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THE DISRUPTION OF BRAIN MICROTUBULES *IN VITRO* BY THE PHOSPHOLIPASE INHIBITOR *p*-BROMOPHENACYL BROMIDE

ALAN J. HARGREAVES,*† ANTHONY P. GLAZIER,* JOHN FLASKOS,‡ FRASER H. MULLINS* and W. GRAHAM MCLEAN*

* Department of Pharmacology and Therapeutics, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K. and ‡ Laboratory of Biochemistry and Toxicology, Faculty of Veterinary Medicine, University of Thessaloniki, 54006, Greece

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Abstract—The influence of *p*-bromophenacyl bromide (pBPAB) and structural analogues on the assembly and Ca^{2+} sensitivity of porcine brain microtubules (MTs) was studied by spectrophotometric measurements *in vitro*. MT assembly was inhibited by 36 μM pBPAB but not by the structural analogues *p*-chlorophenacyl chloride or acetophenone. In the presence of pBPAB, but not structural analogues, the addition of 10 mM Ca^{2+} induced aggregation of polymerized MT protein, whereas a decrease in turbidity (due to MT disassembly) was observed in controls. The effects of pBPAB on both MT assembly and Ca^{2+} sensitivity were blocked by glutathione, but not by *N*-acetyl L-cysteine, *N*-acetyl L-lysine nor L-tyrosine, indicating that a highly reduced sulphydryl group(s) may be involved. Western blotting analyses of drug-treated MTs revealed a form of tubulin with altered electrophoretic characteristics, probably caused by a covalent interaction with pBPAB. MT preparations polymerized in the presence of the drug contained fewer MTs than control samples, the predominant structures being identified as amorphous aggregates of MT proteins. The fact that pBPAB affects MT integrity at an effective anti-inflammatory dose *in vitro* may reflect the involvement of MT disruption in some of the pharmacological effects of this drug. pBPAB is not therefore a suitable tool for studying the specific involvement of phospholipase A_2 in cellular events.

Key words: *p*-bromophenacyl bromide; phospholipase A_2 inhibitor; microtubule assembly

The anti-inflammatory agent pBPAB§ is generally accepted and widely used experimentally as an inhibitor of phospholipase A_2 [1–4]. However, recent work shows that pBPAB can have diverse effects at the cellular level, some of which may not be related to its ability to inhibit phospholipase A_2 [5–10]. In one study it was suggested that pBPAB could act by disrupting the cytoskeleton [7] raising the possibility that this drug might interact directly with MTs, which are known to play a central role in the control of cell division, cell shape, cell surface topography and intracellular transport [11].

Drugs known to interfere with MTs, such as colchicine and its analogues, the Vinca alkaloids and taxol, have been valuable tools in the study of MT functions and in the treatment of a variety of pathological conditions [11, 12]. All of these agents bind to distinct regions of the tubulin molecule and, consequently, they disrupt MTs in different ways [13–19]. Due to the high toxicity of colchicine [20, 21] the Vinca alkaloids and taxol are the preferred compounds in cancer chemotherapy [12]. However, colchicine has been used successfully in the treatment of inflammatory reactions associated with rheumatism and gout [22, 23], suggesting that MTs in themselves represent a potential target for pharmacological intervention in inflammatory disorders.

Our own previous results suggest that at least part of the pharmacological action of pBPAB may involve an interaction with MT proteins [24]. The purpose of the present study was to examine in detail the molecular basis of MT disruption by pBPAB.

MATERIALS AND METHODS

Preparation of MTs. MTs were purified through two cycles of temperature-dependent disassembly and reassembly following the procedure of Shelanski *et al.*[25] with minor modifications [24]. Two-cycle purified MTs were stored as centrifuged pellets, at −80° for up to 2 months. Prior to an experiment, MT pellets were resuspended in MES buffer (100 mM 2-(*N*-morpholino) ethanesulphonic acid, 1 mM $MgSO_4$, 1 mM EGTA, pH 6.8) by glass–Teflon homogenization. This suspension was incubated for 30 min on ice before cold-stable aggregates were removed by centrifugation at 100,000 *g* (av) for 20 min at 4°, leaving the depolymerized MT proteins in the supernatant. The solubilized MT proteins were further diluted in MES buffer to give a protein

† Corresponding author at present address: Department of Life Sciences, The Nottingham Trent University, Nottingham NG11 8NS, U.K. Tel. 0602 418418.

§ Abbreviations: AP, acetophenone; DTNB, dithionitrobenzoic acid; GTP, guanosine triphosphate; MAP, microtubule-associated protein; MTs, microtubules; PAGE, polyacrylamide gel electrophoresis; pBPAB, *p*-bromophenacyl bromide; *p*CPAC, *p*-chlorophenacyl chloride.

concentration of 4–5 mg/mL for use in assembly experiments (see below), unless otherwise indicated.

Turbidimetric measurement of MT assembly. MT formation was measured as the increase in absorbance at 350 nm [26] in a Cecil 550 spectrophotometer over 30 min at 37°, following the addition of 1 mM guanosine triphosphate (GTP; Sigma type II-S). Measurements were made in the presence or absence of pBPAB (10 μg/mL) or its structural analogues *p*-chlorophenacyl chloride (10–40 μg/mL) and acetophenone (10–40 μg/mL). In some experiments, as indicated in the Results, either 1 mM glutathione, 1 mM *N*-acetyl L-cysteine, 1 mM *N*-acetyl L-lysine or 1 mM L-tyrosine were included.

When appropriate, the calcium sensitivity of the polymerized MTs was then assessed by the absorbance change resulting from incubation for 5 min at 37° in the presence of 10 mM $CaCl_2$. Polymerized MTs are normally disassembled under these conditions [27, 28]. Thus, any drug-induced change in their response to Ca^{2+} would be indicative of altered biochemical properties of polymerized MTs.

Electrophoretic and immunological analysis of assembled MTs. After a typical assembly experiment (see above), polymerized MTs were collected by centrifugation at 100,000 *g* (av) for 30 min at 37°. MT pellets were then solubilized by boiling for 5 min in electrophoresis sample buffer, containing 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue. Proteins were then separated by polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) SDS (SDS–PAGE) on a 7.5% polyacrylamide running gel overlaid with a 4% stacking gel [29]. The SDS used in these gels was of the highest purity available from the Sigma Chemical Co. (Poole, U.K.); as a consequence, the α- and β-tubulin subunits migrated as a single Coomassie-stained band.

Electrophoresed proteins were either visualized by staining with Coomassie brilliant blue or, alternatively, they were transferred electrophoretically on to nitrocellulose membrane filters [30]. The resultant western blots were blocked by incubation for at least 1 hr with BSA/TBS (1% w/v bovine serum albumin in 10 mM Tris, 140 mM NaCl, pH 7.4). They were then incubated overnight at 4° with either of the following monoclonal antibodies: YL 1/2, (Seralab, Crawley Down, U.K.) which recognizes tyrosinated α-tubulin [31]; YOL 1/34 (Seralab) a general anti α-tubulin antibody; 6-11B-1 (from Dr G. Piperno) which cross-reacts with acetylated α-tubulin [32]; a β-tubulin monoclonal antibody from Amersham International (Amersham, U.K.). All antibodies were diluted 1/50 in BSA/TBS except anti β-tubulin which was diluted 1/1000.

After three 10 min washes in TBS/Tween (0.05% v/v Tween-20 in TBS) the blots were incubated for 3 hr at room temperature with horseradish peroxidase-coupled rabbit anti-mouse or rat Ig (Dako Ltd, Slough, U.K.), as appropriate, diluted 1/1000 in BSA/TBS. After three further washings with TBS/Tween, antigenic cross-reactivity was detected using 4-chloronaphthol as the substrate [33].

Analysis of MTs by electron microscopy. MTs, polymerized for 30 min in the presence or absence of 10 μg/mL pBPAB, were adsorbed on to carbon-coated 300-mesh electron microscope grids, for 30 sec at room temperature. They were then stained for 5 sec at room temperature with 1% (w/v) aqueous uranyl acetate. After removal of excess stain the samples were air-dried prior to their examination in a Phillips EM series electron microscope.

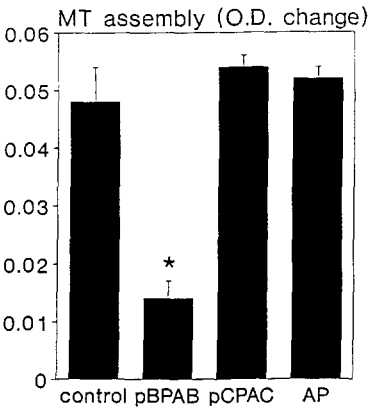

Fig. 1. The effects of pBPAB and structural analogues on brain microtubule assembly. Polymerization of MT proteins (2 mg/mL) in the presence or absence of pBPAB (10 μg/mL), *p*-chlorophenacyl chloride (pCPAC; 10 μg/mL) or acetophenone (AP; 10 μg/mL) was measured turbidimetrically, as the change in absorbance at 350 nm in a spectrophotometer after 30 min incubation with 1 mM GTP. Results are expressed as the mean percentage of control values (±SEM) for each individual experiment (N = 5). Asterisk indicates $P < 0.05$ by paired Student's *t*-test.

Other analytical procedures. Protein was assayed by the method of Lowry *et al.* [34], using BSA as the standard.

RESULTS

In previous work we found that pBPAB inhibited MT assembly by more than 50% at a concentration of 5–10 μg/mL. We also found that the addition of 10 mM Ca^{2+} to preassembled MTs, which induces disassembly in the absence of a drug, caused the aggregation of MT protein into amorphous forms in the presence of pBPAB at 5 μg/mL or higher [24]. The results in Figs 1 and 2 show that similar doses of the structural analogues *p*-chlorophenacyl chloride and acetophenone had no significant effect on either MT assembly (Fig. 1) or the response of polymerized MTs to 10 mM added Ca^{2+} (Fig. 2). These analogues were still ineffective at 4-fold higher doses (not shown). The presence of bromine groups appears, therefore, to be important in the action of pBPAB on MTs.

Electrophoretic analysis of polymerized MTs showed that the pattern of microtubule-associated proteins (MAPs) known to nucleate/stabilize MTs *in vitro* was identical in both untreated and drug-treated MTs. However, an extra (poorly resolved) polypeptide was observed just below the tubulin

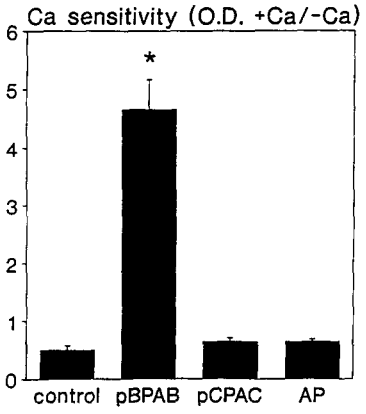

Fig. 2. The effects of pBPAB and structural analogues on the Ca^{2+} sensitivity of polymerized MTs. MTs polymerized as described in Fig. 1, were incubated for 5 min at 37° in the presence of 10 mM $CaCl_2$. Results are expressed as a mean ratio (±SEM) of absorbance change in the absence of Ca^{2+} over that recorded in its presence. Asterisk indicates $P < 0.005$ by paired Student's *t*-test. Slight changes in Ca^{2+} ratios observed with structural analogues were not statistically significant.

band in drug-treated MTs (Fig. 3B). Qualitatively similar gel patterns were observed on gels of total MT protein (not shown).

The possibility that this drug-induced polypeptide could be a modified form of tubulin was examined by probing western blots of equal amounts of control and pBPAB-treated MTs with anti-tubulin antibodies. The new polypeptide cross-reacted with two different monoclonal antibodies YL 1/2 and 6-11B-1 (Fig. 3C and D), which recognize tyrosinated and acetylated forms of α-tubulin, respectively [31, 32]. Another anti α-tubulin antibody YL 1/34 gave the same result (data not shown). However, the modified tubulin polypeptide did not cross-react with a commercial monoclonal antibody that recognizes the β-subunit (Fig. 3E). These data agree with our earlier work showing that pBPAB action is due, at least in part, to a direct effect on tubulin [24].

Further information about the nature of this interaction was then obtained by performing MT assembly and Ca^{2+} sensitivity experiments in the presence of *N*-acetyl L-lysine, *N*-acetyl L-cysteine, glutathione and L-tyrosine. In the presence of 1 mM glutathione the inhibitory effects of pBPAB on MT assembly were almost completely blocked; there was also a substantial reduction in the turbidity increase caused by 10 mM Ca^{2+} in the presence of drug (Table 1). The remaining amino acids had no significant effect on the pBPAB-induced inhibition of MT assembly or on the response of drug-polymerized MTs to Ca^{2+} (Table 1). In the absence of pBPAB neither glutathione nor the free amino acids had any effect. The effects of pBPAB on MT assembly and Ca^{2+} sensitivity may therefore involve a common drug binding site(s).

The binding of pBPAB to tubulin presumably causes a conformational change in the latter which inhibits MT assembly and could also alter the structure of polymerized MTs. The effects of pBPAB on MT structure were therefore studied by electron microscopy of negatively stained MTs. As shown in Fig.4, and in agreement with turbidimetric studies (Fig. 1), there were fewer MTs in samples treated with pBPAB (Fig. 4A) compared with controls (Fig. 4B); the most prominent structures in the former were identified as amorphous aggregates of MT proteins.

DISCUSSION

The results demonstrate that pBPAB is a potent MT-disrupting agent. The inability of two structural analogues of pBPAB, *p*-chlorophenacyl chloride and acetophenone, to inhibit MT assembly or alter Ca^{2+} sensitivity of polymerized MTs provides strong evidence that the effects of pBPAB are specific and dependent on the presence of bromine groups on the phenol ring. The fact that the chlorinated compound has no effect on MTs could be related to differences between the electronegativity and/or molecular size of Cl and Br.

Previous experiments with MAP-free tubulin preparations showed that the effects of pBPAB on Ca^{2+} sensitivity involved a direct interaction of drug with tubulin [24]. Indeed, electrophoretic and immunological analyses of control and drug-treated MTs in the present study demonstrate that a modified form of α-tubulin is obtained in the presence of pBPAB. The detection of the modified α-tubulin after SDS–PAGE is consistent with the possibility that pBPAB binds covalently to this subunit in such a way as to alter its electrophoretic mobility. Although similar changes were not found in β-tubulin or MAPs we cannot rule out the possibility that pBPAB may still bind to these proteins.

Further information about the mechanism of drug binding was obtained by performing turbidity assays in the presence or absence of pBPAB, glutathione and various amino acids. The results showed that the drug-induced inhibition of MT assembly could be blocked by 1 mM glutathione but not by the same concentration of either *N*-acetyl L-cysteine, *N*-acetyl L-lysine or L-tyrosine. The latter three compounds were still ineffective at a concentration of 3.6 mM (not shown), suggesting that the decreased MT formation caused by pBPAB does not simply involve Schiff base formation, binding to any free sulphydryls or non-specific interactions with amino acids. The effect of the tripeptide glutathione, on the other hand, is consistent with the idea that the pBPAB binding site, which causes inhibition of MT assembly and altered Ca^{2+} sensitivity, contains a highly-reduced -SH group and/or more than one amino acid. The exact location of the pBPAB binding site(s) on tubulin is not known. However, based on the above observation and the amino acid sequence data of Postingl *et al.* [35] a potential *accessible* -SH-containing (covalent) binding site for pBPAB on α-tubulin could be residues 304–306 (Lys-Cys-Asp), in which cysteine is flanked by two hydrophilic residues.

Covalent binding of pBPAB to a histidine residue is known to be involved in the inhibition of phospholipase A_2 by this drug [1, 3, 36]. By contrast,

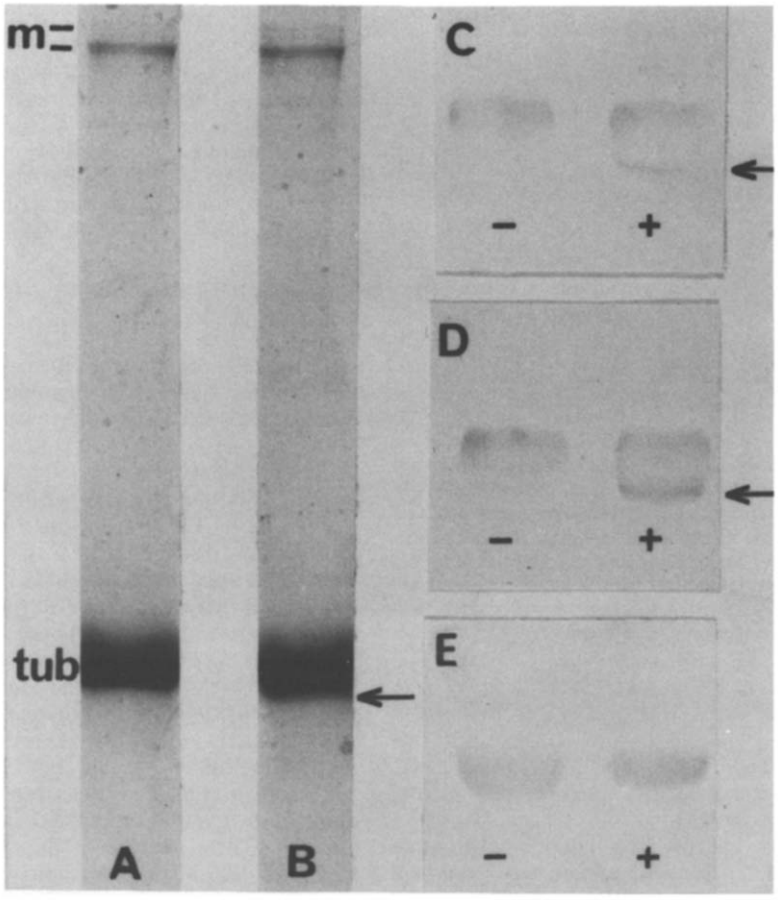

Fig. 3. Electrophoretic and western blotting analyses of MTs polymerized in the presence or absence of pBPAB. MTs polymerized as described in Figs 1 and 2, were collected by centrifugation and the resultant pellets solubilized prior to SDS–PAGE (A, B) and western blotting (C–E) procedures. Shown are: Coomassie-stained electrophoresis gels of MTs polymerized in the presence (B) or absence (A) of pBPAB. Western blots of control (−) and pBPAB (+) polymerized MTs were probed with monoclonal antibodies YL 1/2 (C) and 6-11B-1 (D) against α-tubulin, together with an anti β-tubulin monoclonal antibody (E). The covalently modified form of α-tubulin is indicated by arrows; the bands corresponding to non-modified tubulin (tub) and high molecular weight MAPs (m) are indicated on gel A.

Table 1. The effects of glutathione and amino acids on the disruption of MTs by pBPAB

Additions	MT assembly % Control	MT assembly Significance	Calcium response Ratio	Calcium response Significance
None	56 ± 4	—	2.5 ± 0.8	—
Lysine	56 ± 4	NS	2.8 ± 1.1	NS
Cysteine	53 ± 5	NS	2.3 ± 0.4	NS
Glutathione	82 ± 5	$P < 0.002$	1.2 ± 0.2	$P < 0.05$
Tyrosine	59 ± 5	NS	2.5 ± 0.9	NS

MT assembly and Ca^{2+} sensitivity were measured as described in Figs 1 and 2, in the presence and absence of 10 μg/mL pBPAB with either 1 mM glutathione, 1 mM *N*-acetyl L-cysteine, 1 mM *N*-acetyl L-lysine or 1 mM L-tyrosine. The results are expressed as mean percentages (±SEM, N = 5) of controls containing no pBPAB, in the case of MT assembly, or as the absorbance ratio $+Ca^{2+}/-Ca^{2+}$ in the Ca^{2+} sensitivity test. Statistical significance of data (vs drug only) was estimated by paired Student's *t*-test. NS = not significant.

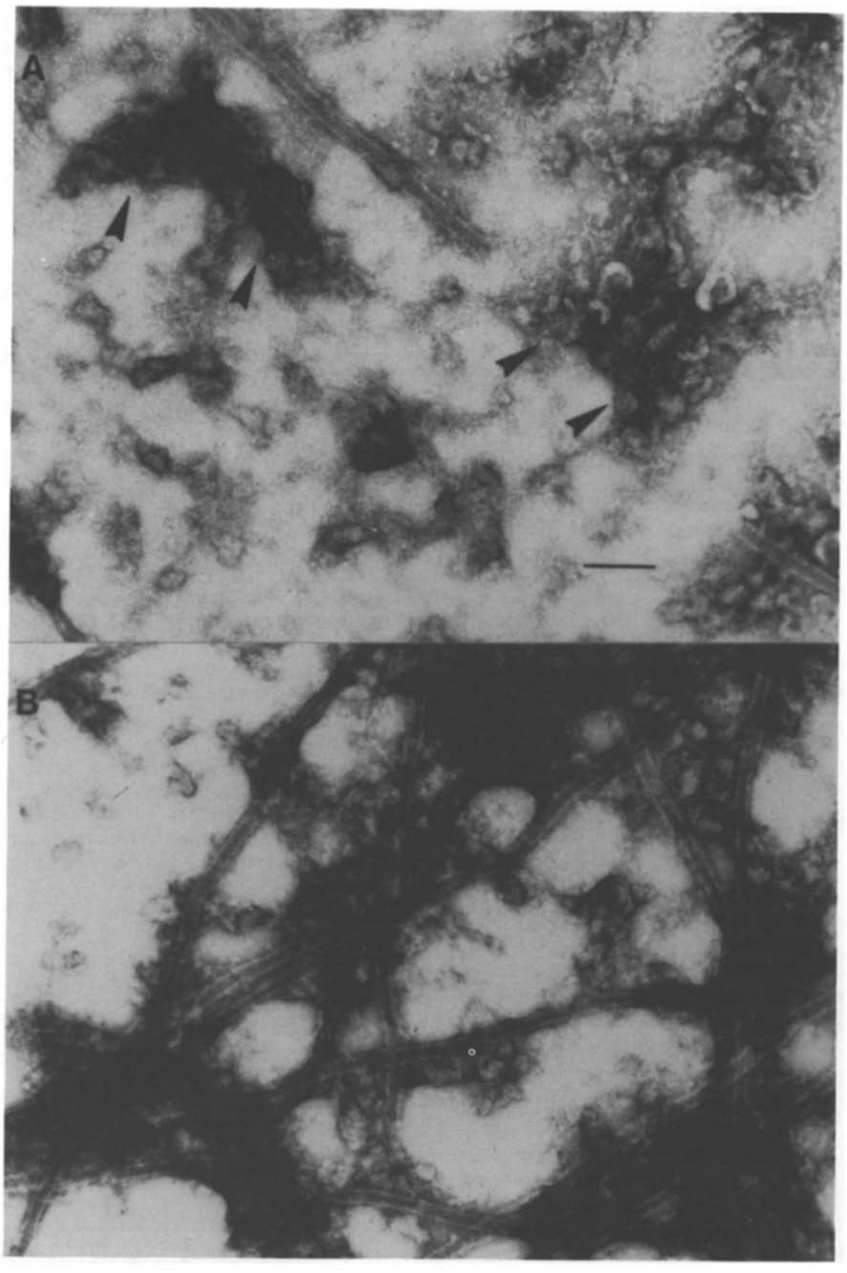

Fig. 4. Electron microscopy of MTs polymerized in the presence and absence of pBPAB. Samples from the experiment described in Fig. 1 were applied to carbon-coated electron microscope grids as described in Materials and Methods. Shown are micrographs of MTs polymerized in the presence (A) and absence (B) of 10 μg/mL pBPAB. Arrow heads indicate the amorphous aggregates that predominated in drug-treated samples. The bar represents 50 nm.

L-histidine was found to have no effect on the inhibition of MT assembly by pBPAB (data not shown), indicating that a specific interaction of pBPAB with a histidine residue on tubulin was unlikely. However, it has been suggested that pBPAB can interact covalently with any nucleophilic group and, *in vivo*, would be expected to alkylate thiol and amine groups of other proteins [3]. Our results indicate that covalent binding to a highly reduced -SH group, but not other nucleophilic groups, is important in the inhibition of MT assembly and alterations in the Ca^{2+} sensitivity of polymerized MTs caused by pBPAB. This idea is supported by the observation that other sulphydryl blocking reagents such as dithionitrobenzoic acid (DTNB) also disrupt MTs [37, 38]. Indeed, our own preliminary data indicate that the binding of DTNB to MT proteins is reduced in the presence of pBPAB. Assuming that all SH blocking is caused by pBPAB binding to tubulin, the complete inhibition of MT

assembly correlates with the binding of approximately 4 moles of drug per mole tubulin dimer. However, further assays with MAP-free tubulin are needed to confirm the exact stoichiometry of tubulin–drug binding.

It is likely that the altered Ca^{2+} sensitivity of MTs is caused by drug-induced conformational changes to tubulin that reveal additional binding sites involved in protein aggregation. However, we cannot rule out the possibility that the Ca^{2+} interacts directly with Br groups of tubulin-bound pBPAB. Indeed, the observation that Ca^{2+} induces aggregation of MAP-free tubulin and that even greater aggregation occurs in the presence of pBPAB [24] is compatible with both possibilities.

In summary, our results demonstrate that the inhibition of MT assembly and the altered Ca^{2+} sensitivity of MTs induced by pBPAB occur through similar mechanisms, involving a direct interaction of drug with α-tubulin subunits. The observation of these effects at or below an effective anti-inflammatory dose may reflect the involvement of MT disruption in the pharmacological action of this drug. It also questions the suitability of pBPAB as a selective inhibitor of endogenous phospholipase A_2 in complex cellular systems, in agreement with other work which shows that pBPAB affects diverse cellular processes [5–10]. Work underway in our laboratory indicates that pBPAB is a strong inhibitor of cell proliferation *in vitro*; our present aim is to confirm the role of tubulin modifications in this process.

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