formed from BHA is unreactive [12] and such radicals only react with another free radical, or oxygen [18], thereby increasing overall scavenging efficiency.

In summary, the resonance-stabilised free radical scavenger butylated hydroxyanisole inhibits alloxan-induced diabetes in mice in a dose and time dependent manner. It is considerably more effective on a molar basis than the conventionally used aliphatic hydroxyl radical scavengers such as dimethylsulfoxide (DMSO), *N,N'*-dimethylurea (DMU), thiourea, or the short chain aliphatic alcohols.

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Activity of benzimidazole carbamates against L1210 mouse leukaemia cells: Correlation with *in vitro* tubulin polymerization assay

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The use of mammalian tubulin as a screen for potential anti-cancer drugs is well documented. Numerous reports on the qualitative correlation between the *in vitro* inhibition of polymerization of tubulin to form microtubules and the anti-cancer activity of vinblastine [1], maytansine [2, 3], podophyllotoxin [4–6], steganacin [6, 7], colchicine [5, 8–10] and their respective analogues have been published. However, this relationship does not extend to the biological actions of all such derivatives, as the analogues of podophyllotoxin, etoposide and teniposide, which do not inhibit the polymerization of tubulin [11] are potent anti-cancer agents, and the colchicine analogues isocolchicine, colchiceine and β-lumicolchicine, have been shown to inhibit purine uptake in cells by a mechanism considered to be microtubule-independent [12].

The activity of benzimidazole carbamates (Fig. 1) as inhibitors of tubulin polymerization *in vitro* has been the subject of a number of reports in the literature [13–15]. This mechanism is thought to account for their wide range of pharmacological actions as antifungal, anthelmintic and antineoplastic agents. In particular, the antitumour activity of nocodazole (Table 1, 21) gives rise to the question of whether the entire class of benzimidazole carbamates possesses similar activity [16, 17].

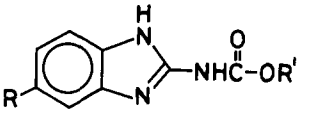

Fig. 1. Structure of benzimidazole carbamates.

Based on the results of a previous study of the structure–activity relationships of benzimidazole carbamates as inhibitors of tubulin polymerization [15], a number of 5(6)-substituted methyl benzimidazole carbamate analogues were tested against L1210 mouse leukaemia cells to determine whether qualitative and quantitative correlation existed between the two assays.

Experimental

Compounds 1–10 and 15 (Table 1) were prepared as described previously [15]. Compounds 11 and 12 were gifts from Hoechst (Australia) and Wellcome (Australia), respectively. Compounds 14 and 21 were gifts from Janssen

Pharmaceutica (Australia). Compound 13 was prepared by transcarbamoylation of Compound 12 in isopropanol/dimethylformamide according to the method of Budesinsky *et al.* [18]. The intermediates for compounds 16–20 were prepared by nucleophilic substitution of 5-chloro-2-nitroaniline with the appropriate phenol with subsequent reduction and cyclization according to the method of Averkin *et al.* [19].

The isolation of tubulin from sheep brain and inhibition studies were carried out as previously described [15]. The concentration of drug required to inhibit the rate of polymerization of tubulin by 50%, the IC_{50}, was obtained from these studies.

L1210 tumour cells from leukaemic mice were incubated for 24 hr in a microcell containing 2 ml of RPI 1640 media with added foetal calf serum. Stock solutions of inhibitors were prepared by appropriate dilutions of a 5×10^{-3} M solution in DMSO with the medium. Twenty to 200 μl of these solutions were added to the microcells to achieve the final concentrations. The assays at each concentration were carried out in duplicate. Because of the narrow range of inhibition, each inhibitor was tested first on a broad concentration range (typically, 1×10^{-5} M, 5×10^{-5} M, 1×10^{-6} M, 1×10^{-7} M, 5×10^{-8} M and 1×10^{-8} M) and then consecutive concentrations showing complete inhibition and no inhibition, respectively, were used as the extreme concentrations for the subsequent experiments.

The number of cells in individual microcells were counted twice and the count averaged to give the number of cells present after 24 hr. Conversion to percentage control growth was calculated using the following formula:

$$\% \text{ of control growth} = \frac{\text{cell count at 24 hr (inhib.)} - \text{cell count at 0 hr}}{\text{cell count at 24 hr (control)} - \text{cell count at 0 hr}} \cdot \times 100.$$

The concentration of inhibitor required to inhibit the rate of growth by 50% compared with the control (the ID_{50}) was calculated from the graph of the percent of control growth vs inhibitor concentration by interpolation at the 50% value. Routine duplication of the ID_{50} determination gave values within ±10% of the observed value with the exception of the methoxy-analogue (1).

Analysis of the correlation of ID_{50} and IC_{50} value was accomplished using the "Funfit" interactive time-sharing programme [15].

Results and discussion

Initial investigations revealed that complete inhibition of cell growth occurred over an extremely narrow range, typically a 2- to 3-fold increase.

The ID_{50}s were highly reproducible (±10%) with the exception of the methoxy-substituent (1). Over 7 determinations the inhibitory activity was observed to vary over a 20-fold concentration range, as such ID_{50} is quoted as a range of observed values. This variation was considered to be due to an inherent insolubility of the compound under the assay conditions. The examples shown in Fig. 2 are typical of the inhibition profiles obtained. For potent inhibitors ($ID_{50} < 0.4$ μM, for example, 3), it was not possible to obtain intermediate concentrations producing between 100% and 5% activity. A similar trend was found by Styles and Garner [20] using carbenazim as an inhibitor of Chang cell survival.

IC_{50} and ID_{50} values for all compounds are shown in Table 1. In general, the ID_{50}s were an order of magnitude lower than the corresponding IC_{50}s. The effect of increasing the straight chain length of the alkyloxy substituent in the 5 (or 6) position of the benzimidazole nucleus (1–3, 5, 7–9) on the ID_{50} was similar to that on the IC_{50}. A 13- to 47-fold increase in anti-leukaemic potency was obtained by increasing the length from methoxy (1) to *n*-propyloxy (3) compared with a 23-fold increase in activity against tubulin. Increasing the substituent length further from *n*-proplyoxy (3) to *n*-octyloxy (9) had no substantial effect on potency in either assay.

Branching of the alkyloxy chain in the β-position (that is, the position adjacent to the atom attached to the benzimidazole nucleus) caused a 4-fold loss of anti-leukaemic

Table 1. Inhibitory activity of benzimidazole carbamates against the polymerization of sheep brain microtubules (IC_{50}) and L1210 leukaemia cells (ID_{50})

Compound No.	R*	IC_{50} (μM)	ID_{50} (μM)
1	OCH_3	57†	2–7
2	OCH_2CH_3	7.4†	0.72
3	$OCH_2CH_2CH_3$	2.4†	0.15
4	$OCH(CH_3)_2$	7.4†	0.65
5	$OCH_2(CH_2)_2CH_3$	3.3†	0.14
6	$OCH_2CH(CH_3)_2$	3.1†	0.29
7	$OCH_2(CH_2)_3CH_3$	2.8†	0.15
8	$OCH_2(CH_2)_4CH_3$	2.2†	0.10
9	$OCH_2(CH_2)_6CH_3$	3.2†	0.12
10	OC_6H_5	3.3†	0.25
11	SC_6H_5	5.4†	0.47
12	SOC_6H_5	NI 100‡	11.3§
13	SOC_6H_5 (R′ = $CH(CH_3)_2$)	30	1.68
14	COC_6H_5	6.1†	0.31
15	$CH(OH)C_6H_5$	70†	2.6
16	α-$OC_{10}H_7$	1.9	0.08
17	β-$OC_{10}H_7$	0.79	0.08
18	OC_6H_4—3′—Cl	1.5	0.14
19	OC_6H_4—3′—Br	1.1	0.07
20	OC_6H_4—4′—C_6H_5	1.5	0.07
21	CO—2′—Thienyl	2.1†	0.07

* For all compounds except compound 13, R′ = CH_3.
† Data obtained from a previous study [15].
‡ NI, not inhibitory at maximum concentration tested.
§ 30% inhibition at 11.3 μM (maximum concentration tested).

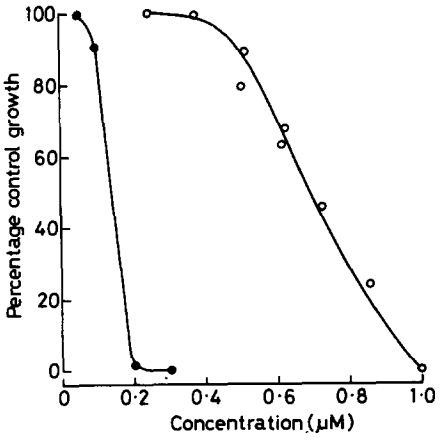

Fig. 2. Relationship between percentage activity of cell growth and concentration of compounds (2) and (3).

activity (compare 3 with 4) while branching in the γ-position caused a 1.6-fold loss of activity. These trends are consistent with the observation that branching in the α- and β-position of the 5(6)-substituent decreased the inhibitory activity in the tubulin assay.

Similarly, the effect of changing the bridging moiety in the 5(6)-position of a number of biaryl substituents (10–14) was similar to that previously observed with tubulin. Reduction of the carbonyl moiety of mebendazole (14) to 13 resulted in a 9- and 11-fold loss of activity in the L1210 and tubulin assays, respectively. Oxidation of the sulphur of fenbendazole (11) to oxfendazole (12) resulted in a greater than 20-fold loss of activity in both assays. Replacement of the methylcarbamate in the 2-position of the benzimidazole nucleus (12) with an isopropyl group (13) dramatically increased activity in both assays.

A number of substituted 5(6)-phenoxy derivatives (16–20) which showed potent inhibitory activity against tubulin polymerization (IC_{50} 0.79–1.9 μM) were also potent inhibitors of L1210 growth with activities comparable to nocodazole (21).

Quantitative correlation of the IC_{50} and ID_{50} values (expressed as their negative logarithms) for the 19 compounds for which full dose–response data were available gave the following regression:

$$pID_{50} = 0.039\ (\pm 0.002)pIC_{50} + 0.112\ (\pm 0.044)$$

92% of the data variance of the regression of pID_{50} values can be explained in terms of the variation of pIC_{50} values. The high colinearity between the L1210 assay and the tubulin polymerization assay indicates that the primary mode of action of benzimidazole carbamates in actively dividing cells is via inhibition of the polymerization of tubulin.

The high quantitative correlation between these assays demonstrates that the selection of potential antitumour agents from this class can be made based on the *in vitro* inhibition of tubulin polymerization. However, it is still necessary to consider the major *in vivo* problems encountered by nocodazole. The variable efficacy of different formulations of the drug may be due to low bioavailability following oral administration, while reduced activity against solid tumours and ependymoblastoma probably reflects its rapid metabolism [16]. Certainly the bioisosteric analogue of nocodazole, mebendazole (14), used commercially as an anthelmintic, has been shown to have low bioavailability in humans [21] and to undergo rapid first pass liver metabolism to the weakly active reduced ketone (15). Hence in selecting benzimidazole carbamates for clinical development, the choice of substituents based on *in vitro* experiments must necessarily consider the expected pharmacokinetic and pharmacodynamic characteristics of the compound.

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