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A Preliminary Assessment of the Structure-Activity Relationship of Benzimidazole-Based Anti-Proliferative Agents

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Abstract

PDK1 is pivotal in the development and progression of several cancers. A 3D pharmacophore was developed for pyrazole derivatives displaying anti-proliferative activity and PDK1 inhibition. The pharmacophore was utilized in the design of benzimidazole analogs. Our preliminary results indicate the pharmacophore should be useful in designing PDK1 inhibitors and anti-proliferative agents.

Keywords

3D Pharmacophore; Benzimidazole; PDK1; Prostate cancer; SAR; Anti-proliferative

INTRODUCTION

With cancer-related fatalities being the second leading cause of death in the US, understanding the activity of effective chemotherapeutic agents is critical to addressing prostate and other cancers. The mechanism of action for a number of chemotherapeutic agents has been attributed to their activity in the PI3K pathway. PDK1 is a significant component of this pathway and is over-expressed in a number of tumor cells [1]. The protein controls apoptosis and other biological features of carcinogenesis through the phosphorylation of protein kinases involved in cell cycle regulation [2,3]. Most notably, the protein mediates phosphorylation of the AGC family of protein kinases (derived from protein kinase A, G and C) which are known to regulate cell growth, differentiation and survival. Studies utilizing knockout and *knockin* mice showed that PDK1 regulates cell size in embryonic stem cells. The studies also showed that the absence of the kinase was detrimental to cell survival [4,5]. In addition, the inhibition of this kinase has been linked to the induction of mitochondrial mediated apoptosis and decreased proliferation in PC-3 cells (human prostate cancer cell line) [6,7]. Due to its pivotal role in the development of cancer and the regulation of cell cycle, PDK1 has been targeted in the development of chemotherapeutic agents for prostate cancer.

The activities of AGC substrates relating to their role in carcinogenesis is strongly dependent on their ability to bind to and be phosphorylated by PDK1. The phosphorylation process can be inhibited by small ligands that are able to bind to the PDK1 active site and displace ATP [8,9]. In the absence of ATP, PDK1 is unable to gain an active conformation and therefore cannot phosphorylate its substrates or enhance the biological mechanisms that lead to the formation and survival of tumors. This connection suggests that designing molecules based on PDK1 might lead to more effective anti-proliferative agents.

Studies of the FDA approved celecoxib molecule (**1**) indicate that the molecule induces apoptosis by inhibiting the PDK1/Akt interaction in a process independent of its cyclooxygenase-2 (COX-2) activity [8]. Expanding the utility of this molecule, Zhu *et al.* created a library of celecoxib derivatives that diminished cell proliferation by inhibiting PDK1 activity in PC-3 cells at low micromolar concentrations. The versatility of their most successful analog, OSU-03013 (**2**), has been documented for a number of tumors. While its mechanism of action has not been fully characterized, the molecule demonstrated the ability to prevent ATP binding in the PDK1 protein. Their work supports the premise that the PDK1 binding/inhibition may result in the pro-apoptotic activity of a compound. Molecular modeling studies conducted for the pyrazole derivatives shows that the molecule forms hydrogen bonds with Ser160 and Ala162. The interaction promotes binding and ATP inhibition in PDK1 and has been exploited in the design of other PDK1 ligands [7, 9–11]. This binding mode was reevaluated for celecoxib and its derivatives to reveal that a more stable protein-ligand complex could be afforded through electronic interaction with Glu209 and Asp223 [12]. The collective work further supports the hypothesis that high affinity binding to PDK1 is directly related to the anti-proliferative activity of the molecule.

While their mechanism of action has not been fully characterized, celecoxib derivatives have the ability to prevent ATP binding in the PDK1 protein. We have evaluated the structural features of the pyrazole derivatives that are believed to lead to the anti-proliferative activity and PDK1 binding affinity. The analysis resulted in the development of a 3D “fuzzy” pharmacophore, which identifies broad structural considerations that are believed to facilitate the high affinity binding of small ligands to the protein. A comparative analysis of binding modes for both the benzimidazole- and the pyrazole-based compounds has been completed. The studies suggest that the pharmacophore would be useful in the design of molecules with similar or improved activity to that of the celecoxib analogs. In addition, it provides a plausible mechanism of action for the anti-proliferative effects of the benzimidazoles and illustrates that the molecules maintain a similar binding mode to that of celecoxib. In this preliminary study we have synthesized benzimidazole derivatives and analyzed the anti-proliferative activity of the molecules. The molecules were designed using a fuzzy pharmacophore based on known anti-proliferative agents whose activity has been attributed to the inhibition of PDK1. In addition, we describe the potential of the benzimidazole derivatives to act as PDK1 ligands by comparing the various docked orientations of the molecules in the x-ray crystal structure of PDK1.

RESULTS AND DISCUSSION

Chemistry

The preparation of several analogs has been accomplished using the synthetic route outlined in Scheme 1. Under microwave irradiation, a 4-substituted aniline derivative (**4**) was coupled to 1-fluoro-2-nitrobenzene (**3**) to give a nitro diaryl amine (**5**). In the domestic microwave, the reaction produces yields greater than 99.5% when the aniline substituent was an electron donating group [13]. However, optimizing the reaction condition was very difficult and relied heavily on the placement of the vessel into the microwave cavity to ensure reproducibility.

When the reaction was conducted in the CEM Discovery System (a single mode industrial microwave able to maintain the temperature and pressure of the reaction mixture) the reaction time was decreased from 20 minutes to 5 minutes in some cases. The CEM reaction gave reproducible results and the crude reaction mixture showed no evidence of side products. The nitro group of the diaryl amine was reduced using zinc and ammonium chloride. After 24 hours, 90–100% of the nitro compound was reduced to the diamine (**6**). Ring formation was afforded from a condensation reaction of the diamine and the acylchloride (**7**) in two steps to give the target molecules **9–11**.

Biological Assay

Compounds **9–11** were analyzed for their ability to inhibit cell proliferation in human prostate cancer cells. All compounds demonstrated an inhibition similar to that of the OSU compounds with low micromolar potencies, Fig. (1). In addition, compound (**9**) was 10- μ M more effective at diminishing proliferation in PC-3 cells than its imidazole counterpart celecoxib (**1**) [8,14]. There appears to be a correlation between the number of aromatic atoms and the activity of the molecule. The *in vitro* assay demonstrates that the benzimidazole template is ideal for the design of anti-proliferation agents. Additional analyses of molecules are ongoing.

Pharmacophore Development and Molecular Design

PDK1 is a central regulatory agent in the formation and progression of many tumors. As such, designing molecules that target the protein is instrumental to the development of anti-cancer drugs. In the present study a fuzzy pharmacophore was generated based on pyrazole derivatives with known PDK1 inhibition. The pharmacophore identified the broad structural consideration that may facilitate a small ligand bind with high affinity to the protein. Of the

35 molecules examined, the five molecules with the highest Gold score were selected to produce the pharmacophore. The alignment of analogous structural features of the selected molecules highlighted three main structural components: 1) an *N*-aryl group with an electronic component to facilitate hydrogen bonding, 2) a region of hydrophobicity, and 3) a large substituent with aromatic character. The x-ray crystal structure of known PDK1 ligands (BX analogs, staurosporine and ATP) indicated that when bound, the ligand forms a hydrogen bond with Ala162 of the protein [10]. As a result, the inhibitor activity of small ligands has been accredited to its ability to interact with Ala162 as it prevents ATP binding. The generated pharmacophore accounts for this showing that the electronic groups of celecoxib derivatives are in close proximity to Ser160 and Ala162, Fig. (2). There is also a potential for this group to interact with the Glu209 and Asp223. The pyrazole ring is appropriately positioned to accept a hydrogen bond from Thr222 (not shown). The hydrophobic contact with the unsubstituted carbon of the pyrazole ring is also highlighted in the pharmacophore. This interaction is said to be key to inducing apoptosis in human prostate cancer PC-3 cells [8]. There is a large lipophilic volume defined by residues 88–96 that accommodates the size of the phenanthrene group (located on C-3). Most significantly, adjacent to this region aromatic residues are perfectly positioned to interact with the phenanthrene group. The existence of the pocket suggests that the degree of aromatic character is more important than the size of the group. That is, a large non-aromatic substituent may diminish binding in this region in comparison to an aromatic substituent of similar size. The CF₃ group represents another hydrophobic contact. The moiety extends away from the hinge region and resides in an area lined with several hydrophobic residues (not shown; Lys163, Ile221, Leu212, and Gly165).

The benzimidazole derivatives were designed utilizing ligand/structure based methodologies facilitated by the fuzzy pharmacophore. The key pharmacophoric features are the electronic nature, size and aromatic character of its substituents. It was conceptualized that the collective hydrophobic area of the CF₃ group and the unsubstituted pyrazole carbon could be replaced by a single entity. Therefore, the benzimidazole group was selected as a replacement for the CF₃ substituted pyrazole ring. While the aromatic sulfonamide moiety was maintained in the novel benzimidazole molecules for direct comparison to celecoxib, the *N*-aryl group was varied as indicated in Table 1. There is not a linear correlation between the lipophilicity of the celecoxib/pyrazole analogs and their ability to inhibit PDK1 kinase activity or proliferation in PC-3 cells. However, plotting the lipophilicity versus size (calculated molar volume) indicated that the celecoxib analogs that displayed the largest PDK1 inhibition and anti-proliferative activity had similar size to lipophilicity ratios (graph not shown). The size to lipophilicity ratios of the benzimidazoles described in this work (**9–11**) are similar to that of OSU-03013, the most active pyrazole compound (**2**, Table 1). The two primary substituents are characterized by their size and polarity. For this reason, the molar volume and the lipophilicity are viewed as key quantitative descriptors of the molecules. While this ratio cannot solely determine the ability of the molecule to effectively inhibit cell proliferation or PDK1 activity, the information collectively with additional data demonstrates the molecules plausible mechanism of action and their similarity to the celecoxib analogs. Providing further support for the use of the benzimidazole group,

overlays of benzimidazole (**9**) aligned with pyrazole (**12**) showed that there is favorable structural correlation between the pyrazole and benzimidazole analogs, Fig. (3).

Three potential binding modes were evaluated for celecoxib (**1**) and the *N*-tolyl substituted benzimidazole (**9**). The binding modes were calculated for the interaction of the sulfonamide group with polar residues in the protein (Ser160/Ala162, Mode 1, Fig. (4); Glu209, Mode 2, Fig. (5); Asp223, Mode 3, Fig. (6)). Based on the calculated binding energies, the PDK1-benzimidazole complex is more stable than the PDK1-pyrazole complex in two of the three binding modes. Therefore, the larger central ring of the benzimidazole molecule should be better accommodated in the active site than the smaller ring of the pyrazole molecule. The reversal of this observation resulted when the sulfonamide group formed a hydrogen bond with Asp223, Fig. (6). This can be attributed to the aromatic substituent moving away from the aromatic pocket designated in the pharmacophore. In Mode 1, the nitrogen of the benzimidazole molecule forms a hydrogen bond with Thr222. However, hydrogen bonding to the central ring of celecoxib was not observed. This potentially is the cause for the enhanced stability of benzimidazole-PDK1 complex over that of the celecoxib-PDK1 complex. Based on the binding energies it appears that the most favorable binding orientation occurs when the hydrogen bonding component interacts with Glu209 in Mode 2. It is believed that Mode 2 represents the ideal benzimidazole-PDK1 binding orientation as it maximizes the pi-stacking interactions and supports the need for large aromatic substituents. Based on reported biological data there should be a direct correlation between PDK1 inhibition and the size of the aromatic substituent, further supporting Mode 2 as the best binding orientation.

CONCLUSION

The fuzzy pharmacophore shows that the benzimidazole molecules should mimic the activity of the pyrazole based compounds. Together with the fuzzy pharmacophore analysis, the *in vitro* assay supports that the benzimidazole template is ideal for the design of anti-proliferation agents. The pharmacophore will have vast utility in the area of computer aided drug design relating to the development of prostate cancer agents and in our research.

EXPERIMENTAL

Synthesis

All reagents and solvents were purchased from Fisher Scientific and Sigma Aldrich. Microwave syntheses were conducted in a CEM Discover LabMate. Mass Spectral data was collected on the Micromass Q-ToF Ultima spectrometer. ¹H NMR data was collected at on Varian INOVA 400 MHz spectrometer. Chemical shifts are expressed relative to tetramethylsilane (TMS) and deuterated acetone. Melting points were determined on the Electrothermal MelTemp Manual Melting Point Apparatus and are uncorrected.

Synthesis of N-(2-Nitrophenyl)-2-naphthalenamine—Potassium fluoride (7 mmol) and potassium carbonate (7 mmol) were combined and ground into a powder. The powder was transferred to a vial and 2-naphthalenamine (7 mmol) was added. 1-fluoro-2-nitrobenzene (7 mmol) was added to the vial and the vial was tapped to facilitate mixing.

The vial was irradiated in a microwave oven (160 °C, 300W, 10 minutes, 29 psi). The mixture was cooled to room temperature before water was added and the organic compound was extracted into ethyl acetate. Sodium chloride was added and the aqueous layer was extracted 3 times with ethyl acetate. The organic solution was then washed twice with 10% HCl. The resulting white solid was filtered and the filtrate was washed 2 times with sodium bicarbonate. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure on the rotary evaporator. The desired compound was eluted by flash chromatography using 1% ethyl acetate/95% petroleum ether. *N*-(2-nitrophenyl)-2-naphthalenamine was obtained in 43% yield as a yellow solid. m.p. 159–161 °C. *m/z* 265.1 (M+H)⁺. ¹H NMR (CDCl₃, 400 MHz) δ 6.79 (t, *J* = 15.2 Hz, 1 H); 7.31 (d, *J* = 8.8 Hz, 1 H); 7.37–7.41 (m, 2H); 7.47–7.51 (m, 2 H); 7.72 (sd, *J* = 1.6 Hz, 1 H); 7.78 (d, *J* = 7.2 Hz, 1 H); 7.84 (d, *J* = 7.6 Hz, 1 H); 7.88 (d, *J* = 8.4 Hz, 1 H); 8.22 (dd, *J* = 10, 1.6 Hz, 1 H); 9.5 (s, 1 H).

Synthesis of 1-(2-naphthalenyl)-2-[4-(methylsulfonyl)phenyl]-1H-benzimidazole (11)—

The *N*-(2-nitrophenyl)-2-naphthalenamine (4 mmol) was dissolved in methanol (15 mL) and transferred to a round-bottom flask. To the solution was added 12N HCl (15 mL) and the suspension was cooled to 0°C before adding tin chloride (22 mmol). The mixture was heated to 60°C and monitored by TLC until completion. Upon completion, the crude mixture was diluted with water and extracted with ethyl acetate. The combined organic extracts were washed with saturated sodium bicarbonate, dried over anhydrous sodium sulfate, filtered and the solvent was removed on the rotary evaporator. The resulting diamine, *N*-(2-aminophenyl) naphthalen-2-amine, was carried to next step without further purification.

4-Carboxybenzenesulfonamide was refluxed in thionyl chloride. Thionyl chloride was removed on the rotary evaporator producing 4-aminosulfonylbenzoyl chloride (**7**) which was carried to the next reaction without further purification. Separately, the *N*-(2-aminophenyl)naphthalen-2-amine (3.8 mmol) was dissolved in tetrahydrofuran (3 mL) and triethylamine (3.8 mmol) was added to the solution. A solution of **7** (3.8 mmol) dissolved in tetrahydrofuran (2 mL) was slowly added. The mixture was stirred at room temperature for 2 hours. The triethylamine and the tetrahydrofuran were removed on the rotary evaporator. The crude mixture was taken up in ethyl acetate (10 mL) and *p*-toluenesulfonic acid was added. The mixture refluxed overnight. The mixture cooled and the resulting precipitate was filtered and washed with ethyl acetate to produce 37% of **11** as a lavender solid. m.p. decomposition at 260 °C. *m/z* 400.1 (M+H)⁺. ¹H NMR (CD₃COCD₃, 400 MHz) δ 6.85 (d, *J* = 8 Hz, 1 H); 7.17–7.23 (m, 2 H); 7.32 (t, *J* = 14.4 Hz, 1 H); 7.43 (t, *J* = 16 Hz, 1 H); 7.56 (t, *J* = 15.2 Hz, 1 H); 7.68–7.73 (m, 6 H); 7.84 (d, *J* = 7.2 Hz, 1 H); 8.11 (d, *J* = 8.4 Hz, a H); 8.17 (q, *J* = 9.6 Hz, 1 H).

Biological Assay [15]

Cell Culture and Drug Treatment—PC-3 human prostate cancer cells were seeded in duplicate 96 well tissue culture plates at 500 cells/well in 80-μL Dulbecco's minimal essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and nonessential amino acids and 1% equimolar mixture of streptomycin-penicillin. The flasks

were incubated in a humidified 95% air/5% CO₂ atmosphere. The cells were grown for at least 24 hours to ensure that they were in the early log phase of growth. Cells were then treated with 20-μL drug solutions (stock 10 mM drug solution in DMSO was diluted in DMEM containing 5% FBS such that final DMSO concentration was less than 1%) of appropriate final concentration by serial dilution. Untreated control cells received 20-μL DMEM containing 5% FBS. Six wells of each plate received the same drug concentration. Thus, each data point and error bars are an average and standard deviation of readings from 12 wells treated with identical drug concentration.

DNA Assay—After appropriate time of incubation, DNA assay was performed. For DNA assay, each culture plate was washed with 25% PBS and incubated in deionized double distilled water for at least one hour at room temperature. Plates were frozen at −70 °C and thawed/equilibrated to room temperature. Hoechst 33258 DNA binding dye was then added to each well in 200-μL of high salt TNE buffer (10 mM Tris, 1mM EDTA, 2 M NaCl [pH 7.4]) at a final concentration of 6.7-μg/mL. After further incubation at room temperature for 2 hours under protection from light, culture plates were scanned on the CytoFluor 2350™ scanner using the 360/460 nm filter excitation and emission set. Readings from all plates were normalized to the fluorescence of control untreated cells and all data were expressed as the percent control.

Fuzzy Pharmacophore [16]

Structural alignment of the molecules was accomplished using the flexible alignment tool of MOE with default settings and the MMFF94. The molecules were docked in the crystal structure of PDK-1 in complex with ATP (PDB code 1H1W) using the GOLD software. The fuzzy pharmacophore was then generated for the docked structures with the highest GOLD score using *Pymol* and the *Liquid* software plug-ins.

Structural Overlay

The B3LYP hybrid functional with a double-zeta valence-polarization DFT basis set was used to perform DFT geometry optimizations of the benzimidazole and pyrazole derivatives on a 32-processor Xeon cluster using the NWChem computational chemistry program for parallel computers [17]. The benzimidazole and pyrazole derivatives were superimposed using the ‘superpose’ function in Tinker [18]. The alignment was based on the atoms shown in Fig. (7).

Docking

The crystal structure of PDK-1 complexed with ATP (PDB code 1H1W) was subjected to the deletion of heteroatoms (phosphoserine, glycerol and sulfate ions), and the addition of polar hydrogens. Missing atoms were added to Gln73, Arg75, Arg238, Glu303, Lys304, Glu348, Lys357, and the missing residues Glu233, Ser234, Lys235 and Gln236 were added using DS Visualizer v1.6 and the Extensible Computational Chemistry Environment [19]. The Gasteiger partial atomic charges and the gas-phase minimum energy structure were computed with MOE using the MM94x force field and default parameters [20]. MOE was used to designate the rotatable bonds in the ligand and predict its bound conformations in the binding cavity. The docked protein-ligand structures with the most favorable hydrogen

bonding to Ser160/Ala162, Glu209, Asp223 were then placed in a cubic solvent box and energy minimized in the same manner as the ATP-bound structure.

Calculation of Binding Energies and QSAR Parameters

Binding affinities were calculated using the MM94x force field implemented in MOE by taking the difference between the energy of the bound system and the isolated protein and ligand using the optimized protein-ligand complexes described above. In all cases, the binding energies indicate favorable ligand binding.

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Fig. (1).

The curve shows the dose dependent effects of benzimidazole analogs on the proliferation in PC3 cells. The concentrations required for the inhibition of cell growth was less than 25- μM for the compounds tested.

Fig. (2).

Fuzzy Pharmacophore - Overall description of the potential interactions of the molecule with PDK1 when docked in the ATP binding pocket of the protein. Green = hydrophobic interactions; Red = hydrogen bond acceptor; Blue = hydrogen bond donor. Polar residues (Ser160, Ala162, Glu209, and Asp223) are positioned to interact with the polar *N*-aryl substituent of the molecule. The aromatic residues are in close proximity to the aromatic substituent of the molecule.

Fig. (3).
Overlays of benzimidazole compound **9** (pink) aligned with celecoxib analog **12** (green).
The molecules are aligned along the central ring.

Fig. (4).

Model1 describes the binding mode when hydrogen bonding occurs between the sulfonamide groups of celecoxib (**1**) and benzimidazole analog (**9**), A and B respectively, and Ser160 and/or Ala162. The aromatic group is positioned near Leu88 and Val96. The nitrogen of the benzimidazole can form a hydrogen bond with Thr222 of the protein.

Fig. (5).

Mode 2 describes the binding mode when hydrogen bonding occurs between the electronic groups of celecoxib (**1**) and benzimidazole analog (**9**), A and B respectively, and Glu209. The aromatic group is near Val96 and Leu212.

Fig. (6).

Mode 3 describes the binding mode when hydrogen bonding occurs between the electronic groups of celecoxib (**1**) and benzimidazole analog (**9**), A and B respectively, and Asp223. The aromatic group is near Val96 and Leu212.

Fig. (7).
The overlay was accomplished by aligning the atoms of the rings as indicated.

Scheme 1.

Reagents: a) KF, K₂CO₃, MW; b) Zn, NH₄Cl, anhydrous CH₃OH, ; c) N(CH₂CH₃)₃, THF, room temp.; d) Ethyl acetate, .

Table 1

Listed are the Structures of Selected Pyrazole Derivatives and the Designed Benzimidazole Derivatives. The Experimental Anti-Proliferative Activity and the Calculated QSAR Descriptors (Molar Volume and Lipophilicity) of Compounds are also given

No.	Compound	Molar Volume (cm ³ /mol)	cLogP	PC3 IC ₅₀ (μM) ¹	No.	Compound	Molar Volume (cm ³ /mol)	cLogP	PC3 IC ₅₀ (μM) ²
1		314.88	2.99	30	9 ¹		336.75	3.09	24
2		403.38	5.40	3	10		389.50	4.29	20
12		400.12	5.41	32	11		368.75	4.05	10

Values obtained from reference [8].

Values extracted from the dose dependent curve Fig. (1).