Table 2. Drug sensitivity of several cell lines to colchiceineamides

| Cell line | $LC_{50}^{*}$ ($\mu$M) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | VI | VII | IX | XI |
| P388 | 0.029 | 0.029 | 0.029 | 0.036 | 0.048 | 0.24 | 0.32 | >10 | 0.025 |
| P388/ADR | 0.32 | 0.3 | 0.32 | 0.54 | 0.32 | 3.2 | 3.6 | >10 | 0.34 |
| J774.2 | 0.035 | 0.035 | 0.044 | 0.158 | 0.053 | 1.0 | 1.0 | >10 | 0.035 |

$LC_{50}$, drug concentration that inhibits cell division by 50% after 72 hr.

treating each cell line are described in the following publications: J774.2 [10] and P388 and P388/ADR [11].

*Results and discussion*

The analogs studied are outlined in Table 1. As can be seen, for tubulin samples that were incubated with equiconcentrations of drugs for the same length of time, it is clear that substitution of the 10-methoxy group did not reduce the activity of the drug as long as the alkyl moiety was short. In fact, methyl (II) and ethyl (III) amines were even more potent than colchicine. Propyl (IV) and isopropyl (V) were comparable to colchicine, while larger alkyl groups, from butyl (VI, VII and VIII) derivatives and larger, had almost no inhibitory potency on tubulin polymerization. The dimethylhydrazine (XI) derivative was as potent as colchicine. With higher concentrations of these analogs or longer incubation times prior to GTP or taxol introduction, less polymer was assembled.

Studies on the competition of each colchicine derivative and [$^3$H]colchicine are summarized in Fig. 1. The results clearly indicate that all the analogs tested competed with colchicine for the same binding site on tubulin and their relative affinities to this site dictated their poisoning potencies on tubulin assembly, as seen in Table 1.

Several drug-treated cell lines showed a decrease in their viability and proliferation, and this was compatible with their effect on tubulin assembly. Table 2 summarizes the $LC_{50}$ for three cell lines. The $LC_{50}$ for the doxorubicine-resistant line (P388/ADR) was about one order of magnitude higher than the $LC_{50}$ for the native P388 line, due to cross-resistancy to colchicine and its analogs. An immunofluorescence study with antibody directed against tubulin performed on CHO cells treated with several of these drugs (not shown) revealed the disappearance of the microtubule cytoskeleton. These results demonstrate that the colchicine analogs appear to function on cell lines in a manner similar to the parent compound.

To summarize, we have presented a series of colchicine derivatives which guard the C-10 position from being hydrolyzed. The potencies of these drugs as tubulin assembly inhibitors were reciprocal to the size of the amino substituent. These drugs can also serve as precursors for further modification of the colchicine molecule.

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## *In vivo* binding of [1-$^{14}$C]methylisocyanate to various tissue proteins

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Cyanates have been reported to be antisickling agents which bind to the N-terminal valine of $\alpha$- and $\beta$-chains of haemoglobin [1, 2]. *In vivo* carbamylation of various tissue proteins, including brain proteins by cyanate, has been reported [3, 4]. Isocyanates, the reactive isomers of cyanates, also bind with proteins [2], and methylisocyanate (MIC) has been shown to be an effective antisickling agent *in vitro* [5]. Use of cyanate as an antisickling agent *in vivo* is limited due to its high toxicity [6]. There is a paucity of literature on MIC binding to various tissue proteins after

it crosses the "blood–tissue barrier". We report here that [1-$^{14}$C]MIC can carbamylate various tissue proteins, contributing to its toxicity, following intraperitoneal administration or inhalation exposure.

*Materials and methods*

All common chemicals used were of analytical grade procured from Aldrich (USA) and BDH (England). [1-$^{14}$C]Acetic anhydride was supplied by Bhabha Atomic Research Centre, Bombay (India).

*Synthesis of [1-$^{14}$C]MIC.* [1-$^{14}$C]MIC (sp. act. 42 $\mu$Ci/mmol, chemical purity >95% by GLC) was prepared in our laboratory by refluxing [1-$^{14}$C]acetyl chloride with sodium azide in the presence of a phase-transfer catalyst in a modified procedure [7, 8] and redistilling the product in a microdistillation assembly. Acetyl chloride was obtained by reaction of thionyl chloride with [1-$^{14}$C]acetic anhydride (5 mCi, sp. act. 108.7 mCi/mmol) after premixing it with 1.5 ml of cold material.

*Administration of [1-$^{14}$C]MIC.* Female Wistar rats weighing 150 ± 2 g (unless otherwise mentioned) were used in the present study. [1-$^{14}$C]MIC was suspended in dimethyl sulfoxide (DMSO) for i.p. administration, MIC being stable up to 24 hr in DMSO as monitored by GLC. The $LD_{50}$ (i.p.) of MIC in female rats was 18.99 ± 1.2 mg (SE) as determined by the "Up and down" method of Dixon [9]. For inhalation experiments with [1-$^{14}$C]MIC, rats were exposed for 30 min in an all-glass static chamber of 21.5 liter capacity [$LC_{50}$ (30 min) of MIC for rats is 1.04 mg/l; $t_{99} < 10$ sec].

*Preparation of haemolysate.* Blood was collected from the orbital sinus in heparinised centrifuge tubes. Hemolysate was prepared by the method of Ronald and Donald [10]. Blood (1 ml) was centrifuged and, after removing plasma, packed cells were washed four times with normal saline and finally were lysed with 1 ml of distilled water. Cells in distilled water were kept for 10 min at 4° and thereafter were shaken vigorously on a vortex mixer for 2 min. Chloroform (1 ml) was then added to the mixture which was then centrifuged at 3000 rpm for 20 min. The uppermost layer containing haemoglobin was taken for the determination of radioactivity after decolorising with 30% hydrogen peroxide (as bleaching agent). Haemoglobin was measured by a cyanmethaemoglobin method [11].

*Determination of free and protein-bound radioactivity in tissues.* Brain, liver, kidney and lung tissues were homogenized in 7% trichloroacetic acid (TCA) at 4° and centrifuged at 3000 rpm to separate the precipitate. The pellets were washed with cold acetone to remove non-specifically bound radioactivity [12]. Supernatant fractions were kept aside for the determination of free radioactivity, washed pellets were solubilized in alcoholic potassium hydroxide (20% KOH in 70% ethanol), and radioactivity was determined in an LKB 1211 Rack-Beta Liquid scintillation counter. The composition of the scintillation mixture was the same as that described by Itoh and Quastel [13]. A quench correction was applied to each sample.

*Determination of protein carbamylation.* Precipitation of globin and preparation of 3-methyl 5-isopropyl hydantoin from carbamylated globin was done by the method of Manning *et al.* [14]. Total blood proteins were precipitated by adding 7% of TCA to 0.5 ml of blood at 4°. TCA precipitates of tissues were used for hydantoin preparation. Hydantoin derivatives from total blood proteins and tissue proteins were prepared by the same method as described earlier. After evaporating hydantoin containing ethyl acetate, the residue was suspended in 0.1 ml of distilled water, and radioactivity was determined.

*Results and discussion*

Results in Table 1 show the presence of both free and protein-bound radioactivity in brain, liver, kidney and lung, 30 min after i.p. administration of [1-$^{14}$C]MIC at 30 and 15 mg per kg dose levels. Maximum protein-bound radioactivity was present in liver and a minimum in brain. On the following days (1, 4 and 10) with a 15 mg/kg dose level (i.p.), protein-bound radioactivity decreased in liver, kidney and lung but, even on day 10, a considerable amount of protein-bound radioactivity was still present. There was no substantial change in protein-bound radioactivity in brain during the course of the present study (Table 1). Radioactivity was also determined in the hemolysate at both dose levels (Table 1). As the hemolysate mainly contained haemoglobin, the observed incorporation would mostly be due to haemoglobin-bound radioactivity. There was retention of radioactivity in hemolysate until day 4 after administration (15 mg/kg, i.p.) of [1-$^{14}$C]MIC. As MIC was injected intraperitoneally, maximum protein binding was observed in liver. Distribution to other tissues appears to have been due to passive diffusion of MIC followed by protein binding (Table 1) as results correlate with the blood flow to the tissues investigated.

Isocyanates readily form covalent bonds with N-terminal free -$NH_2$ groups of proteins by acylation reaction, irrespective of group R, i.e. in this case the methyl group [2]. Reaction of isocyanate with thiol groups is reversible [2]. However, near stoichiometric binding of 2-chloroethyl-isocyanate with the thiol active site of glutathione reductase and stable enzyme inhibition has been reported [15]. The significance of binding of isocyanates with —OH and —COOH groups of proteins *in vivo* is not clear. MIC, being a highly reactive electrophile, can bind to various tissue and blood proteins, especially to the N-terminal -$NH_2$ group of proteins. Carbamylation of the N-terminal valine of sickle cell haemoglobin by cyanate and MIC bound valine can form 3-methyl 5-isopropyl hydantoin on heating with concentrated HCl, which can be extracted with ethyl acetate [5, 14]. Work carried out in our laboratory showed that, when rats and rabbits were exposed by inhalation to MIC, it bound to haemoglobin. A hydantoin derivative of MIC bound N-terminal amino-acid valine of haemoglobin has been detected as 3-methyl 5-isopropyl hydantoin by GLC, by comparing with chemically synthesized 3-methyl 5-isopropyl hydantoin.* As MIC can bind to various N-terminal amino acids of proteins and can form corresponding hydantoin derivatives on heating with HCl [2], the radioactivity extracted with ethyl acetate arose mostly from the hydantoin derivative of carbamylated N-terminal amino acids of tissue proteins, total blood proteins and haemoglobin.

The carbamylation of globin, total blood proteins and liver proteins was obtained by [1-$^{14}$C]MIC (39 mg/kg, i.p.; Table 2). Results show that radioactivity in the hydantoin derivative of total blood proteins was higher than that of corresponding carbamylated globin, indicating that other blood proteins were also carbamylated by [1-$^{14}$C]MIC. Similar results were obtained with the 15 mg/kg, i.p., dose level also (data not presented). Approximately 26.6% of protein-bound radioactivity from liver tissue was extracted in the hydantoin fraction (Table 2). As the radioactivity can also be distributed in non-hydantoin products arising from binding with sites other than N-terminal -$NH_2$ groups of proteins such as thiol [15], the complete protein-bound radioactivity was not recovered in the hydantoin fraction. The nature of other binding sites has not been established by us.

Experiments were also carried out on the tissue distribution of radioactivity from [1-$^{14}$C]MIC following its

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Table 1. Distribution of intraperitoneally administered [1-$^{14}$C]MIC in various rat tissue and blood hemolysate

| Dose (mg/kg) | Radioactivity injected ($\mu$Ci) | Sacrifice time after i.p. injection | Tissue radioactivity (dpm $\times 10^{-3}$/g wet wt) | | | | | | | | Radioactivity in hemolysate (dpm $\times 10^{-3}$/100 mg haemoglobin) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Brain | | Liver | | Kidney | | Lung | | |
| | | | F | PB | F | PB | F | PB | F | PB | |
| 30 | 3.31 | 30 min | 4.41 ± 0.12 | 1.97 ± 0.25 | 10.90 ± 1.03 | 27.34 ± 10.45 | 13.51 ± 2.51 | 6.87 ± 0.53 | 9.40 ± 1.23 | 12.74 ± 1.21 | 3.69 ± 1.76 (7) |
| 15 | 1.66 | 30 min | 2.40 ± 0.14 | 2.39 ± 0.37 | 7.32 ± 0.79 | 11.67 ± 0.95 | 9.23 ± 0.13 | 7.54 ± 0.21 | 5.85 ± 0.63 | 5.55 ± 1.32 | 2.34 ± 0.47 (4) |
| 15 | 1.66 | 1 day | 1.54* ± 0.23 | 2.27§ ± 0.47 | 1.50* ± 0.22 | 6.30† ± 1.54 | 1.84* ± 0.32 | 4.19* ± 0.79 | 1.55* ± 0.39 | 3.98§ ± 1.01 | 1.13† ± 0.43 (4) |
| 15 | 1.66 | 4 days | ND | 2.22§ ± 0.52 | ND | 2.01* ± 0.30 | ND | 3.68* ± 1.05 | ND | 2.50† ± 0.31 | 1.56§ ± 0.52 (5) |
| 15 | 1.66 | 10 days | ND | 1.91‡ ± 0.06 | ND | 1.10* ± 0.52 | ND | 1.43* ± 0.11 | ND | 0.73* ± 0.22 | Not determined |

Abbreviations: F, Free; PB, protein-bound radioactivity and ND, no detectable radioactivity. In all the experiments, animals of uniform weight were used (150 ± 2 g). Results are expressed as mean ± SD of a quadruplicate number of animals except in the determinations of blood hemolysate radioactivity, where the number of animals used is given in parentheses. Weight (in grams) of tissues for 30-min values (15 mg/kg): brain, 1.06 ± 0.07; liver, 3.53 ± 0.34; kidney, 1.19 ± 0.04; and lung, 1.65 ± 0.51.

*–‡ The significance of differences of values obtained on different days was compared to the initial 30-min values (15 mg/kg) using Student's $t$ test: *$P < 0.001$; †$P < 0.01$; and ‡$P < 0.05$; §not significant.

Table 2. Radioactivity in hydantoin fraction for determination of carbamylation of globin, total blood proteins and liver proteins

| | Radioactivity | |
|---|---|---|
| | (dpm × $10^{-3}$) | (dpm × $10^{-3}$/g tissue wet wt) |
| Globin (0.5 ml blood) | 0.69 ± 0.53 | |
| Per 100 mg haemoglobin | 1.06 ± 0.82 | |
| Total blood proteins (0.5 ml blood) | 1.04 ± 0.62* | |
| Bound radioactivity in liver proteins | | 113.32 ± 13.43 |
| Hydantoin fraction derived from carbamylated liver proteins | | 30.15 ± 14.22 |

Results from a typical experiment are presented as mean ± SD. Four rats weighing 135 ± 2 g were used. [1-$^{14}$C]MIC was administered intraperitoneally (39 mg/kg; 3.36 $\mu$Ci/rat).

* Not significant when compared to the value of globin (0.5 ml blood), using Student's *t*-test.

Table 3. Distribution of radioactivity in various rat tissue following exposure to [1-$^{14}$C]MIC by inhalation

| Dose | Sacrifice time interval after exposure | Tissue radioactivity (dpm × $10^{-3}$/g wet wt) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Brain | | Liver | | Kidney | | Lung | |
| | | F | PB | F | PB | F | PB | F | PB |
| 1 $LC_{50}$ (30 min) | 30 min | 0.55 ± 0.16 | 2.23 ± 0.62 | 2.13 ± 1.22 | 11.04 ± 05.54 | 2.69 ± 0.92 | 3.54 ± 1.06 | 7.56 ± 1.89 | 5.50 ± 2.42 |
| 1 $LC_{50}$ (30 min) | 1 day | ND | 4.02* ± 1.06 | 0.73† ± 0.21 | 3.63* ± 0.62 | ND | 2.80† ± 0.65 | ND | 2.46* ± 0.53 |

Rats were exposed for 30 min to [1-$^{14}$C]MIC (1 $LC_{50}$ = 1.04 mg/l, 23 mg [1-$^{14}$C]MIC in a 21.5-liter all-glass static chamber). Abbreviations: F, Free; PB, protein-bound radioactivity; and ND, no detectable radioactivity. Weight of rats = 128 ± 2 g. Results are expressed as mean ± SD of quadruplicate animals. Significance of difference of values was obtained using Student's *t* test.

* $P < 0.05$.

† Not significant.

Table 4. Radioactivity in protein-bound form from various tissues and corresponding hydantoin fractions following exposure to [1-$^{14}$C]MIC by inhalation

| Tissue | Animal number | Protein-bound radioactivity (dpm × $10^{-3}$/g wet wt) | Radioactivity in hydantoin fraction (dpm × $10^{-3}$/g wet wt) |
|---|---|---|---|
| Brain | 1 | 2.34 | NS* |
| | 2 | 2.95 | NS |
| Liver | 1 | 5.95 | 3.77 |
| | 2 | 18.24 | 4.38 |
| Kidney | 1 | 4.71 | 0.68 |
| | 2 | 5.25 | 1.14 |
| Lung | 1 | 11.92 | 4.82 |
| | 2 | 9.61 | 3.25 |

Rats were exposed to 2 $LC_{50}$, [1-$^{14}$C]MIC in a 21.5-liter all-glass static chamber for 30 min. $LC_{50}$ (30 min) = 1.04 mg/l, $t_{99} < 10$ sec. Weight of rats: 148 g each.
* Not significant.

exposure by inhalation (1 $LC_{50}$ for 30 min). The results showed a similar pattern of protein binding as was observed in the case of i.p. administration (Table 3). In brain, protein-bound radioactivity increased 1 day after exposure. The reason for that could not be accounted for in the present study. Marked recovery of radioactivity in the hydantoin fraction from carbamylated tissue proteins was observed (Table 4) except from carbamylated brain proteins, where there was no significant recovery. Brain, weighing around 0.6 g, was used (another half of brain was used for protein-bound radioactivity determination) for hydantoin preparation and, due to low protein-bound radioactivity, recovery might not have been possible. No significant binding of radioactivity was observed in globin or total blood proteins from inhalation-exposed rats. This may have been due to fast transport of MIC to various tissues where significant carbamylation was observed. Lee [5] also suggested the possibility of fast transport of cyanate to organs during the reaction time required for binding to globin. MIC, being a highly reactive electrophile, may bind reversibly to blood proteins (other than $NH_2$ groups where binding is irreversible), transported and released to various organs. It has been demonstrated that carbamylation of thiol groups by cyanate can serve as an intermediate for the formation of stable N-carbamylated complex [2].

MIC is hydrolysed to methylamine in aqueous medium [16]. In the present study, the isocyanate carbon of MIC was labelled ($CH_3$ —N = $^{14}$C = 0); therefore, resulting methylamine did not contain radioactivity. The observed radioactivity in various tissue proteins would arise largely due to covalent binding of MIC to tissue proteins. This has also been substantiated by experiments in which hydantoin derivatives from globin, blood proteins and tissue proteins were prepared and radioactivity was determined.

In summary, the present study shows covalent binding of [1-$^{14}$C]MIC to brain, liver, kidney and lung proteins, when administered intraperitoneally and also by inhalation. Evidence for carbamylation of globin and blood proteins by intraperitoneally administered MIC is also presented, and results suggest the crossing of the "blood–tissue barrier" by MIC in its "active form".

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