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

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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 prostastic acid phosphatase (PAP) as an enzyme overexpressed in prostate cancer and secreted in the extracellular space. Using AutoDock 3.0 software, the prodrug ammonium 2-(2′-phosphoryloxyphenyl)-6-iodo-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 IQ2-OH derivative; (125IQ2-OH) was shown (1, 3, 5). These studies also indicated that (125IQ2_p, its radioiodinated analogue (125IQ2_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-iodo-4-(3′H)-quinazolinone (125IQ2_p, and its radioiodinated analogue (125IQ2_p) were 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; (6) the in vitro incubation of 127IQ2_p/125IQ2_p derivatives with several ALP-expressing human and mouse tumor cell lines results in the efficient and rapid formation of the corresponding water-insoluble derivatives 127IQ2_OH/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 dose, whereas similar injection into normal tissues (e.g., muscle) leads to little measurable hydrolysis 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 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 (AP; EC 3.1.3.1), a hydrolase with monophosphoesteratic 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-iodo-4-(3′H)-quinazolinone (IQ2_p), and its radioiodinated analogue (IQ2_p) were synthesized, purified, and characterized (1, 5), and their ALP-mediated hydrolysis to the water-insoluble, fluorescent 2-(2′-hydroxyphenyl)-6-iodo-4-(3′H)-quinazolinone (IQ2_p, and its radioiodinated analogue (IQ2_p) were 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 vivo incubation of 127IQ2_p/125IQ2_p derivatives with several ALP-expressing human and mouse tumor cell lines results in the efficient and rapid formation of the corresponding water-insoluble derivatives 127IQ2_OH/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 dose, 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). Consequently, we recognized that the

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 levels 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).

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1 http://www.cancer.org
synthesis of its stannylated quinazolinone precursor (SnQ₂-P) produces a mixture of two compounds, SnQ₂-P and SnQ₂-P(I) (~1:1 ratio), whose radioiodination leads to the formation of ¹²⁵I-Q₂-P and its cyclic isoform ¹²⁵I-Q₂-P(I), respectively. Both iodinated molecules have been docked onto ALP, and the data indicate that the calculated binding energies for ¹²⁵I-Q₂-P are more favorable than for its cyclic isoform (3). Furthermore, it has been shown that pure ¹²⁵I-Q₂-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 IQ₂-P and IQ₂-P(I) was achieved, and binding free energy (ΔG, a measure of binding strength), intermolecular interactions, and binding affinity in terms of the inhibition constant (Kᵢ) were obtained. We also incubated the prodrugs ¹²⁷I-Q₂-P and ¹²⁵I-Q₂-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 (¹²⁵I-Q₂-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
LSGraph version 1.5 (Life Science Graph, IT.Omics, Lille, France) and Ingenuity Pathways Analysis (IPA) version 2.0 (Ingenuity Systems, 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). 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 and minimized into the optimal conformation with its module MM2 [molecular mechanics method to final root-mean-square (RMS) gradient of 0.100 Å 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 phosphoramidic group in IQ2-P(I)).

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\begin{array}{lll}
\Delta G [\text{kcal/mol}] & -11.90 & -13.39 (-12.86) ^* \\
K_i [\text{at } T = 298.15 \text{K}] & 1.89E-09 & 1.52E-10 (3.77E-10) ^* \\
IC_{50} [\text{units/µL}] & 0.0036 \pm 0.0003 (0.029 \pm 0.008) ^* \\
\end{array}
\]

*corresponding value for ALP (6).

Fig. 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, His17 is ideally positioned for nucleophilic attack on phosphorus atom and Asp258 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−) or monoanionic (IQ2−) 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 Å: 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− and IQ2−(I) are shown in Fig. 2. Binding energy (ΔG) and binding constants (Ki) 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 123IQ2-P to drug 125IQ2-OH in solution. The ammonium 2-(2-phosphorylaminophenyl)-6-[125/127I]-iodo-4-(3,6-diamino-2-phenylindole

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-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 has been shown to catalyze the dephosphorylation of SnQ2-P(I) to SnQ2-P and the production of IQ2-P(I) to IQ2-P by human prostate tumor cells and normal mammalian cells assessed by fluorescence microcopy and autoradiography. The following cell lines were used: LNCaP [American Type Culture Collection (ATCC)], a cell line isolated from a 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 [123I]IQ2-P (0.1 mg/mL, fluorescence microscope studies) or [127I]IQ2-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/light).
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 IQ2-P and its cyclic analogue IQ2-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 IQ2-P in size and in the presence of aromatic pharmacophores and a phosphorus-containing bulky anionic group. BABPA is also a potent PAP inhibitor (IC50 4 nmol/L; refs. 17, 23), and the predicted (calculated) binding constant of IQ2-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 Arg11, Arg15, Arg79, His12, and His257 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 (∆G) and the binding constant (Kd), 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 Arg11, Arg15, Arg79, His12, His257, and Asp258. The benzylamino and benzyl moieties of the docked compound are within van der Waals distance of Ile18 and Trp174 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 His257 as seen in the crystal structure complex or in other phosphate-based inhibitors (24, 25).

The PAP inhibitor BABPA and our compound IQ2-P share some chemical-structural similarities that suggest that IQ2-P may be a
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 radioiodinated derivative, exists as two isomers (SnQ2-P and SnQ2-P(I)) whose radioiodination 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 phosphoramic group becomes chemically adverse to nucleophile attack when IQ2-P(I) is docked into the active site of PAP with the phosphoramic 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(I) 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 radioiodinated prodrug 125IQ2-P to radioiodinated 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 radioiodinated quinazolinone derivatives during transit/journey 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 purposefully 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 radioiodinated 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 levamisole, 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

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**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 /no</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.
1See refs. 33, 34.
2See 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$Q_2$-OH molecules remain cell bound was confirmed when tumor cells were incubated with $^{125}$I$Q_2$-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 $^{125}$IQ$_2$-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>
<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>5</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 $^{125}$IQ$_2$-P showing hydrolysis of prodrug and precipitation of $^{125}$IQ$_2$-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 (pHi) of tumor cells is acidic. However, it has become apparent that the pHi is in fact either neutral or slightly alkaline (40–42), whereas the extracellular pH (pHe) is acidic (43, 44). Furthermore, it has also become evident that high lactate levels, the presumed cause of the decrease in pHe, are associated with, and a predictor of, an increase in the likelihood of metastases (45–47). Consequently, extracellular proteins, such as secreted PAP and glycosylphosphatidylinositol-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 (~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 Kᵢ and IC₅₀ 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 EMICT approach.

In conclusion, using advanced computational data-mining and modeling methods, we have identified PAP as a suitable target for the EMICT technology being developed in our laboratories. The water-soluble prodruk IQ2-P has been docked in silico to the crystal structure of PAP. The iodinated PAP substrate (125IQ2-P and 127IQ2-P) has been synthesized. The incubation of 125IQ2-P with PAP in solution leads to the formation of its dephosphorylated analogue 125IQ2-OP. 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 prodruk and the formation of water-insoluble, fluorescent IQ2-OP 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 (123I-SPECT and 124I-PET) and treating (131I) prostate tumors and their metastases. In addition, the proposed EMICT 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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