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Interaction of Pseudolaric Acid B with The Colchicine Site of Tubulin

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Abstract

We purified pseudolaric acid B (PAB) from the root and stem bark of *Pseudolarix kaempferi* (Lindl.) Gordon. Confirming previous findings, we found that the compound had high nanomolar IC₅₀ antiproliferative effects in several cultured cell lines, causing mitotic arrest and the disappearance of intracellular microtubules. PAB strongly inhibited tubulin assembly (IC₅₀, 1.1 μM) but weakly inhibited the binding of colchicine to tubulin, as demonstrated by fluorescence and with [³H]colchicine. Kinetic analysis demonstrated that the mechanism of inhibition was competitive, with an apparent K_i of 12–15 μM. Indirect studies demonstrated that PAB bound rapidly to tubulin and dissociated more rapidly from tubulin than the colchicine analog 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone, whose complex with tubulin is known to have a half-life of 17 s at 37 °C. We modeled PAB into the colchicine site of tubulin, using the crystal structure 1SA0 that contains two αβ-tubulin heterodimers, both bound to a colchicinoid and to a stathmin fragment. The binding model of PAB revealed common pharmacophoric features between PAB and colchicinoids, not readily apparent from their chemical structures.

1. Introduction

The natural world has provided a seemingly limitless number of compounds that interfere with the functioning of microtubules. These compounds all bind to the primary component of these organelles, the heterodimer tubulin, at a number of distinct binding sites [for a review, see ref. 1]. Recently, there have been at least two reports [2, 3] of the interaction with tubulin of a structurally unique agent named pseudolaric acid B (PAB; structure shown in Fig. 1). PAB is the most active constituent extracted from the bark of *Pseudolarix*

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Dedication: This paper is dedicated to the memory of Dr. Arnold Brossi.

Conflict of interest statement: The authors declare that there are no conflicts of interest.

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kaempferi and is characterized by high nanomolar to low micromolar cytotoxicity against a number of cell lines. In their initial description of the antitubulin activity of PAB, Wong et al [2] concluded that the compound weakly inhibited the binding of [³H]colchicine to tubulin and that PAB was thus a colchicine site agent. In contrast, Tong et al. [3] examined colchicine binding to tubulin using the enhanced fluorescence that occurs when colchicine binds to tubulin [4]. They were unable to detect an inhibitory effect of PAB on colchicine binding and concluded that PAB must bind to an alternate, but undefined, site on tubulin.

We, too, had isolated PAB from *P. kaempferi* and had begun to study its interaction with tubulin when the reports of Wong et al. [2] and Tong et al. [3] came to our attention. Our initial findings were more in agreement with the former group rather than the latter, and this led us to study the interaction of PAB at the colchicine site in greater detail.

In this report, we show that PAB has the kinetic properties of a competitive inhibitor of [³H]colchicine binding. We also investigated the effects of PAB on the fluorescence of colchicine bound to tubulin and found that PAB-induced inhibition could be demonstrated, especially at higher PAB concentrations. This led us to investigate the mechanism of PAB binding to tubulin by indirect studies, and these indicated that PAB rapidly bound to and dissociated from tubulin, accounting for both its weak inhibitory effects and the difficulty of documenting inhibition by the fluorescence method.

Finally, because substantial structural differences exist between PAB, colchicine, and other colchicine site agents (selected structures in Fig. 1), determining the binding mode of PAB to tubulin should enhance our understanding of the pharmacophoric features of PAB responsible for its inhibitory activity. To this end, we employed the crystal structure consisting of two $\alpha\beta$ -tubulin heterodimers, both bound to colchicinoid molecules and to a stathmin fragment [5], as a template for modeling PAB bound in the colchicine site.

2. Materials and methods

2.1. Materials

Electrophoretically homogeneous bovine brain tubulin was purified as described previously [6]. [³H] Colchicine was purchased from Perkin-Elmer (Boston, MA) and podophyllotoxin from Sigma-Aldrich (St. Louis, MO). Combretastatins A-2 and A-4, 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone (MTPT), and thiocolchicine were generous gifts, respectively, of Drs. George R. Pettit (Arizona State University), Thomas J. Fitzgerald (Florida State University), and Arnold Brossi (National Institute of Diabetes and Digestive and Kidney Diseases). All cell lines were supplied by the cancer drug screening group of the Developmental Therapeutics Program at the National Cancer Institute at Frederick (Frederick, MD), except for the HOP-18 human lung cancer cell line, which was obtained from the Developmental Therapeutics Program repository at the National Cancer Institute at Frederick (Frederick, Maryland), and the human Burkitt lymphoma CA46 cell line and the *Potorus tridactylis* PtK2 cell line, both of which were obtained from the American Type Culture Collection (Manassas, VA).

2.2. Purification and structure determination of PAB

The air-dried root and trunk barks of *P. kaempferi* (Lindl.) Gorden were purchased from Qiping Local Herbal Medicine Market at Guangzhou, Guangdong, China. The powdered bark (10 kg) was extracted three times with 95% ethanol at room temperature. After evaporation of the pooled solvents, the residue (ca. 500 g) was suspended in 1 liter of 5% NaHCO₃ and extracted with ethyl acetate to afford a neutral ethyl acetate-soluble fraction. The remaining aqueous solution was brought to pH 6 with 5% HCl and extracted with ethyl acetate to obtain an acidic ethyl acetate-soluble fraction. The ethyl acetate fractions were

combined and subjected to silica gel column chromatography. The column was eluted with a gradient solvent mixture of petroleum-ethyl acetate (4:1 to 2:1), followed by CHCl₃-methanol (10:1 to 0:1). Four major subfractions were obtained by pooling fractions with similar TLC patterns. Subfraction 3 showed significant cytotoxicity against a panel of human cancer cell lines in vitro and was further chromatographed on a silica gel column by elution with CHCl₃-methanol (19:1) to afford pure PAB (4.5 g) after re-crystallizations from petroleum ether-acetone (4:1) and from methanol. The purity of the PAB was 98.5% by HPLC. Its structure was confirmed by comparison of its mass and nuclear magnetic resonance spectral data with those in the literature [7].

The properties of PAB are as follows: colorless needles; melting point, 139-140 °C; molecular formula, C₂₃H₂₈O₈; ESI-MS (positive mode): *m/z* 887 [2M+Na]⁺, 869 [2M+Na-H₂O]⁺, 455 [M+23]⁺; ESI-MS (negative mode): *m/z* 863 [2M-H]⁻, 431 [M-H]⁻; ¹H NMR (CDCl₃, δ) 7.24 (1H, brd, H-15), 7.2 (1H, m, H-8), 6.54 (1H, dd, J = 15.1 and 11.4 Hz, H-14), 5.90 (1H, d, J = 15.1 Hz, H-13), 3.70 (3H, s, OCH₃), 3.3 (1H, m, H-3), 3.06 (1H, dd, J = 13.5 and 6.3 Hz, H-5), 2.86 (1H, dd, J = 15.5 and 6.3 Hz, H-6), 2.73 (1H, dd, J = 15.0 and 8.8 Hz, H-9), 2.58 (1H, ddd, J = 15.0, 4.0, and 1.8 Hz, H-9), 2.1 (1H, m, H-6), 2.11 (3H, s, OCOCH₃), 1.94 (3H, d, J = 1.2 Hz, CH₃-17), 1.8 (2H, m, H-1), 1.7 (2H, m, H-2), 1.7 (1H, m, H-5), 1.58 (3H, s, CH₃-12); ¹³C NMR (**6**) 33.2 (C-1), 24.2 (C-2), 49.2 (C-3), 90.0 (C-4), 30.6 (C-5), 20.0 (C-6), 134.4 (C-7), 141.6 (C-8), 27.6 (C-9), 55.2 (C-10), 83.6 (C-11), 28.4 (C-12), 144.4 (C-13), 121.6 (C-14), 138.6 (C-15), 127.8 (C-16), 12.5 (C-17), 173.2 (C-18), 168.0 (C-19), 172.8 (C-20), 52.0 (CH₃O), 21.7 (CH₃CO), 169.4 (CH₃CO).

2.3. Biochemical Methods

The binding of [³H]colchicine to tubulin was measured with DEAE-cellulose filters (Whatman, Ltd, Maidstone, UK), as described in detail by Verdier-Pinard et al. [8]. Incubation times and other specific conditions are described for the individual experiments. Fluorescence measurements of the binding of nonradiolabeled colchicine to tubulin were performed with a Photon Technology International (Birmingham, NJ) fluorimeter, as described by Bhattacharyya and Wolff [4]. Tubulin was the last component added to the reaction mixtures, which were transferred individually to a cuvette warmed to 37°C. Fluorescence readings were taken after 30 min. Excitation was at 353 nm, and emission was read at 430 nm.

2.4. Cytological Methods

Cells were grown in microtiter plates in RPMI 1640 medium with 5% fetal bovine serum at 37 °C in a 5% CO₂ atmosphere for 96 h. Protein was parameter measured by the standard National Cancer Institute sulforhodamine B technique, in which cell protein is the parameter quantitated [9].

2.5. Molecular Modeling

The Maestro 9 (Schrödinger, LLC, New York, NY) modeling software running on a Dell (Round Rock, TX) Precision 690 with Red Hat (Raleigh, NC) Enterprise Linux 4 was used to perform the modeling studies. Simulations were performed *in vacuo* using a distance-dependent dielectric with a nonbonded interaction limited to within 13 Å in an OPLS 2005 force-field. Minimizations involved up to 500 steps of Polak-Ribière conjugate gradient. The 1SA0 crystal structure of tubulin in complex with *N*-deacetyl-*N*-(2-mercaptoacetyl)-colchicine and a stathmin fragment [5] was selected as the template for docking studies and was prepared as previously described [10]. A conformation of the PAB structure was generated and energy minimized. PAB was then oriented in the colchicine site pocket of the 1SA0 crystal structure, using the best pharmacophoric alignment of its stereoelectronic features to that of bound colchicine. The tubulin-PAB complex was subjected to stepwise

refinement. First, with tubulin fixed in Cartesian space, the conformation of PAB in the colchicine site was energy minimized. This was followed by energy minimization of the complex in which all atoms were unconstrained but were limited to movements of less than 0.5 Å. In the final stage, PAB was extracted from the pocket and re-docked into the colchicine site using the Glide program set to its highest precision setting. This resulted in a binding pose with a binding Gscore of -6.7, which indicates favorable binding affinity.

3. Results and discussion

Our initial studies with PAB in cells agreed with those of Wong et al. [2] and Tong et al. [3]. Using combretastatin A-4 as our control antitubulin agent, we obtained IC₅₀ values for PAB against the growth of three human cancer cell lines (breast carcinoma MCF-7, melanoma MDA-MB-435, and Burkitt lymphoma CA46) ranging from 260 to 690 nM versus values from 5 to 20 nM for combretastatin A-4. In CA46 cells treated with 500 nM PAB, we obtained a mitotic index of 57%, versus 3.5% in untreated cells. We also observed in *Potorus tridactylis* PtK2 cells complete disruption of the microtubule cytoskeleton with a sufficiently high concentration of PAB.

As an inhibitor of tubulin assembly, PAB was quite active, yielding an IC₅₀ of 1.1 μM, identical with the value most recently obtained for combretastatin A-4. We used the “standard condition” described previously [11], in which every reaction component was shown to alter the IC₅₀ obtained. It is therefore not unexpected that both Wong et al. [2] and Tong et al. [3] found higher IC₅₀ values (5-10 μM) than we did. We also examined, in comparison with the slow binding colchicine, whether preincubating tubulin with PAB would result in a reduction of the polymerization IC₅₀. With colchicine, the IC₅₀ for assembly decreased over 3-fold, from 7.6 μM to 2.3 μM with a 15 min preincubation at 30 °C in the absence of the GTP required for assembly. With PAB, there was no change in the IC₅₀, whether or not there was a tubulin-compound preincubation. Since tubulin assembly begins within approximately 1-1/2 min of initiating the temperature jump from 0 to 30 °C, this implies rapid binding of PAB to tubulin.

In our initial study of the effect of PAB on the binding of 5.0 μM [³H]colchicine to 1.0 μM tubulin, we found a relatively weak effect on the reaction, with 4% and 31% inhibition at 1.0 and 5.0 μM PAB, respectively, as compared with the 90% and 99% inhibition with 1.0 μM and 5.0 μM combretastatin A-4, respectively. This was, however, stronger inhibition than that reported by Wong et al. [2] (no inhibition with 10 μM and about 50% inhibition with 50 μM PAB, respectively), probably reflecting different reaction conditions. Although somewhat surprising, the discrepancy between inhibitory effects on tubulin assembly and colchicine binding is not infrequently observed, probably reflecting the difference between a catalytic (assembly) and a stoichiometric (colchicine inhibition) reaction. We have, however, noted on several occasions that cytotoxicity in a series of analogs correlates better with inhibition of ligand binding than inhibition of assembly [for an example of a colchicine site series, see ref. 12], so the weak inhibition of colchicine binding by PAB, relative to combretastatin A-4, is concordant with the relative cytotoxicity of the two compounds.

Considering the distinct chemical structures of PAB and colchicine (Fig. 1), we thought it important to determine the mechanism of inhibition by PAB using standard kinetic analysis [13]. The main panel of Fig. 2A shows a Lineweaver-Burke double reciprocal (1/s vs 1/v) plot of inhibitory data obtained with several concentrations of [³H]colchicine and with either 0, 10, 20, 30, or 40 μM PAB. The intercept of the data curves is on the ordinate, as expected for competitive inhibition [13], but, as noted previously [14], with tubulin, the intercepts for both competitive and noncompetitive inhibition (the latter on the negative abscissa) are very close to the origin. We therefore felt it was worthwhile to convert the data to alternate

display methods. We have generally used [14] the Hanes plot [13] for this purpose, and this data conversion is shown in the left-hand inset of Fig. 2A. In the Hanes plot, the data for competitive inhibition should yield a family of parallel curves, when s is plotted against s/v , and this was the case with PAB. With [^3H]colchicine, in contrast to [^3H]vinblastine [14], the parallel curves generated in the Hanes format by competitive inhibitors are often close to horizontal, as is the case in the left-hand inset of Fig. 2A. We therefore also transformed the data into the Woolf format [13], with v/s plotted against v . In this format, competitive inhibition yields a set of curves with negative slopes that intercept the ordinate relatively far from the origin, and this was the case with our PAB data (right-hand inset of Fig. 2A). Finally, these data were plotted in the Dixon format [13], i vs $1/v$, and the data were most consistent with an apparent K_i for PAB of 12–15 μM vs [^3H]colchicine (Fig. 2B). For comparison, using the same reaction conditions, apparent K_i values of 0.12 and 0.5 μM were previously obtained for combretastatin A-4 and podophyllotoxin, respectively [15].

We next wanted to evaluate the effect of PAB on the fluorescence of colchicine when it binds to tubulin [4], since Tong et al. [3] reported, using a preformed tubulin-colchicine complex, that no displacement of colchicine occurred after 60 min at 37 °C with 25 μM PAB. That is to say, there was no reduction in the fluorescence of the tubulin-colchicine complex during the time course of the experiment. In contrast, when tubulin was added to mixtures of colchicine and either PAB or podophyllotoxin, significant inhibition of fluorescence development occurred with both compounds, increasing with inhibitor concentration (Fig. 3). As would be expected from the apparent K_i values summarized above, inhibition by podophyllotoxin was substantially greater than inhibition by PAB. Just over 50% inhibition occurred with 5 μM podophyllotoxin, whereas 50% inhibition would require over 100 μM PAB.

In actuality, the failure of PAB to displace colchicine from the tubulin-colchicine complex is entirely expected from the properties of the colchicine binding reaction [16]. For example, we found that the half-life of the tubulin-colchicine complex is 27 h at 37 °C and 70 h at 30 °C [17]. One would predict that a compound like PAB, which binds rapidly to the colchicine site (see above) and dissociates rapidly (see below), would have negligible ability to displace colchicine from tubulin during the hour-long incubation period used by Tong et al. [3]. On the other hand, PAB should be able to inhibit colchicine binding when added simultaneously to tubulin with colchicine, provided the incubation period is short (we used a 10 min incubation in our [^3H]colchicine studies and a 30 min incubation in our fluorescence studies).

In a previous study [18], we had found that inhibition of [^3H]colchicine binding varied with incubation time. In particular, we found with agents that bound and dissociated rapidly that inhibition decreased as incubation time increased. As a corollary, we found that if colchicine site compounds were prebound to tubulin and subsequently [^3H]colchicine was added, the amount of colchicine bound increased as the incubation time increased. If several agents were examined simultaneously, the rate at which colchicine bound (expressed as relative inhibition of colchicine binding) reflected the relative dissociation rates of the compounds from tubulin.

Such a study with PAB is presented in Fig. 4, in which PAB is compared with thiocolchicine, MTPT, and combretastatin A-2, three compounds that had been previously evaluated by this method [18]. Thiocolchicine was included because its affinity for tubulin is greater than that of colchicine. It both binds more rapidly to tubulin and dissociates more slowly [17, 19]. As a consequence, even after a prolonged incubation (20 h), little colchicine binds to tubulin preincubated with thiocolchicine, and even after 8 h the apparent inhibition of [^3H]colchicine binding was over 90%. In complete contrast, the compound MTPT,

consisting only of the A and C rings of colchicine, with its binding parameters well-established by fluorescence [20-22], is known to bind to and dissociate rapidly from tubulin. Accordingly, in the study presented in Fig. 4, tubulin that was preincubated with MTPT rapidly developed the ability to bind [³H]colchicine, and, by 4 h, inhibition of colchicine binding had decayed to about 50% of that observed with tubulin incubated without a potential inhibitory compound. As in the earlier study [18], combretastatin A-2 was intermediate in its effects between thiocolchicine and MTPT, with 50% loss of inhibition of colchicine binding occurring at about 8 h.

When PAB was examined in this assay (Fig. 4), preincubating the compound with tubulin indicated even faster dissociation than occurred with MTPT. Inhibition of colchicine binding by 50% occurred at 5-10 min. Since the half-life of the tubulin-MTPT complex measured by fluorescence is only 17 s at 37 °C [21], the presumptive half-life of the tubulin-PAB complex must be much shorter. However, it must also be stressed that the data obtained in the experiment of Fig. 4 are dependent on association as well as dissociation rates, since all compounds were present in the reaction mixture during the entire time course of the assay. Nonetheless, the data of Fig. 4 are consistent with extremely rapid dissociation of PAB from its complex with tubulin. The instability of the complex, as compared with the tubulin-colchicine complex, undoubtedly contributed to the inability of Tong et al. [3] to detect an inhibitory effect of PAB on colchicine binding.

It was also of interest to try and determine whether there were any β -tubulin isotype differences in the interaction of PAB with the colchicine site. Although there is substantial clinical and cytological evidence that β III-tubulin overexpression is associated with resistance to taxoids and vinca alkaloids [reviewed in ref 23], little is known about tubulin isotype interactions with colchicine site agents from a clinical perspective. Ludueña and colleagues [24, 25], using bovine brain tubulin preparations enriched for specific β -tubulin isotypes by antibody affinity chromatography, demonstrated differences in affinity of $\alpha\beta$ II, $\alpha\beta$ III, and $\alpha\beta$ IV (the predominant β -isotypes in bovine brain tubulin) for colchicine [24] and nocodazole [25], a colchicine site compound. For colchicine, there is almost an 8-fold difference in the binding rate constants, with $\alpha\beta$ IV binding most rapidly and $\alpha\beta$ III least rapidly. There is an even greater difference in affinity constants: that of $\alpha\beta$ IV was almost 28 times that of $\alpha\beta$ III and 14 times that of $\alpha\beta$ II. For nocodazole, the greatest affinity is for $\alpha\beta$ IV, the least (a 5-fold difference) for $\alpha\beta$ III. In terms of isotype content of bovine brain tubulin [26], ignoring the rare $\alpha\beta$ I (3%), $\alpha\beta$ IV is the least common (13%) and $\alpha\beta$ II the most common (58%), while the proportion of $\alpha\beta$ III is 25%. Thus, the highest affinity for colchicine and nocodazole in bovine brain tubulin is observed for the relatively rare $\alpha\beta$ IV.

Since antibody purified isotypes are not generally available and difficult to prepare, our approach to this question was to use twelve human cancer cell lines maintained by the Developmental Therapeutics Program (eleven in the ongoing drug screening program, one from the repository) that had been characterized by Hiser et al. [27] for their β -tubulin isotype content as well as their sensitivity to paclitaxel and vinblastine. We examined these twelve cell lines for their sensitivity to PAB, and our results, as well as the pertinent data of Hiser et al. [27], are summarized in Table 1. As previously noted by Hiser et al. [27], no obvious correlation is observed between any isotype and sensitivity to either paclitaxel or vinblastine. In particular, note that the cell lines with the highest proportions of β III-tubulin, although in all cases relatively small amounts of β III-tubulin were detected, were not the cell lines most resistant to either paclitaxel (COLO-205 cells) or vinblastine (HCT-15 cells). However, for both paclitaxel and vinblastine, the range of sensitivities was about 70-fold between the most sensitive and the most resistant cell lines.

For PAB, we found a much narrow range of sensitivities among these 12 cell lines. The least sensitive cell lines (HOP-18 and SK-OV-3 cells) had IC_{50} values of 1.2 μM , and the most sensitive (MDA-MB-231 cells) had an IC_{50} of 0.26 μM , for a range of 4.6. Within this narrow range, however, there was no obvious sensitivity correlation with either the rarer isotypes (βII and βIII) or the more common isotypes (βI and $\beta IVa+b$), as is shown in Fig. 5. We conclude, as did Hiser et al. [27], that factors, such as drug uptake, in addition perhaps to β -tubulin isotype content, have significant effects on cell sensitivities to antitubulin agents.

In order to delineate the essential binding interactions of PAB with tubulin, we modeled the binding mode of PAB using the 1SA0 crystal structure of tubulin [5] as a template. Fig. 6 shows the superimposition of the PAB binding pose with that of colchicine derived from the 1SA0 crystal structure. The models show that both PAB and colchicine occupy similar conformational space at the colchicine site, but that the carboxylate side chain of PAB extends beyond the *N*-methyl amide group of colchicine to form novel interactions. The carboxylate group of PAB forms hydrogen bonds to the $\beta Lys252$ ϵ -amino side chain and to the $\alpha Asn101$ side chain NH_2 moiety. This is in contrast to colchicine, which does not extend to this region of the $\alpha\beta$ -subunit interface.

The models also revealed common stereoelectronic features between PAB and colchicinoids. As we previously described [10], the 2-methoxy oxygen of the colchicine phenyl A ring is potentially hydrogen bonded to the $\beta Cys239$ sulfhydryl group and, thus, establishes a key pharmacophoric feature for ligand activity at the colchicine site. As depicted in Fig. 6A, the vinyl-substituted methyl ester group of PAB is nearly superimposable onto the colchicine 2-methoxy group. As a result, like the 2-methoxyl oxygen of colchicine, the carbonyl oxygen of the vinyl-substituted ester in PAB potentially forms a hydrogen bond to the $\beta Cys239$ sulfhydryl group. At the same time, the methyl group of this PAB ester may form similar hydrophobic contacts as the 2-methoxy methyl group of colchicine. (For clarity, the pharmacophoric poses of colchicine and PAB are reiterated separately in Fig. 6B, and the preceding corresponding regions of the two compounds are highlighted in Fig. 6B, as are additional correspondences described below.) Additionally, as previously described [10], the tropolone-attached methoxy group of colchicine contains a key pharmacophoric center in its hydrophobic methyl functionality. As shown in Fig. 6, the bridgehead ester group of PAB has its methyl group nearly superimposed onto the tropolone methoxy methyl group of colchicine, indicating a second common pharmacophoric feature between PAB and colchicinoids. Finally, the tropolone carbonyl oxygen of colchicine was also previously identified as a key hydrogen bond acceptor in its binding to tubulin, being hydrogen bonded to the $\alpha Val181$ backbone NH moiety. As Fig. 6 shows, the carbonyl oxygen of the bridgehead ester of PAB nearly superimposes on the tropolone carbonyl oxygen of colchicine, indicating a third common pharmacophoric feature of PAB that is analogous to that of colchicinoids. In addition, the tropolonic methyl group of colchicine and the bridgehead ester methyl moiety nearly superimpose, representing a fourth common pharmacophoric element shared by the two compounds. In sum, the binding model of PAB revealed common pharmacophoric features between PAB and colchicinoids that are not apparent based on analysis of their distinct chemical structures.

In summary, we have shown that PAB is a competitive inhibitor of the binding of colchicine to tubulin with an apparent K_i of 12-15 μM . PAB, based on indirect evidence, binds rapidly to and dissociates extremely rapidly from the colchicine site. Based on a comparative study with MTPT, whose complex with tubulin has a known half-life of 17 s at 37 °C, it is likely that the half-life of the tubulin-PAB complex at 37 °C is less than one second. Molecular

modeling demonstrated that PAB fit readily into the colchicine site, in agreement with the biochemical findings.

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Fig. 1. Molecular structures of PAB, colchicine, thiocolchicine, MTPT, podophyllotoxin, and combretastatins A-4 and A-2.

Fig. 2.

PAB is a competitive inhibitor of the binding of [^3H]colchicine to tubulin. Incubation was for 10 min at 37 °C. Reaction mixtures contained 1.0 μM (0.1 mg/ml) tubulin, which was the last component added to the reaction mixtures. A. Kinetic analysis of binding data. Symbols: no PAB, ○; 10 μM PAB, △; 20 μM PAB, ●; 30 μM PAB, ○; 40 μM PAB, ◇; 40 μM PAB, ▲. Main panel: Lineweaver-Burk plot. The abscissa units are μM^{-1} colchicine. The ordinate units are $1/\text{pmol colchicine bound}$. Left inset: Hanes plot. The abscissa units are μM colchicine. The ordinate units are $\mu\text{M colchicine}/\text{pmol colchicine bound}$. Right inset: Woolf plot. The abscissa units are $\text{pmol colchicine bound}/\mu\text{M colchicine}$. The ordinate units are $\text{pmol colchicine bound}$. B. Dixon plot for determination of K_i . Symbols: 2 μM colchicine, △; 3 μM colchicine, ●; 4 μM colchicine, ○; 5 μM colchicine, ◇. The abscissa units are $\mu\text{M PAB}$. The ordinate units are $1/\text{pmol colchicine bound}$.

Fig. 3. Inhibition of colchicine binding, measured by fluorescence, by PAB ($\circ$) and podophyllotoxin ($\bullet$). Fluorescence study was performed following the procedure described by Bhattacharyya and Wolff [4]. Reaction mixtures contained $1\ \mu\text{M}$ tubulin, $5\ \mu\text{M}$ colchicine, and PAB or podophyllotoxin at the indicated concentrations. The symbol Δ represents the fluorescence observed with only colchicine in the reaction mixture.

Fig. 4.

Comparison of compound dissociation rates from tubulin, measured indirectly by determining subsequent inhibition of binding of [^3H]colchicine. Reaction mixtures (5 ml) contained the components described previously that stabilize tubulin [8, 18], 1.0 μM tubulin (0.1 mg/ml), and inhibitory compounds at 10 μM : thiocolchicine (◇), combretastatin A-2 (Δ), MTPT (●), or PAB (○). The reaction mixtures were incubated at 37 °C for 30 min and chilled on ice. Aliquots (0.1 ml) of each reaction mixture were distributed into tubes, and 200 pmol of [^3H]colchicine (10 μl) were added to each tube. Samples were then incubated for the indicated times in triplicate at 37 °C. At each time point, the amount of [^3H]colchicine bound to tubulin was determined, and the reaction mixtures with inhibitor were compared with the control containing only [^3H]colchicine, with the data expressed as the percentage of inhibition of colchicine binding.

Fig. 5. Comparison of cell line sensitivity to PAB with their content of $\alpha\beta$ I-tubulin (A) and $\alpha\beta$ IVa +b-tubulin. The specific values are shown in Table 1.

Fig. 6.

Common pharmacophores of colchicine and PAB, based on docking studies. A. Docked pose of PAB in the colchicine site, superimposed onto that of colchicine derived from the 1SA0 crystal structure [5]. Tubulin is rendered in ribbon, with amino acids within 4 Å shown as thin sticks. Hydrogen bonding amino acids are depicted as thick sticks, with carbon atoms from the α - and β -subunits colored purple and grey, respectively. Hydrogen bonds are highlighted by yellow dashed lines. PAB and colchicine are shown in stick with carbon atoms colored green and brown for PAB and colchicine, respectively. The α -tubulin backbone is tinted light purple, while the β -tubulin backbone is grey. Helices are shown as thick ribbons, and β -sheets as thin strands. Nitrogen atoms are blue, oxygen red, and sulfur yellow. Tubulin hydrogen atoms are white, while those of colchicine and PAB are not shown. B. Binding conformations of colchicine and PAB extracted from the models. Colors as in Panel A. Corresponding pharmacophoric features, as discussed in the text, are indicated. Hydrogen bond acceptors and hydrophobic features are shown as red and blue dashed circles, respectively.

Table 1
Correlation of sensitivity to PAB to β -tubulin isotype content in 12 cultured human cancer cell lines

Tumor type	Cell line	β I	β II	β III	β IV a+b	Paclitaxel ^a	Vinblastine ^a	PAB ^b
Relative amount of β -tubulin isotype (%) ^a -----IC ₅₀ (nM) -----								
Colon carcinoma								
	COLO-205	36.0	0	0	64.0	28	0.7	850 $\pm$ 70
	HCT-15	61.8	0	0.2	38.0	4.6	28	530 $\pm$ 100
Breast carcinoma								
	BT-549	65.9	0.6	6.4	27.1	4.9	1.1	440 $\pm$ 200
	T-47D	85.7	0	0	14.3	1.5	9.3	490 $\pm$ 10
	MCF-7	39.1	0	2.5	58.4	1.0	0.4	550 $\pm$ 70
	MDA-MB-231	76.1	0	0	23.9	0.4	1.6	260 $\pm$ 60
Lung carcinoma								
	A-549	71.9	0	1.6	26.5	2.7	1.7	1,100 $\pm$ 200
	HOP-18	63.2	1.5	5.0	30.3	0.7	0.5	1,200 $\pm$ 300
Melanoma								
	MALME-3M	84.4	3.8	5.1	7.0	4.4	0.7	420 $\pm$ 50
	SK-MEL-2	73.1	1.4	1.5	24.0	3.9	0.9	330 $\pm$ 40
Ovarian carcinoma								
	OVCAR-3	97.0	0	0	3.1	1.5	2.5	610 $\pm$ 7
	SK-OV-3	85.6	0	0.8	13.6	3.3	1.6	1,200 $\pm$ 40

^a Data from ref. 27.

^b Average of at least two determinations $\pm$ standard deviation.