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Discovery of novel 2-aryl-4-benzoyl-imidazole (ABI-III) analogues targeting tubulin polymerization as antiproliferative agents

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Abstract

Novel ABI-III compounds were designed and synthesized based on our previously reported ABI-I and ABI-II analogs. ABI-III compounds are highly potent against a panel of melanoma and prostate cancer cell lines, with the best compound having an average IC₅₀ value of 3.8 nM. They are not substrate of Pgp and thus may effectively overcome Pgp mediated multidrug resistance. ABI-III analogs maintain their mechanisms of action by inhibition of tubulin polymerization.

Keywords

Melanoma; prostate cancer; multidrug resistance; tubulin polymerization inhibitor; antiproliferative activity

Introduction

Due to their critical role in mitosis and cell division, tubulin polymerization represents an excellent cancer drug target, with several clinical drugs developed (e.g., paclitaxel, docetaxel and vinblastine).^{1, 2} However, one major problem for current tubulin inhibitors is the development of drug resistance in cancer patients. P-glycoprotein (Pgp)-mediated multidrug resistance is one major reason for failure of treatment by paclitaxel and vinblastine.^{3, 4} Accordingly, the search for novel tubulin inhibitors that can overcome multidrug resistance has intensified in recent years; and as a result, a number of active compounds have been identified.^{5–13} Our search has focused on the discovery and optimization of novel tubulin inhibitors with high potency and acceptable pharmacologic and pharmacokinetic properties.^{9, 10, 12–16}

We previously reported the discovery of ABI-I/II (2-aryl-4-benzoyl-imidazole, Figure 1) analogs with potent antiproliferative activity against a panel of melanoma and prostate cancer cell lines.^{12, 13} We modified the A and B rings of these analogs by introducing different functional groups to explore their effects on the activity.¹³ The A ring modification with a bulky 2-(3',4',5'-trimethoxyphenyl)-indole ring (Figure 1, **21b**) displacing a phenyl ring led to significantly decreased activity; B-ring modification by methylation or benzylation on the imidazole NH resulted in similar or decreased activity for *N*-methylated

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Supporting Information Available: Synthesis of **6b–e**, NMR and LC-MS data are available via the internet at <http://pubs.acs.org>.

and *N*-benzylated compounds, respectively. Fused A/B ring (Figure 1, ABI-II) resulted in much lower activity for compounds with substitution on the position-6 of the indole ring, while compounds with substitution on position-5 showed moderate activity.

Since indole is a very important building block for many biological active agents including tubulin inhibitors,^{17–19} after analyzing the structure-activity-relationships (SAR) of ABI-I and ABI-II analogs, we designed and attempted to synthesize compound **21a** (Figure 1) with an un-substituted indole as A ring. However, due to the lack of selectivity with the benzoylation reaction, we only obtained **21b** with an extra 3',4',5'-trimethoxyphenyl on the indole 2-position.¹³ This compound showed only moderate activity, probably due to the extra bulkiness from the 3',4',5'-trimethoxyphenyl group in the indole 2-position which may not fit into the binding pocket of tubulin. Our continued efforts resulted in the discovery of compound **6a** with a 3-indole as A ring (Figure 1). We found **6a** demonstrated significantly improved potency with an average IC₅₀ value of 3.8 nM in five tested cancer cell lines. Encouraged by this discovery, we performed a focused SAR study on the indole ring (Figure 1, ABI-III) by (a) varying the X in the indole A-ring (X=NH, O, S); or (b) retaining the indole nitrogen but introducing a methyl or a bulkier phenylsulfonyl group on the indole nitrogen atom. In this paper we report the synthesis and biological testing for these highly potent compounds on both resistant and parental cancer cell lines. We confirmed the mechanism of action of these novel compounds is through the inhibition of tubulin polymerization. In addition, we performed *in vitro* assays of Pgp function to further demonstrate that these new analogs are not substrate of Pgp and thus can effectively overcome Pgp-mediated multidrug resistance that are common to several existing anti-mitotic drugs.

Results and Discussion

Chemistry

The synthesis of compound **6a–c** is outlined in Scheme 1. Briefly, the indole-3-carboxaldehyde compound **1** was protected by the phenylsulfonyl group on the indole NH to afford *N*-protected indole-3-carboxaldehyde **2**. Compound **2** was reacted with glyoxal and ammonium hydroxide to generate the 2-aryl-1*H*-imidazole compound **3**. Protection of the imidazole NH on compound **3** with phenylsulfonyl provided the intermediate **4**, which was coupled with 3,4,5-trimethoxy benzoyl chloride in the presence of tert-butyl lithium to produce compound **5**. The benzoylation occurred in imidazole position 4 because of less steric hindrance in the two tautomers as confirmed by 2D NMR studies (supplementary S8–15). Removal of the protecting group from compound **5** by sodium hydroxide or by tert-butylammonium fluoride (TBAF) yielded compound **6a** and **6b** respectively. **6a** was further methylated by methyl iodide to produce the dimethylated compound **6c**.

Compounds **6d** and **6e** were synthesized following the route outlined in Scheme 2. The benzofuran (**8d**) or benzothiophene compound (**8e**) was converted to compound **9(d, e)** in the presence of ammonium hydroxide and glyoxal to construct the imidazole scaffold. The imidazole ring of compound **9(d, e)** was protected by a phenylsulfonyl group to achieve *N*-protected compound **10(d, e)**. Finally, compound **6(d, e)** was generated through a one-pot reaction by coupling **10(d, e)** with 3,4,5-trimethoxy benzoyl chloride followed by treating with tert-butylammonium fluoride to remove the protecting group.

Antiproliferative Activities of ABI-III in Melanoma and Prostate Cancer cells

The antiproliferative activity of these compounds was evaluated in two human metastatic melanoma cell lines (A375 and WM164) and three human prostate cancer cell lines (LNCaP, PC-3, and Du 145) using the methods described previously.^{12, 20} Colchicine was

used as a positive control. The ability of these new analogs to inhibit the growth of cancer cell lines is summarized in Table 1. The 3-indole compound **6a** showed high activity (3.8 nM; unless specified, the IC₅₀ value for each compound is the average of all five cancer cell lines), identifying this compound as the most potent compound in the ABI family (I/II/III) to date. The bulky phenylsulfonyl group in the 3-indole ring (**6b**) resulted in 50-fold lower activity (196.2 nM), suggesting the size of the substituent on the indole NH may play an important role in determining activity. Consistent with this hypothesis, the dimethylated 3-indole compound **6c** showed very good potency (39.8 nM), indicating that a smaller group on either the imidazole- or indole-NH was well tolerated. The benzofuran compound **6d** and benzothiophene compound **6e** showed relatively strong activity (41.2 nM, 35.0 nM for **6d** and **6e** respectively), although it is about 10-fold less active than the 3-indole compound **6a**, implying that the replacement of the 3-indole NH with either an oxygen or a sulfur does not provide any beneficial effect on the activity.

Effects of ABI-III Compound on Paclitaxel Resistant Cell Line

Paclitaxel resistance is a major mechanism accounting for therapeutic failures for the clinical use of paclitaxel and vinblastine.^{21–23} Our previous ABI-I/II analogs were shown to effectively overcome paclitaxel resistance.¹³ We hypothesized the ABI-III analogs modified based on ABI-I/II scaffold would retain the ability to circumvent paclitaxel resistance mechanisms. To support our hypothesis, we examined the antiproliferative activity of compounds **6a–e** in a paclitaxel resistance cell line: PC-3/TxR, with paclitaxel as positive control.²⁴ In the PC-3/TxR cell line, more than 200 genes are upregulated in addition to Pgp overexpression, which may represent additional paclitaxel drug resistance mechanisms.²⁴ As indicated in Table 2, compounds **6a–e** showed equal potency on both the parental (PC-3) and its paclitaxel resistant cell line (PC-3/TxR) with resistance indices ranging from 0.9 to 1.1. While paclitaxel showed picomolar activity (0.4 nM) in the parental PC-3 cells, it demonstrated significantly lesser potency (188.0 nM) in the PC-3/TxR cell line with a resistance index of 437. These results clearly indicated that ABI-III analogs can overcome paclitaxel-resistance mechanisms and suggest that they may provide a therapeutic advantage over paclitaxel.

ABI-III Compound **6a** Is Not a Substrate of Pgp

The results from paclitaxel resistant cells not only confirmed our hypothesis that the ABI-III compounds can overcome paclitaxel resistance, but also guided us to perform the Pgp-glo assay to examine the effect of compound **6a** on Pgp ATPase. In the Pgp-glo assay, ATP was incubated with recombinant human Pgp and then unmetabolized ATP in the presence of test compound was detected as a luciferase-generated luminescent signal. The decreases in luminescence after drug treatment reflect ATP consumption by Pgp. ATP consumption by Na₃VO₄ is attributed to minor non-Pgp ATPase activities in this system. The changes of luminescence of compound treated samples from the one of Na₃VO₄ treated samples were plotted to illustrate the stimulation or inhibition of Pgp ATPase activity by compound treatment (Figure 2). Verapamil, a well-known Pgp substrate, stimulated Pgp ATPase activity resulting in significantly decreased luminescence ($P < 0.05$). However, no significant difference in luminescence changes was observed between the vehicle control treated group and **6a** treated groups (up to 1 μ M), suggesting that **6a** is neither a stimulator nor an inhibitor for Pgp ATPase. The result from Pgp-glo assay strongly indicates that **6a** is not a substrate of Pgp, and can at least partially explain the ability of ABI-III compounds on overcoming paclitaxel resistance.

Tubulin Polymerization Assay on ABI-III Compounds

We hypothesized that ABI-III compounds maintain their mechanism of action by inhibiting tubulin polymerization based on the structure similarity of ABI-III and ABI-I/II analogs. To confirm our hypothesis, we conducted *in vitro* tubulin polymerization assays on ABI-III analogs. Bovine brain tubulin (>97% pure) was incubated with compounds **6a–e**, at concentrations of 5 and 10 μ M. Colchicine at 5 μ M was used as a positive control. Compounds **6a–e** inhibited tubulin polymerization in a dose-dependent manner (Figure 3). Complete inhibition of tubulin polymerization was observed after 20 min of treatment with **6a** (Figure 3A) at 10 μ M, while 70% inhibition was achieved by **6a** at 5 μ M. Compound **6b** (Figure 3B) inhibited tubulin polymerization by 67% and 85% at 5 and 10 μ M, respectively. Compound **6c** (Figure 3C) inhibited 65% and 80% of tubulin polymerization at 5 and 10 μ M, respectively, while 30% and 60% inhibition of tubulin polymerization was observed for compound **6d** (Figure 3D) at 5 and 10 μ M, respectively. Compound **6e** (Figure 3E) at 10 μ M inhibited tubulin polymerization to an extent of 30%, but had no effect on tubulin polymerization 5 μ M (same as control DMSO). These results clearly indicate that the ABI-III compounds are strong inhibitors of tubulin polymerization and the inhibitory effects are roughly proportional to the relative antiproliferative potency for **6a** (IC_{50} =3.8 nM), **6b** (IC_{50} =196.2 nM), **6c** (IC_{50} =39.8 nM), **6d** (IC_{50} =41.2 nM), and **6e** (IC_{50} =35.0 nM).

Molecular modeling studies on 6a

Tubulin polymerization assay suggest that ABI-III compounds exert their effects by inhibiting tubulin polymerization. To better understand how ABI-III analogs interact with tubulin, we investigated the theoretical binding mode of **6a** at colchicine binding site in tubulin dimer using Schrodinger 2011 molecular modeling suite (Schrodinger, Inc., New York, NY). We tried to dock compound **6a** and **21b** into two different tubulin crystal structures (PDB ID code: 1SA0 or 3HKD). However, compound **21b** did not fit the binding pockets in either crystal structures due to the extra bulkiness provided by the 3',4',5'-trimethoxyphenyl on the indole 2-position, which may explain the low activity of this compound (2.9 μ M).¹³ Compound **6a** demonstrated excellent binding in these models with glide scores of -7.42 and -10.50 for 1SA0 and 3HKD, respectively. This result is consistent with our previous observation that TN16-tubulin complex (3HKD) fits ABI scaffold better than the colchicine-tubulin complex (1SA0).¹³

The overview of binding site of **6a** and TN-16 in 3HKD is showed in Figure 4A in which the mesh indicates residue surface within 4 angstrom from TN-16. This binding pocket is located on the interface between α and β -subunit of tubulin dimer and extended inside to the nucleoside-binding domain the β -subunit.^{25, 26} Figure 4B illustrated the close view of the potential binding pose. Generally, **6a** (orange tube model) overlapped very well with TN-16 (green wire model). The 3-indole group of **6a** penetrates deeply into the bottom of the pocket. The interaction was strongly stabilized by two hydrogen bonds: the first one provided by the indole NH and ASN167 in β -S5; the second formed between imidazole NH and VAL238 in β -H7, while the native ligand, TN-16, has only one hydrogen bond with VAL238 in β -H7 in this modeling study. The 3, 4, 5-trimethoxybenzoyl group (C ring) of **6a** extends toward the α/β interface, similar to the mode of active ABI-II compound.¹³ The C ring moiety contributes to the binding interaction by forming the third hydrogen bond between one of its methoxyl groups and the hydroxyl group of THR197 in α -T5, which further enhances the interaction between **6a** and the tubulin dimer. The three observed hydrogen bonds along with the possible hydrophobic interaction provided by the indole A ring and phenyl C ring, can at least partially explain the high potency of **6a**.

Conclusion

Novel ABI-III analogs were synthesized and a focused SAR study was performed. The ABI-III compounds were highly potent against melanoma and prostate cancers *in vitro*. The 3-indole compound **6a** was identified as the most potent ABI analog. The ABI-IIIs were not substrates of Pgp, and demonstrated equal potency on both a paclitaxel resistant cancer cell line and its matching parental cell line. Thus they can effectively overcome Pgp-mediated multidrug resistance and paclitaxel resistance. Mechanism of action studies suggest that the ABI-III compounds exert their effect by inhibiting tubulin polymerization. Molecular modeling provides insights into the binding of ABI-III analogs in tubulin. In summary, novel ABI-III analogs represent a potent, new class of tubulin inhibitors that hold great potential for development into more effective therapeutics for treatment of paclitaxel resistant cancers.

Experimental Procedures

All reagents for the synthesis were purchased from commercial sources and were used without further purification. Moisture-sensitive reactions were carried out under an argon atmosphere. NMR spectra were obtained on an Agilent Inova-500 MHz spectrometer (Agilent Technologies Inc., Santa Clara, CA). Mass spectral data was collected on a Bruker ESQUIRELC/MS system (Bruker Daltonics, Billerica, MA) equipped with an ESI source. The purity of the final compounds was analyzed by an Agilent 1100 HPLC system (Santa Clara, CA). HPLC condition: 90% methanol at flow rate of 1.0 mL/min using a Luna-PFP 5 μ m column (250 $\times$ 4.6 mm) purchased from Phenomenex (Torrance, CA) at ambient temperature. UV detection was set at 280 nm. Purities of the compounds were established by careful integration of areas for all peaks detected and 95%.

2-(1*H*-indol-3-yl)-1*H*-imidazol-4-yl(3,4,5-trimethoxyphenyl) methanone (**6a**)

To a solution of (1-(phenylsulfonyl)-2-(1-(phenylsulfonyl)-1*H*-indol-3-yl)-1*H*-imidazol-4-yl)(3,4,5-trimethoxyphenyl)methanone **5** (0.66 g, 1 mmol) in ethanol (40 mL) and water (4 mL) was added sodium hydroxide (0.4 g, 10 mmol) and stirred overnight under refluxing condition in darkness. The reaction mixture was diluted by 50 mL of water and extracted by ethyl acetate (200 mL). The organic layer was dried over magnesium sulfate and concentrated. The residue was purified by flash column chromatography (hexane: ethyl acetate 1:1) to give a yellow solid. Yield: 60%. Mp 210–212°C. ¹H NMR (CD₃OD, 500 MHz) δ 8.31 (d, *J*=6.5 Hz, 1 H), 7.99 (s, 1 H), 7.90 (s, 1 H), 7.48–7.52 (m, 3 H), 7.24–7.28 (m, 2 H), 4.00 (s, 6 H), 3.93 (s, 3 H). MS (ESI) calcd for C₂₁H₁₉N₃O₄ 377.1, found 400.1 [M + Na]⁺. HPLC: t_R 4.33 min, purity >99%.

The synthesis and chromatographic data for **6b–d** and their intermediates are detailed in supplementary materials.

Cell Culture and Cytotoxicity Assay

Melanoma cell lines (A375 and WM-164) and prostate cancer cell lines (LNCaP, DU 145, and PC-3) were purchased from ATCC (American Type Culture Collection, Manassas, VA). Paclitaxel-resistant PC-3/TxR, and its parental cell lines were gifts from Dr. Evan Keller at the University of Michigan. Melanoma cells were cultured in DMEM and prostate cancer cells were cultured in RPMI 1640 (Cellgro Mediatech, Inc., Herndon, VA) supplemented with 10% FBS. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were exposed to different concentrations of a test compound for 48 h (fast growing melanoma cells) or 96 h (slow growing prostate cancer cells) in three to five replicates. Cell numbers at the end of the drug treatment were measured by the

sulforhodamine B (SRB) assay using a plate reader (DYNEX Technologies, Chantilly, VA). Percentages of cell survival versus drug concentrations were plotted, and the IC₅₀ values were obtained by nonlinear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA).

Pgp ATPase assay

Pgp ATPase assay was conducted using the Pgp-Glo Assay System (Promega, Madison, WI) according to the manufacturer's protocol. The luminescence of the sample reflects the ATP level in the sample, which is negatively correlated with the activity of Pgp ATPase and was detected using a Synergy 4 Hybrid Multi-Mode Reader (BioTek Instruments, Winooski, VT). Accordingly, the stimulator of Pgp ATPase will result in significantly lower signals than untreated samples. Na₃VO₄, a selective inhibitor of Pgp without Pgp ATPase activity, and verapamil, a known Pgp substrate with the ATPase activity stimulation, were used as control substrates as provided by Pgp-Glo™ Assay System.

In Vitro Microtubule Polymerization Assay

Bovine brain tubulin (0.4 mg) (Cytoskeleton, Denver, CO) was mixed with 5, 10 μM of the test compounds or colchicine at 5 μM (positive control) and incubated in 110 μL of general tubulin buffer (80 mM PIPES, 2.0 mM MgCl₂, 0.5 mM EGTA, and 1 mM GTP) at pH 6.9. The absorbance at 340 nm was monitored every 1 min for 20 min by the SYNERGY 2 microplate reader (Bio-Tek Instruments, Winooski, VT). The spectrophotometer was set at 37 °C for tubulin polymerization.

Molecular Modeling

All molecular modeling studies were performed with Schrodinger Molecular Modeling Suite 2011 (Schrodinger LLC, New York, NY) running on a Dell Linux workstation. We selected tubulin complex with TN16 (PDB code: 3HKD) as our modeling system because of the structure similarity between ABI-III analogs and TN16. ABI-IIIs were built and prepared using the Ligprep module, and they were docked into the TN16 site by the Glide module in the Schrodinger Suite. The best docking complexes were subject to restricted molecular dynamics to release any strains by using the MacroModel module with OPLS-2005 force field. The ligand and its surrounding residues within 15 Å were allowed to move freely while the outer atoms are frozen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ABI	2-aryl-4-benzoyl-imidazoles
MDR	multidrug resistance
Pgp	P-glycoprotein

SAR	structure-activity relationships
TMS	tetramethylsilane
TBAF	tert-butylammonium fluoride
RT	Room temperature

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Figure 1.
Structures of ABI-I/II/III.

Figure 2. Effect of **6a** on Pgp ATPase activity. Change in luminescence compared to 100 μ M Na_3VO_4 treated samples was plotted (Mean $\pm$ SD, $n=3$). **6a** showed similar effect as vehicle control on Pgp ATPase activity, indicating **6a** is not substrate for Pgp. * $p<0.05$.

Figure 3.

Effect of ABI-III compounds on tubulin polymerization *in vitro*. Tubulin (0.4 mg/assay) was exposed to 5, 10 μ M ABI-III compounds (vehicle control, 5% DMSO) 6a (panel A), 6b (panel B), 6c (panel C), 6d (panel D), 6e (panel E) or colchicine (positive control, at 5 μ M). Absorbance at 340 nm was monitored at 37 $^{\circ}$ C every minute for 20 min.

Figure 4.
Binding modes of 6a in the colchicine binding site of tubulin.

Scheme 1.
Synthesis of **6a–c**

Scheme 2.
Synthesis of **6d** and **6e**

Table 1

In vitro growth inhibitory effects of ABI-III compounds on melanoma and prostate cancer cells.

Structure	ID	X	Y	IC ₅₀ ±SEM (nM)(n=4)					
				A375	WM164	LNCaP	PC-3	DU 145	Average
6a	NH	NH	3.2±1.2	5.3±2.0	2.8±0.6	3.7±0.3	3.9±1.0	3.8
	6b	N-PhSO ₂	NH	414.0±35.4	166.2±18.9	107.0±17.5	105.0±2.1	189.0±13.5	196.2
	6c	N-CH ₃	N-CH ₃	18.8±3.6	24.2±4.1	35.9±3.3	40.7±5.1	76.5±4.1	39.8
	6d	O	NH	53.4±12.5	73.6±15.2	14.1±6.2	33.3±2.1	45.0±9.6	41.2
	6e	S	NH	41.0±5.2	45.7±9.3	29.1±3.9	20.8±0.5	51.2±3.5	35.0
Colchicine				20.6±3.6	29.0±5.2	16.3±4.0	11.5±0.1	11.2±1.1	17.7

Table 2

Antiproliferative activity on ABI-III analogs on paclitaxel resistant cell line.

ID	IC ₅₀ ±SEM (nM)(n=4)				
	6a	6b	6c	6d	6e
PC-3	3.7±0.3	33.3±2.1	20.8±0.5	40.7±5.1	105.0±2.1
PC-3/TxR	3.7±0.8	27.6±9.8	22.4±5.1	42.8±11.9	98.6±6.3
RI*	1	0.9	1.1	1	0.9
					437

Resistance indices (RI) were calculated by dividing IC₅₀ values on paclitaxel resistant cell lines PC-3/TxR by IC₅₀ values on the matching sensitive parental cell line PC-3. The larger the RI value, the more resistant the drug.