$S(-)$-TMQ is considerably more potent than $R(+)$-TMQ as a stimulant of β-adrenoceptor systems including lipolysis, relaxation of trachea, and chronotropic activity in heart. Furthermore, the blockade of antigen-sensitized histamine release by the TMQ analogs and the reversal of drug inhibition by propranolol are further evidence for involvement of β-adrenoceptors for this series of compounds. In this regard, Tsuzurahara *et al.* [7] demonstrated that the blockade of histamine release by $S(-)$-TMQ in rat peritoneal mast cells is directly related to elevations in cyclic-3′,5′-adenosine monophosphate and β-adrenoceptor activation.

The rank order of potency of TMQ analogs and isoproterenol for inhibition of antigen-induced histamine release was (−)-TMQ > (−)-isoproterenol > *erythro*-α-methylTMQ = *threo*-α-methylTMQ > *N*-methylTMQ > α-dimethylTMQ. With the exception of *threo*-α-methylTMQ, this rank order is identical to that reported for the activation of β_2-adrenoceptors in guinea pig trachea [5]. Previously, we observed a reduction in potency of TMQ analogs for the stimulation of β-adrenoceptors in guinea pig lung parenchymal versus tracheal strips, and suggested that these more lipophilic analogs of TMQ may bind to nonspecific (or nonreceptor) sites in lung parenchyma [5]. Similar to lung parenchymal strips, the minced lung pieces represent a heterogeneous cell population, and an increased nonspecific binding of *threo*-α-methylTMQ may account for the difference in rank order of potency of this compound in these two pharmacological systems. Further, the ability of propranolol to reverse the inhibitory effect of *threo*-α-methylTMQ on antigen-stimulated histamine release suggests that this drug, like the other TMQ analogs, produces its effect by the stimulation of β-adrenoceptors.

In summary, we conclude that the methyl-substituted TMQ analogs retain significant *in vitro* antiallergic activity and interact with a high degree of stereoselectivity and potency in β-adrenoceptor systems. Further evaluation of these TMQ analogs *in vivo* may provide useful leads to the development of agents for treatment of pulmonary and/or hypersensitivity disorders.

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The activity of UDP-glucuronyltransferase, sulphotransferase and glutathione-*S*-transferase in primary cultures of rat hepatocytes

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Primary cultures of hepatic parenchymal cells are widely used to study mechanisms of cytotoxicity and carcinogenesis of xenobiotics under defined conditions *in vitro* [1, 2]. The cytotoxic and carcinogenic potency of chemicals depends upon the balance between activation and detoxification processes in the cells. Activation is often associated with the cytochrome P-450 mixed function oxidase (MFO)* system, while the Phase II conjugation reactions are usually considered to detoxify xenobiotics. Alterations in the balance between these two processes during culture may limit the application of cultured hepatocytes to studies of cytotoxicity and carcinogenesis.

Very little is known about the maintenance of conjugation reactions during primary culture of hepatocytes, whereas MFO activity is known to decline to low levels within the first 24–48 hr of culture [3–5]. We have measured the activity of UDP-glucuronyltransferase (GT, EC 2.4.1.17) and sulphotransferase (ST, EC 2.8.2) in cultured hepatocytes using the model compounds 1-naphthol and

* Abbreviations used: GT UDP-glucuronyltransferase, ST sulphotransferase, GSH reduced glutathione, MFO mixed function oxidase, CDNB 1-chloro-2,4-dinitrobenzene, HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

Table 1. Conjugation of 1-naphthol (N) and phenolphthalein (P) during culture

Time in culture (hr)	P glucuronidation		N glucuronidation		N sulphation		Ratio of glucuronidation/ sulphation of N	
	20 μM	100 μM	20 μM	100 μM	20 μM	100 μM	20 μM	100 μM
24	57 ± 8 (5)	56 ± 8 (6)	41 ± 4 (3)	48 ± 4 (5)	77 ± 12 (3)	53 ± 3 (3)	1.13 (3)	2.15 (3)
48	134 ± 25 (5)	136 ± 19 (6)	112 ± 13* (5)	124 ± 14 (4)	53 ± 6 (3)	36 ± 2 (3)	4.54 (3)	5.80 (3)
72	230 ± 24 (7)	154 ± 19 (7)	191 ± 9* (5)	161 ± 22 (7)	27 ± 10† (5)	28 ± 4† (4)	9.29 (5)	14.34 (4)

Conjugation was measured in whole cells in Krebs–Henseleit buffer pH 7.6. Freshly isolated cells were incubated in suspension (10^6/ml). For cultured cells the substrates were added to the monolayer of cells in the Petri dish. Conjugates were measured by HPLC [10].

Results for glucuronidation and sulphation are expressed as the % of freshly isolated cell activities, and means ± SE mean are shown, with the number of experiments in parentheses. Fresh cell values for glucuronidation (expressed per mg total cell protein) were:

P 20 μM 0.32 ± 0.04 nmol/min/mg ($N = 5$); P 100 μM 0.41 ± 0.04 nmol/min/mg ($N = 5$); N 20 μM 0.57 ± 0.06 nmol/min/mg ($N = 4$); N 100 μM 1.08 ± 0.23 nmol/min/mg ($N = 4$). * $P < 0.05$ by one way analysis of variance. Significance levels refer to differences between the enzyme activities at 24 hr and at 48 hr and 72 hr.

Fresh cell values for sulphation (expressed per mg total cell protein) were:

N 20 μM 0.36 ± 0.04 nmol/min/mg ($N = 4$); N 100 μM 0.34 ± 0.01 nmol/min/mg ($N = 3$). † $P < 0.05$ by one way analysis of variance. Significance levels refer to differences between the enzyme activities at 72 hr and in fresh cells.

Fresh cell ratios of glucuronidation/sulphation of N were 1.64 (3) at 20 μM and 3.59 (3) at 100 μM.

phenolphthalein, which are substrates for a 3-methylcholanthrene inducible isozyme and a phenobarbitone inducible isozyme of GT respectively [6]. In addition, the levels of reduced glutathione (GSH) and the GSH conjugation of 1-chloro-2,4-dinitrobenzene (CDNB), a substrate for several forms of GSH-*S*-transferase [7], were measured.

Materials

Flow Laboratories (Irvine, Scotland) supplied the William's E medium, the foetal calf serum and antibiotics. Collagenase was from Boehringer Mannheim (F.R.G.) and the remainder of the chemicals used were supplied by Sigma (St. Louis, MO).

Methods and results

Hepatocytes from male Sprague–Dawley rats (180–220 g) were prepared (90% viable by Trypan blue exclusion) and cultured on collagen coated Petri dishes as described previously [8, 9]. The medium used was William's E medium, supplemented with 5% foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml fungizone. Viability of monolayer cultures was assessed by the lactate dehydrogenase (LDH) activity of the medium, and expressed as a percentage of the total LDH activity per dish after addition of 0.1% (v/v) Triton X-100.

The conjugation reactions were all carried out using whole cells in Krebs–Henseleit buffer, pH 7.4, containing 10 mM *N*-2-hydroxymethyl-piperazine-N^1-2-ethanesulfonic acid (HEPES). GT and ST activity were measured at 20 μM and 100 μM 1-naphthol and phenolphthalein and GSH conjugation at 50 μM CDNB. Formation of sulphate and glucuronic acid conjugates from both substrates was detected directly using ion-pair reversed-phase high pressure liquid chromatography (HPLC) [10]. CDNB-GSH conjugation was quantified spectrophotometrically [7]. Cytochrome P-450 samples were prepared as described previously [11] and measured by the method of Omura and Sato [12]. GSH content of the cells was measured fluorimetrically [13] and protein determination was by the method of Lowry *et al.* [14].

The statistical significance of the results was analysed by one way analysis of variance using Bonferroni probabilities [15].

Table 1 shows that the activity of GT decreased to about 50% of fresh cell values by 24 hr in culture. By 72 hr, however, the activity was 2-fold initial fresh cell values with both substrates. Phenolphthalein was not sulphated to a significant extent even in freshly isolated cells, so sulphation was quantified only with 1-naphthol. In contrast to GT activity, sulphation declines slowly throughout the 72 hr in culture. As a consequence of the different behaviour of ST and GT activities in culture, the ratio of glucuronidation to sulphation is constantly changing in the cultured hepatocytes. The concentration of GSH in the cells, and the activity of GSH-S-transferase appear to be maintained for at least 72 hr in the cultured hepatocytes (Table 2).

Table 2. Reduced glutathione (GSH) levels and GSH conjugation of 1-chloro-2,4-dinitrobenzene (CDNB)

Time in culture (hr)	GSH content (% initial fresh cell values)	GSH–CDNB formation (% initial fresh cell values)
24	85 ± 2 (3)	153 ± 25 (4)
48	71 ± 9 (3)	169 ± 26 (4)
72	80 ± 11 (3)	118 ± 5 (4)

GSH levels were measured by fluorimetry [13]. GSH conjugation of CDNB was measured in whole cells using 10^6/ml freshly isolated hepatocytes in suspension. The CDNB was added to the monolayer of cells in the Petri dish to measure conjugation during culture. The results are shown as mean ± SE mean, with the number of experiments in parentheses.

Fresh cell GSH content was 42.3 ± 2.4 nmol/mg protein (N = 8) and GSH–CDNB formation was 1.1 ± 0.1 nmol/min/mg cell protein (N = 4).

Table 3. The effect of cycloheximide on metabolism and viability in cultures

	P-GT 100 μM	N-GT 100 μM	N-ST 100 μM	Cyt P450	GSH	Viability
			(% initial fresh cell values)			(% dead cells)
72 hr control	184	179	38	25	84	21
72 hr 10^{-6} M cycloheximide	67	92	40	29	72	11
72 hr 10^{-5} M cycloheximide	42	51	41	37	81	28

Cycloheximide was added to the culture medium after 48 hr and the effect assessed after 24 hr treatment.

GT and ST activity towards phenolphthalein (P) and 1-naphthol (N) were measured in whole cells by adding the substrates to the monolayer in the Petri dish.

Cytochrome P450 content was measured in cell homogenates by the method of Omura and Sato [12] and GSH content was measured fluorimetrically [13]. Viability (% dead cells) was estimated by NADH penetration measured by lactate dehydrogenase activity.

The results are means of duplicate experiments; variation was less than 15% for all measurements.

Cycloheximide, an inhibitor of protein synthesis, was added (10^{-6} M and 10^{-5} M) at 48 hr in culture and the effect determined at 72 hr. Table 3 shows that ST activity, the content of cytochrome P-450 and GSH in the cells, and cell viability were relatively unaffected after the 24 hr exposure to cycloheximide. However, the increase in GT activity with both 1-naphthol and phenolphthalein was prevented by the presence of cycloheximide.

Discussion

Maintenance of cytochrome P-450 in primary cultures of hepatocytes has been shown to depend on medium composition [11], with a modified Earle's medium being the most effective, followed by William's E medium. The conjugation reactions appear to be maintained to similar extents in both of these media (results not shown), and for the present study the hepatocytes were cultured in William's E medium.

GT, ST and GSH-*S*-transferase activities behave differently in cultured rat hepatocytes. GSH-*S*-transferase, measured with CDNB, is stable for 72 hr. CDNB is conjugated by several forms of rat GSH transferase, A, AA, B (ligandin) and C [16] and Croci and Williams have previously shown that GSH conjugation towards CDNB is stable for 24 hr in rat hepatocyte cultures, whereas that towards 1,2-dichloro-4-nitrobenzene (DCNB) is not [16]. It would thus appear that the isozymes of GSH-*S*-transferase have differential stabilities during culture, analogous to previous observations with the isozymes of cytochrome P-450 [17].

ST activity towards 1-naphthol was the most labile of the three enzyme activities measured, declining steadily over the entire 72 hr in culture. ST activity also appears to be labile in many immortal mammalian cell lines. Wiebel *et al.* found that phenol ST is more labile than GT in several cell lines including a rat hepatoma cell line, mouse and hamster kidney cells and a human lung carcinoma cell line [18].

During the first 24 hr of culture GT activity decreased to approximately 50% of that in freshly isolated hepatocytes. Previous reports of GT activity in rat hepatocyte cultures demonstrated a decrease after 24 hr [5, 19] using testosterone, 4-nitrophenol and phenolsulfophthalein as substrates. At present the reason for this instability in GT maintenance over the first 24 hr in culture is not known.

The 4-fold increase in GT activity occurring between 24 and 72 hr in culture could be due either to induction of the enzyme requiring *de novo* enzyme synthesis or to activation of the membrane bound transferase by removal of membrane constraint. To distinguish between these possibilities cycloheximide was added to the culture medium after 48 hr in culture and was shown to prevent the increase in GT activity. These results suggested that the increase in GT activity was due to protein synthesis. We have previously reported an apparent induction in the activity of the MFO system under similar conditions [20]. The increase in GT activity is thought to reflect de-differentiation of the hepatocytes in culture and development of a pre-neoplastic pattern of drug metabolizing activities [21]. Many attempts have been made to modify culture conditions in order to improve the expression of drug metabolizing enzymes and preliminary results indicate that co-culture of rat hepatocytes with another rat liver epithelial cell line may prevent this increase in GT activity.*

The successful use of cultured hepatocytes as a model system for toxicity studies necessitates the maintenance of both MFO and conjugating enzyme activities at levels found on initial isolation from the liver. The data presented here indicate that the balance between the different pathways of conjugation is continuously altering during hepatocyte primary culture. This effect, combined with changes in oxidative enzyme activity, will result in altered potential for detoxification of xenobiotics.

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Methylglyoxal bis(butylamidinohydrazone), a new inhibitor of polyamine biosynthesis that simultaneously inhibits ornithine decarboxylase, adenosylmethionine decarboxylase and spermidine synthase

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Aliphatic polyamines, putrescine, spermidine and spermine, seem to be essential for cell growth, and they are considered to serve as control intermediates in cellular responses to hormones, growth factors and other environmental signals [1]. For this reason there has been a great deal of interest in determining the regulatory mechanisms of polyamine biosynthesis. Additionally, considerable efforts have also been made to develop and examine inhibitors for polyamine biosynthesis. These inhibitors appear to be clinically important in the treatment of cancer [2], and they are useful in determining the functions of the polyamines in normal cell growth and differentiation [3].

The most widely used inhibitors of polyamine biosynthesis are difluoromethylornithine, an enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC; EC 4.1.1.17) [4] and methylglyoxal bis(guanylhydrazone) (MGBG), a potent inhibitor of putrescine-activated adenosylmethionine decarboxylase (AdoMetDC; EC 4.1.1.50) [5]. MGBG is used very often in polyamine researches [6, 7], but the severe side effects [8, 9] are compromising its usefulness.

In view of the obvious advantages afforded by more specific inhibitors of polyamine synthesis, we discovered dicyclohexylamine [10] and recently synthesized *N*-chlorosulfonyl-dicyclohexylamine [11] as the inhibitors for spermidine synthase. These compounds have been proven effective in altering polyamine metabolism in several mammalian and bacterial systems [11–15].

In the present paper we report that methylglyoxal bis(butylamidinohydrazone) (MGBB) exerts its inhibitory effects on three different enzymes in the polyamine biosynthetic pathway.

Materials and methods

Chemicals. MGBB was synthesized as described elsewhere [16], principally according to the method previously published [17]. DL-[1-^{14}C]ornithine (sp. act. 57.6 mC$_i$/mmole), *S*-adenosyl-L-[carboxy-^{14}C]methionine (sp. act. 58 mC$_i$/mmole) and *S*-adenosyl-L-[methyl-^{14}C]methionine (sp. act. 53.6 mC$_i$/mmole) were purchased from New England Nuclear Corp. (Boston, MA). Decarboxylated *S*-adenosylmethionine, both unlabeled and labeled in the methyl group, was prepared by the action of AdoMetDC from *Escherichia coli* (strain B) and purified by chromatography on Dowex-50-H$^+$ and paper electrophoresis [18]. All other chemicals were products of Nakarai Chemicals Ltd.

Enzyme preparations. ODC from Ehrlich ascites tumor cells [19], AdoMetDC from rat liver [20] and spermidine and spermine synthases from rat ventral prostate [10] were prepared as described in previous publications. Protein was determined by the method of Bradford [21] using bovine serum albumin as a standard.

Enzyme assays. The activities of ODC [22], AdoMetDC [23], spermidine and spermine synthases [24] were measured as described earlier.

Results and discussion

MGBB showed the inhibition of ODC, AdoMetDC and spermidine synthase activities. To our surprise, this compound, a derivative of AdoMetDC inhibitor MGBG, inhibited ODC more sensitively than AdoMetDC. The effect of the concentration of ornithine on the inhibition of ODC by MGBB is shown in Fig. 1. This inhibition was competitive with ornithine, and the calculated K_i for MGBB was 3.5 μM. The K_m value for ornithine was estimated to be 0.33 mM.

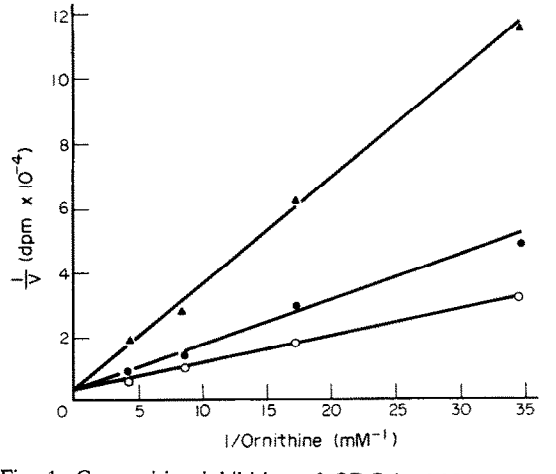

Fig. 1. Competitive inhibition of ODC by MGBB with ornithine as the variable substrate. ODC activity was assayed in the absence (○) or presence of 2 (●) or 10 μM (▲) MGBB, with 0.029–0.232 mM ornithine and 72 μg enzyme protein.