metastatic prostate carcinoma; PC-3 (ATCC), a human prostatic adenocarcinoma cell line derived from a metastatic bone site; 22RV1 (ATCC), a human prostate carcinoma epithelial cell line; and HMEC (Cambrex Corp., East Rutherford, NJ), a human mammary epithelial cell line. The cells were trypsinized, suspended in medium, and seeded onto glass slides. After an overnight incubation at 37°C, the medium was removed, and the cells were reincubated with 125IQ2-P (0.1 mg/mL, fluorescence microscope studies) or 125IQ2-P (10 μCi/mL, autoradiography). At various times (1–48 h), the cells were observed under a fluorescence microscope, and the density of the green crystals formed was semiquantitatively recorded (0: no fluorescent crystals; +1: a few scattered fluorescent crystals; +2 to +6 increasing numbers of crystals; +5 cells completely covered with crystals). In addition, the cells were repeatedly washed in PBS after the 24- and 48-h incubations and then fixed in ice-cold ethanol. For fluorescence microscopy, the washed/fixed cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI a nuclear stain) and the distribution and intensity of fluorescent crystals were observed and compared with those before washing of the cell monolayers. For autoradiography, the slides were dipped vertically into Kodak 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, counterstained with DAPI, and viewed under a microscope (fluorescence/white).

Results and Discussion

Data mining. Our method of data mining using LSGraph and IPA, complemented by Gene Ontology and PubMed, to search through literature, protein databases, and knowledge bases identified 375 proteins involved in the extracellular space in prostate cancer. The large size of the list was due to Gene Ontology filtering within the context of the cellular components “extracellular region” and “membrane” and the inclusion of functionally related neighbors using LSGraph and IPA. The primary purpose of the data mining was to determine new target phosphatases showing, in analogy to alkaline phosphatase–based EMCIT (1–5), the capacity to catalyze the conversion of water-soluble, nonfluorescent IQ2-P to water-insoluble, fluorescent IQ2-P(OH). Using the combined data-mining strategy based on LSGraph and IPA, PAP was identified as a suitable candidate for further study (Fig. 1).

Human PAP possesses protein tyrosine phosphatase activity and is secreted as a glycosylated homodimer in the seminal fluid of the prostate gland (17). Like most phosphomonoesterases, it hydrolyzes a wide spectrum of substrates, including alkyl, aryl, and other phosphate derivatives (17, 18). Despite its acidic pH preference for optimal activity (contrary to the alkaline pH preference of ALP), it was nevertheless considered likely to catalyze the dephosphorylation of IQ2-P to IQ2-P(OH). Whereas PAP is known to dephosphorylate the epidermal growth factor receptor at an intracellular site of prostate carcinoma cells (19), it has also been observed to be secreted by epithelial cells into the prostate gland seminal fluid (20). Using our data-mining method, PAP was identified as an enzyme localized in the extracellular space rather than in the cytoplasm, associated with the Gene Ontology term “extracellular region” (in Entrez Gene for human PAP: gene name ACPP) or “extracellular space” (in Entrez Gene for mouse PAP: gene name Acpp; or in UniProt: primary accession number Q8CEO8). Once PAP was identified in LSGraph, it was then situated by IPA in the prostate “cancer” network as an extracellular phosphatase. The successful identification of PAP as an extracellular EMCIT candidate is attributed to the data-mining strategy used (2). This method has the advantages of the following features of knowledge-based applications LSGraph and IPA: (a) LSGraph retrieval capability of all cited entities from PubMed abstracts, (b) selection of entities associated with Gene Ontology terms “extracellular region” and “membrane”, and (c) final overlay of extracellular proteins on the IPA knowledge base “disease” network “cancer.” Although PAP is
considered an intracellular enzyme, it is clear that our data mining allows us to classify it as an extracellular protein as well. This notion is supported by the often reported presence of PAP in sera of patients (11–16, 21, 22).

**Molecular docking.*** Six crystal structures of PAP have been resolved and deposited in RCSB PDB (four human and two rat structures). The three-dimensional complex of PAP with the known inhibitor BABPA (PDB ID: 1ND5) was selected for docking of previously designed and characterized IQ$_2$P and its cyclic analogue IQ$_2$P(I) (5). The PAP-BABPA complex was resolved at the docking-acceptable atomic resolution of 2.9 Å. Docking into the PAP structure reproduced the PAP-BABPA crystallographic complex and, thus, provided an estimate of docking accuracy. Furthermore, the PAP-BABPA complex was selected because the BABPA structure is similar to that of IQ$_2$P in size and in the presence of aromatic pharmacophores and a phosphorus-containing bulky anionic group. BABPA is also a potent PAP inhibitor (IC$_{50}$ 4 nmol/L, refs. 17, 23), and the predicted (calculated) binding constant of IQ$_2$P with PAP can be compared with the predicted and experimentally determined binding constant of BABPA. In addition, the conformation of the PAP active site originating from the PAP-BABPA complex (which during the simulation remains rigid) emulates the active site of the ligand-containing protein rather than the empty and open active site of the apoenzyme structure.

The active site of PAP contains positively charged residues Arg$_{11}$, Arg$_{15}$, Arg$_{79}$, His$_{12}$, and His$_{257}$ that create the crown-like structure able to coordinate the phosphorus group (17). Because the active site of PAP is closed and not exposed to the solvent, we have measured the “tightness” of the binding by calculating the free energy of binding ($\Delta G$) and the binding constant ($K_d$), both predictions derived from the AutoDock algorithm. BABPA was successfully docked to, and oriented at, the position determined in the crystalized complex with PAP within 1 Å RMSD of distances of docked versus crystal ligand atoms (Fig. 2). The docking results indicate that the phosphonic group of BABPA complexes with Arg$_{11}$, Arg$_{15}$, Arg$_{79}$, His$_{12}$, His$_{257}$, and Asp$_{258}$. The benzylamino and benzyl moieties of the docked compound are within van der Waals distance of Ile$_{18}$ and Trp$_{174}$ as in the case of the crystallographic complex. Furthermore, the docking position of the phosphorus atom of the phosphonic group of BABPA is close to the crucial His$_{12}$ as seen in the crystal structure complex or in other phosphate-based inhibitors (24, 25).

The PAP inhibitor BABPA and our compound IQ$_2$P share some chemical-structural similarities that suggest that IQ$_2$P may be a

![Figure 3. HPLC profiles of $^{125}$IQ$_2$P-$^{125}$IQ$_2$P(I) mixture, synthesized from SnQ$_2$P-SnQ$_2$P(I) mixture, before (A) and after (B) 5-min incubation (37°C, pH 7.4) with PAP showing complete hydrolysis of $^{125}$IQ$_2$P and absence of hydrolysis of $^{125}$IQ$_2$P(I). C and D, kinetics of PAP-mediated hydrolysis of $^{125}$IQ$_2$P synthesized from 100% pure SnQ$_2$P to $^{125}$IQ$_2$-OH (5 min, 37°C, pH 7.4) as determined by HPLC quantitative analysis.](image-url)
promising ligand. BABPA contains a three-atom bridge, including a secondary amine atom between its two phenyl rings, which has some analogy to the structure of IQ2-P. When IQ2-P is docked into the active site of PAP, the phosphate group is placed within the phosphate-binding pocket and its quinazolinone moiety interacts with hydrophobic residues (Fig. 2B). The phosphorus atom is at a favorable distance for nucleophile attack by His12, leading to the dephosphorylation of IQ2-P to IQ2-OH, in agreement with the theoretical axial attack of P by this nucleophile (24, 25). The iodine atom of the quinazolinone points outward from the active site and does not hinder the binding to PAP. Moreover, the position of IQ2-P results in 11 hydrogen bonds (two more than the BABPA complex). In fact, the resulting binding free energy of IQ2-P (−13.39 kcal/mol) is more favorable than that of the potent inhibitor BABPA (−11.90 kcal/mol). The estimated ΔG and K values are shown in Fig. 2. Overall, the docking position of IQ2-P and the binding free energy compared with those of BABPA predict that we have discovered a prodrug with higher affinity than BABPA, which should be an excellent substrate for PAP (Fig. 2).

Recently, we realized that ammonium 2-(2'-phosphoryloxyphenyl)-6-tributylstannyl-4-(3H)-quinazolinone, the intermediate used to synthesize the radiiodinated derivative, exists as two isomers (SnQ2-P and SnQ2-P(I)) whose radiiodination leads, respectively, to 125IQ2-P and 125IQ2-P(I) (Fig. 2), each having different in vitro and in vivo biological activities (5). To determine the mechanism underlying the molecular interaction and binding of IQ2-P(I) to PAP, the docking of this isomer was also accomplished using the same simulation conditions as for IQ2-P. Due to its cyclization, IQ2-P(I) shows some different properties. For example, the cyclic amine atom becomes a tertiary amine rigidly anchored between two rings; this loss of molecular flexibility probably decreases the chance of ligand-protein accommodation. Furthermore, the phosphoramidic group becomes chemically adverse to nucleophile attack when IQ2-P(I) is docked into the active site of PAP with the phosphoramidic group anchored in the crown (Fig. 2C). Moreover, IQ2-P(I) is more distally positioned from the crown of arginines and histidines, and consequently, the distance of the N atom of His12 is too great for axial attack on the phosphorus atom (>4.14 Å). Consequently, the calculated binding energy of IQ2-P(I) is significantly lower than that of BABPA and IQ2-P (see table in Fig. 2). These in silico predictions are in line with our experimental findings showing both efficient PAP-mediated hydrolysis of IQ2-P and lack of hydrolysis of IQ2-P(I) (Fig. 3A and B). In previous studies (3), the interaction/binding between the two isomers and ALP had also been examined using in silico molecular modeling and docking techniques. Docking data show that IQ2-P fits the active binding site of ALP favorably and interacts with the catalytic amino acid Ser92, which plays an important role in the hydrolysis process, whereas IQ2-P(I) does not. These observations are also in line with our experimental findings showing that IQ2-P is readily dephosphorylated by ALP to water-insoluble IQ2-OH whereas IQ2-P(I) is not (5).

Kinetics of PAP-dependent conversion of radiiodinated prodrug 125IQ2-P to radiiodinated drug 125IQ2-OH. Phosphatases, such as PAP and ALP, are often elevated in the sera of patients with prostate cancer and certain other diseases (11, 12, 26–31). To ascertain that these phosphatases will not lead to significant hydrolysis of the radiiodinated quinazolinone derivatives during transit/sojourn of these compounds in the blood and normal tissues of an animal (i.e., before their specific hydrolysis by the PAP-overexpressing prostate tumor cells), HPLC was used to determine the minimal PAP concentration necessary to initiate the hydrolysis of IQ2-P to IQ2-OH. In these studies, the 125IQ2-P-PAP incubation time was purposely kept brief (5 min) because our previous studies (5) indicate that the residence time of this compound in blood is short (~10 min). When 125IQ2-P was incubated with various concentrations of PAP, a dose-dependent disappearance of this PAP substrate was observed (Fig. 3C and D).

Simultaneously, a single new peak appeared with a retention time matching that of 125IQ2-OH. After integrating the peak areas, the percentage conversions of 125IQ2-P to 125IQ2-OH were quantified and a plot of PAP concentration (abscissa) versus formation of 125IQ2-OH (ordinate) furnishes a sigmoidal line (IC50, 3,600 units/L). The minimal PAP concentration needed to hydrolyze 125IQ2-P is ≥300 units/L, a value much higher than that reported in the blood of patients with prostate cancer (median, 7 units/L; ref. 32). Consequently, these radiiodinated quinazolinone derivatives are not expected to be hydrolyzed by PAP in the circulation after their i.v. injection into prostate cancer patients. Similarly, the minimal ALP concentration needed to hydrolyze 125IQ2-P is ≥1,000 units/L (IC50, 23,000 units/L; ref. 5), a value that is ~2-fold larger than the 500 units/L found in the blood of other-than-prostate cancer patients (26–31).

Hydrolysis of 125IQ2-P/125IQ2-OH by prostate cancer cells. Recently, we showed that the in vitro incubation (37°C, pH 7.4) of water-soluble nonfluorescent quinazolinone IQ2-P with various viable ALP-expressing human and mouse tumor cell lines (breast, colorectal, lung, ovarian, rhabdomyosarcoma, and teratocarcinoma) led to its hydrolysis and the formation of large, fluorescent, water-insoluble IQ2-OH crystals, whereas no hydrolysis occurred when tumor cells were incubated in the presence of levisamino, a specific inhibitor of ALP (3, 5). In the current studies, we determined the ability of three human prostate cancer cell lines (LNCaP, PC-3, and 22Rv1) to hydrolyze IQ2-P and 125IQ2-P, respectively, to IQ2-OH and 125IQ2-OH. At a minimum, there are three factors that may prevent the dephosphorylation of IQ2-P after its incubation with prostate cancer cells: (a) the pH optima of PAP (~6) and ALP (~7) are very different; (b) unlike many tumor cell types, prostate cancer cells are known to express PAP and not ALP (2); and (c) PAP secretion of two of these cell lines (PC-3 and 22Rv1) is either unknown or currently controversial (33–35). Regardless, the in vitro incubation (37°C, pH 7.4) of the three prostate cancer cell lines with IQ2-P leads to its dephosphorylation (Table 1) in a time-dependent manner and the formation of many fluorescent water-insoluble IQ2-OH crystals (Table 2; Fig. 4A, Before washing). Most of these very large (up to 20 μm long) crystals are washed

### Table 1. PAP expression and hydrolysis of IQ2-P by mammalian cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PAP</th>
<th>IQ2-P hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>Yes*</td>
<td>Yes</td>
</tr>
<tr>
<td>PC-3</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>22Rv1</td>
<td>?</td>
<td>Yes</td>
</tr>
<tr>
<td>HMEC</td>
<td>?</td>
<td>No</td>
</tr>
</tbody>
</table>

*See refs. 36–38.
†See refs. 33, 34.
‡See ref. 35.
| Not known. |
away when the cells are rinsed in PBS and fixed in alcohol. A few crystals, however, are often seen associated with the cells (Table 2; Fig. 4A, After washing). That some of these precipitated $^{125}$I$\text{Q}_2$OH molecules remain cell bound was confirmed when tumor cells were incubated with $^{125}$I$\text{Q}_2$P and processed by autoradiography (Fig. 4B). No hydrolysis (no fluorescent IQ$_2$OH crystal formation) was observed when human mammary epithelial cells (HMEC) were incubated with IQ$_2$P (Fig. 4C) or when $^{125}$I$\text{Q}_2$P was incubated in medium without cells for up to 48 h. Similar findings (minimal fluorescence or its absence) were also obtained after a 24-h incubation of normal mouse tissues (kidneys, liver, and spleen) with IQ$_2$P (5). Taken together with the data showing that (a) IQ$_2$P docks very favorably into PAP (Fig. 2) and is readily hydrolyzed by PAP (Fig. 3) and (b) prostate cancer cell lines are not known to express ALP, we hypothesize that this dephosphorylation is mediated by PAP secreted by each of the three prostate tumor cell lines used [LNCaP cells are known to express and secrete endogenous PAP (36–38); PC-3 cells have been reported to have either elevated (33, 34) or undetectable (35) levels of PAP; and 22Rv1 cells have an unknown PAP status]. If this prediction is correct, it would indicate that all three cell lines secrete PAP.

In 1930, Warburg (39) was the first to report the remarkable extent to which tumor cells are able to convert notable amounts of carbohydrates into lactic acid. Until recently, it was assumed that

### Table 2. Semiquantification of IQ$_2$OH crystals in prostate cancer cell lines

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>LNCaP</th>
<th>PC-3</th>
<th>22Rv1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre wash</td>
<td>Post wash</td>
<td>Pre wash</td>
</tr>
<tr>
<td>1</td>
<td>+3</td>
<td>ND</td>
<td>+1</td>
</tr>
<tr>
<td>3</td>
<td>+4</td>
<td>ND</td>
<td>+2</td>
</tr>
<tr>
<td>6</td>
<td>+4</td>
<td>ND</td>
<td>+3</td>
</tr>
<tr>
<td>24</td>
<td>+5</td>
<td>+4</td>
<td>+4</td>
</tr>
<tr>
<td>48</td>
<td>+5</td>
<td>+4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

---

**Figure 4.** A, hydrolysis of IQ$_2$P induced by *in vitro* incubation with viable human prostate cancer cell lines LNCaP, PC-3, and 22Rv1 showing dephosphorylation of prodrug and crystallization of IQ$_2$OH before (left, low power) and after (right, high power) washing. B, autoradiography of 22Rv1 cells previously incubated with $^{125}$I$\text{Q}_2$P showing hydrolysis of prodrug and precipitation of $^{125}$I$\text{Q}_2$OH (intense grains associated with cell membranes). C, no hydrolysis of IQ$_2$P induced by *in vitro* incubation with viable human normal cells (HMEC). Cell nuclei counterstained blue with DAPI.
the intracellular pH (pH_i) of tumor cells is acidic. However, it has become apparent that the pH_i is in fact either neutral or slightly alkaline (40–42), whereas the extracellular pH (pH_e) is acidic (43, 44). Furthermore, it has also become evident that high lactate levels, the presumed cause of the decrease in pH_e, are associated with, and an increase in the likelihood of metastases (45–47). Consequently, extracellular proteins, such as secreted PAP and glycosphosphatidylinositol-anchored ALP, as well as therapeutic molecules unable to permeate into cells, such as IQ2-P, are exposed to the acidic environment of solid tumor masses. As the pH optimum for PAP is ~6–7 (48) and that for ALP is >9 (49), it follows that the rate of hydrolysis for phosphorylated substrates within the interstitial fluid is likely to be much higher in PAP-expressing tumors. In addition, the relative ALP-IQ2-P and PAP-IQ2-P binding energies and predicted K_i and IC_{50} values are lower for PAP (see table within Fig. 2), supporting the view that prostate cancer (i.e., PAP-expressing tumors) may be a better candidate than ALP-expressing tumors for the EMCIT approach.

In conclusion, using advanced computational data-mining and modeling methods, we have identified PAP as a suitable target for the EMCIT technology being developed in our laboratories. The water-soluble prodrug IQ2-P has been docked in silico into the crystal structure of PAP. The iodinated PAP substrate [125I]IQ2-P and [125I]IQ2-P have been synthesized. The incubation of [125I]IQ2-P with PAP in solution leads to the formation of its dephosphorylated analogue [125I]Q2-OH. Similarly, the in vitro incubation of IQ2-P with several human prostate tumor cell lines (but not normal cells) results in the hydrolysis of this water-soluble, nonfluorescent prodrug and the formation of water-insoluble, fluorescent IQ2-OH crystals, many of which are attached to these prostate cancer cells. It is our hope that these quinazolinone-based radiopharmaceuticals will eventually be developed into a novel, noninvasive method for imaging [125I]-SPECT and [124I]-PET and treating [131I] prostate tumors and their metastases. In addition, the proposed EMCIT approach may (a) function as a prognostic marker for the noninvasive sensing of precancerous, cancerous, and metastatic signatures of prostate cancers in individual patients; (b) move meaningful intervention to a much earlier point in cancer progression; (c) provide a technique for evaluating the early response of individual tumors to therapy, thus facilitating selection of effective treatment by allowing rapid identification of ineffective treatments whose side effects might not be balanced by expected benefits; and (d) allow detection, diagnosis, staging, and treatment to be closely coupled.

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