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Isothiazolone biocides: enzyme-inhibiting pro-drugs

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Summary

Previous investigations indicate that there are basic differences in the antimicrobial activity of the chlorinated and non-chlorinated isothiazolones. These differences appear to result from the formation of a highly reactive thio-acyl chloride tautomer by the chlorinated isothiazolones. In the present investigations the effects of the isothiazolones upon the activity of yeast alcohol dehydrogenase are examined in the presence and absence of various potential neutralisers of their activity. Results indicate that thiol containing compounds may both neutralise and, at lower concentrations, potentiate the inhibitory activity of 5-chloro-*N*-methylisothiazolone. Such potentiation was not observed when the experiments were repeated with benzisothiazolone. The presence of thiol at concentrations which potentiate antimicrobial action enhanced the inhibitory activity of 5-chloro-*N*-methylisothiazolone towards alcohol dehydrogenase when ethanol-limited. This was not observed with benzisothiazolone challenged enzyme.

Introduction

Isothiazolone biocides such as benzisothiazolone (BIT, Formula I), *N*-methylisothiazolone (MIT, Formula II) and 5-chloro-*N*-methylisothiazolone (CMIT, Formula III) are widely used as industrial biocides (Singer, 1976; Andrykovitch and Neihof, 1987). Mixtures of CMIT and MIT form the basis of the Kathon™ range of biocides (Rohm and Haas Inc.) (Zeelie and McCarthy, 1983; Law et al., 1984, 1987). BIT (the basis of the Proxel range of biocides, ICI plc) is not recommended for pharmaceutical, cosmetic and toiletry preparations since it is a skin sensitiser. Although the active components of Kathon are potent sensitisers (> 25 μg/ml; Weaver et al., 1985) and Ames positive (Monte et al., 1983), it is nevertheless widely used in this range of products and has given rise to an increasing number of documented cases of contact dermatitis (De Groot and Herxheimer, 1989); the associated risk, however, being negligible for rinse off products. Mutagenic data from a wide range of studies indicate Kathon to be safe for its designated uses (Scribner et al., 1983). The positive genotoxicity found for Kathon in the Ames test and for mouse lym-

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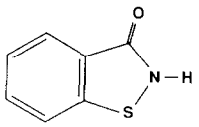
phoma cells needs, however, to be evaluated against these negative findings in other systems.



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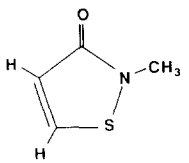

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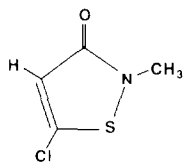

(III)

The antimicrobial activity of all isothiazolones is strongly antagonised by thiol-containing materials (Fuller et al., 1985; Collier et al., 1990a) with which the biocides interact oxidatively (Collier et al., 1990b,c). As for other thiol interactive agents isothiazolones are not noted for their bactericidal properties, however, CMIT, but not BIT or MIT, is fungicidal (Collier et al., 1990a). Such studies have shown the activities of CMIT, but not BIT or MIT, to be neutralised by the presence of histidine and valine in addition to thiol-containing peptides such as glutathione. Patterns of growth inhibition for CMIT and morphological changes associated with its growth inhibitory action at concentrations beneath the MIC suggest possible inhibition of the initiation of DNA replication.

Oxidative interaction of isothiazolones with most thiol-containing compounds (e.g., cysteine, glutathione) causes the formation of disulphides which further interact to give thiol dimers (e.g., cystine, glutathione disulphide) and reduced, ring-opened forms of the biocides (mercaptoacrylamide or mercaptobenzamide). These forms provide accessible thiols which may further interact with isothiazolone to give isothiazolone dimers (Collier et al., 1990b). NMR spectral studies have indicated that the mercaptoacrylamide of CMIT tautomerises to give a highly reactive thioacyl chloride, capable of rapid reaction, not only with thiols but also with amines and water. Such reactivity probably accounts for much of the enhanced biological activity of CMIT and CMIT-containing products such as Kathon (Collier et al., 1990c).

Glutathione (GSH) is present in many microbial cells at relatively high concentrations (> 10 mM; Owens and Hartman, 1986) and will probably be the major target for isothiazolone action, disrupting intracellular redox balance. Some isothiazolone will interact with other thiol-containing materials such as enzymes. Such interaction will either be direct or through tautomerisation to the thio-acyl chloride (Collier et al., 1990b). In this paper we evaluate enzyme inhibition by isothiazolones and their potential, in conjunction with GSH and dithiothreitol, to act as pro-drugs.

Materials and Methods

Enzymes and reagents

Yeast alcohol dehydrogenase (ADH) and NAD were obtained from Sigma, Poole, U.K. BIT was kindly donated by ICI Biocides, Manchester, U.K. CMIT was synthesised according to the methods described by Collier et al. (1990a). All other reagents were obtained from either Sigma or BDH, Poole, U.K. and were the purest grade available.

Alcohol dehydrogenase activity

Phosphate buffer (25 °C, 50 mM, pH 7.0), ethylenediaminetetraacetic acid (EDTA, 30 mM), substrate (either NAD or ethanol), isothiazolone biocide and enzyme (0.01 units) were mixed in quartz cuvettes at 25 °C and the reaction started, after 5 min and 60 min incubation, by the addition of ethanol (0.001–1.0 mM) or NAD (0.001–1.0 mM), as appropriate. The reactions were followed at 25 °C by the change in absorbance at 340 nm. Initial experiments were conducted to determine the Km of both NAD (0.19 mM) and ethanol (0.23 mM) in such reactions. Ethanol-limited and NAD-limited reactions of the enzyme were subsequently studied utilising concentrations of $5 \times$ Km and $<$ Km in the pre-incubation mixture and final reactant, respectively. Where

appropriate, potential neutralisers/activators of the isothiazolones were included with the phosphate buffer.

Results and Discussion

Alcohol dehydrogenase inhibition by isothiazolones

The inhibitory effects of various BIT and CMIT concentrations upon ethanol-limited and NAD-limited ADH activity were expressed from initial reaction rates after 5 min and 60 min pre-incubation of the reaction mixture in the presence and absence of dithiothreitol (DTT, 0.39 mM). Patterns of inhibition suggested that the isothiazolones inhibit ADH by an apparent mixed or general non-competitive inhibition (Engel, 1981), suggesting that the free enzyme is more susceptible to the inhibitor than the enzyme-substrate complex. This suggests that the primary target(s) of BIT and CMIT are group(s) within ADH which are masked, in situ, by substrate. Lineweaver-Burk slopes were plotted against isothiazolone concentration (Engel, 1981) in order to give estimates of K_i (Table 1). Results indicate ADH to be more sensitive to CMIT when NAD-limited than ethanol-limited after prolonged pre-incubation (60 min). In the presence of DTT, however, inhibition of the ethanol-limited reaction was significantly enhanced. After 60 min preincubation the NAD-limited reaction was protected in the presence of DTT. These results suggested that the thio-acyl chloride tautomer of CMIT may have additional target groups at the ethanol binding site or with ethanol itself. Enhancement ratios upon DTT addition suggest its mercaptoacrylamide (Collier et al., 1990b) to be 5–15 times more active towards ADH than CMIT.

Patterns of ADH-inhibition by BIT suggest, as for CMIT, mixed or general non-competitive inhibition. As predicted, addition of DTT quenches the activity of BIT, confirming the relative inactivity of its mercaptobenzamide.

Time-dependency of ADH-inhibition and isothiazolone-potentiation

Thiol-interactive enzyme inhibitors are typically slow acting. The progress of the inhibition by BIT (0.47 μM) and CMIT (0.12 μM) of NAD-limited, ethanol-limited and saturated zero-order ADH-mediated reactions was determined through variation of the pre-incubation time (Figs 1a and 2a). These concentrations were chosen since they gave similar levels of ADH-inhibition (50% at 30 min). For both agents the inhibitory activity increased with pre-incubation time up to and beyond 60 min. Within this general trend CMIT was significantly faster acting with 50–80% of the inhibitory action being achieved within 5 min. Results reaffirm the observations of relative sensitivities for NAD- and ethanol-limited reactions (above). Additionally, for CMIT the progress of the inhibitory effect towards NAD-limited reactions was markedly faster than the others suggest-

TABLE 1

K_i values for CMIT and BIT against ADH activity under conditions of NAD^+ limitation or ethanol limitation, with and without DTT

Isothiazolone	K_i values (μM isothiazolone)			
	NAD^+ limitation		Ethanol limitation	
	5 min	60 min	5 min	60 min
CMIT	1.28	0.03	0.220	0.070
CMIT + DTT	1.20	0.20	0.015	0.015
Enhancement ratio [a]	(1:1)	(1:7)	(15:1)	(5:1)
BIT	3.13	0.25	1.77	0.19
BIT + DTT	7.22	0.49	0.36	0.35
Enhancement ratio [a]	(1:2)	(1:2)	(5:1)	(1:2)

[a] Enhancement ratios express $K_i : K_i$ (DTT).

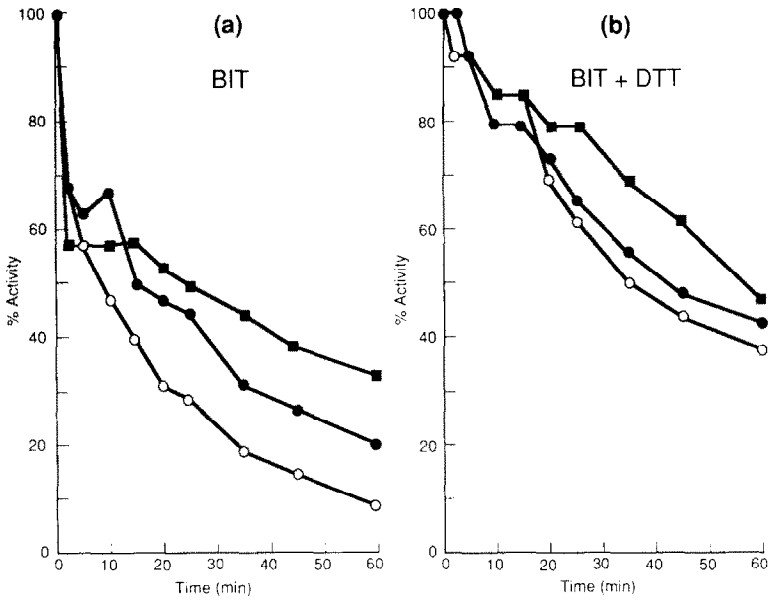

Fig. 1. Inhibition of yeast alcohol dehydrogenase (ADH) by BIT (0.47 μM) in (a) the absence of dithiothreitol and (b) the presence of dithiothreitol (0.016 μM). Enzyme reactions were either fully saturated with ethanol and NAD^+ (●), or were ethanol-limited (■) or NAD^+-limited (○) in the presence of an excess of the other. Enzyme activities were determined by addition of NAD^+ to aliquots of the reaction mixture removed at various times up to 60 min.

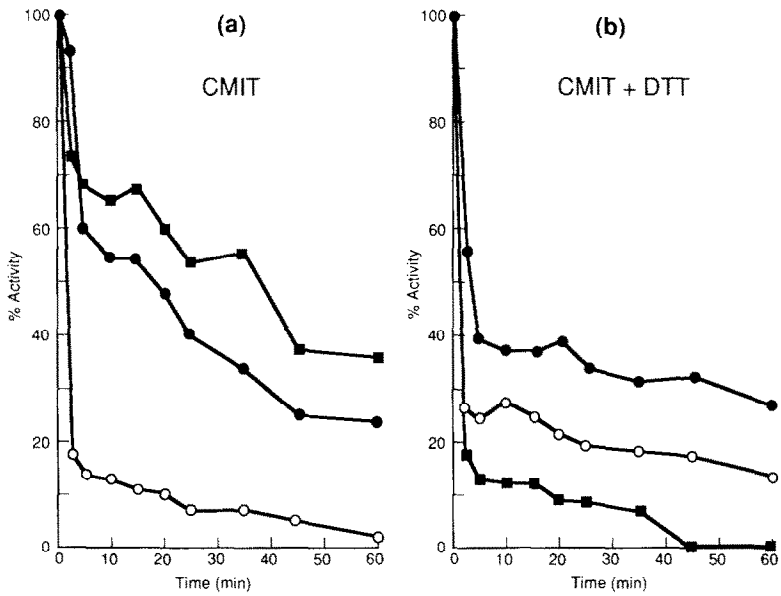

Fig. 2. Inhibition of yeast alcohol dehydrogenase (ADH) by CMIT (0.12 μM) in (a) the absence of dithiothreitol and (b) the presence of dithiothreitol (0.0039 μM). Enzyme reactions were either fully saturated with ethanol and NAD^+ (●), or were ethanol-limited (■) or NAD^+-limited (○) in the presence of an excess of the other. Enzyme activities were determined by addition of NAD^+ to aliquots of the reaction mixture removed at various times up to 60 min.

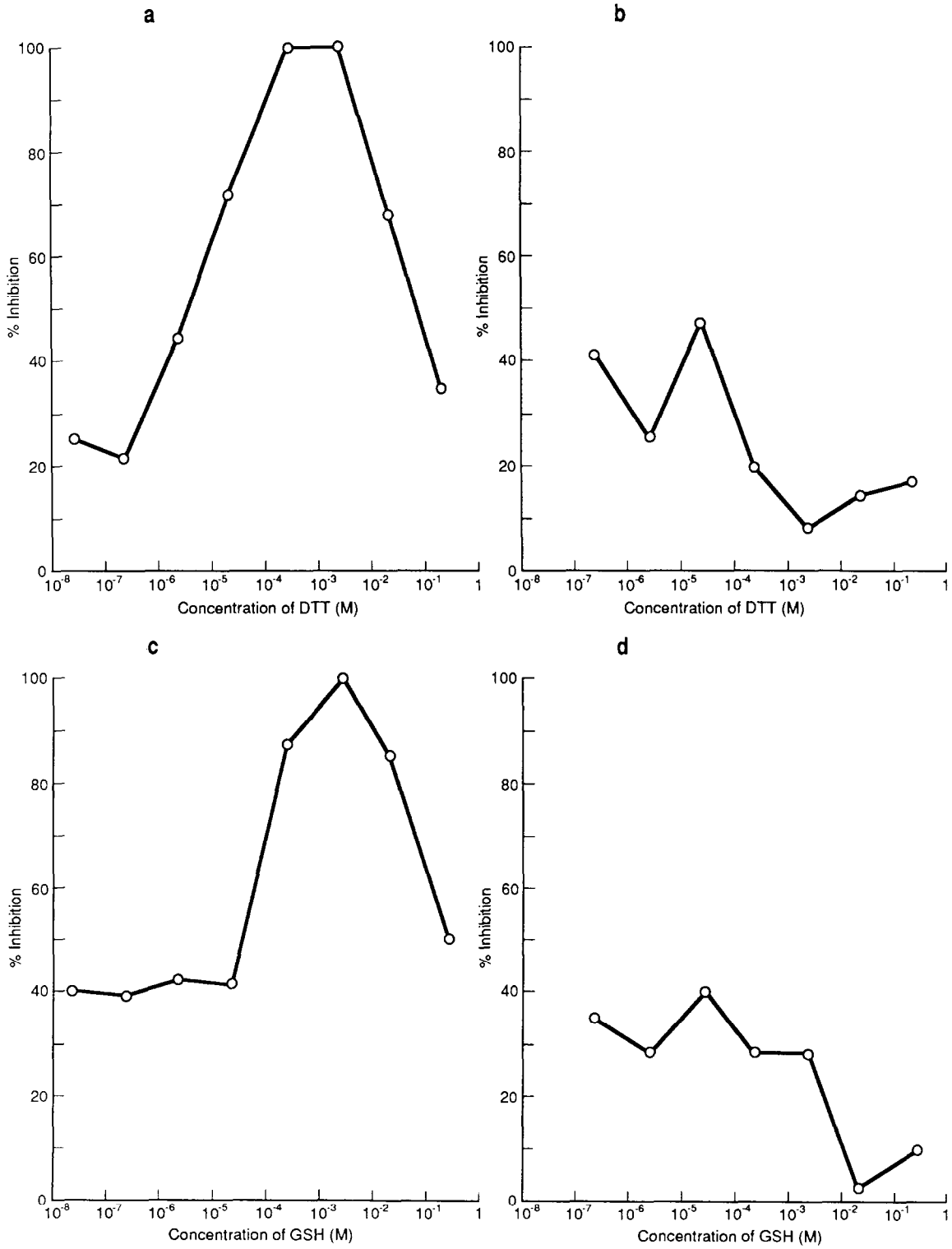

Fig. 3. Effects of varying concentrations of dithiothreitol and glutathione upon the inhibition of yeast alcohol dehydrogenase by CMIT (0.20 μM) and BIT (0.19 μM). a, CMIT plus dithiothreitol; b, BIT plus dithiothreitol; c, CMIT plus glutathione; and d, BIT plus glutathione. Enzyme, biocide and amino acid were incubated for 5 min prior to addition of NAD^+. Reactions contained excess substrates to produce zero order kinetics.

ing the NAD^+ binding site to be more sensitive. Addition of DTT at 5 times the isothiazolone concentration quenched the action of BIT with respect to the extent of the inhibitory response but not to its rate of onset. For CMIT action similar additions enhanced the rate of onset of the inhibition in all cases yet reduced the level of inhibition attained under conditions of ethanol excess. When ethanol concentrations were rate-limiting, however, significant enhancements in the overall level of inhibition were obtained. This supports the results presented in Table 1 which suggested a potential of the CMIT-derived mercaptoacrylamide to react with ethanol and the free-ethanol binding site of ADH.

Neutralisation and potentiation of isothiazolone activity

Previously we have reported on neutralisation of the growth-inhibitory action of CMIT, not only by presence of thiols such as DTT but also by the presence of amino-acids such as valine and histidine (Collier et al., 1990a). Protection by non-thiol amino-acids was suggested to result from quenching of the mercaptoacrylamide formed by subcellular interaction of CMIT. Use of purified enzymes, such as ADH, enables the effects of such amino-acids to be studied in the absence of exogenous thiol. Various concentrations (0.039 μM to 0.39 M) of glutathione, valine, histidine or DTT were included in NAD-limited reaction mixtures and residual isothiazolone activities assayed after challenge with BIT (0.2 μM) or CMIT (0.2 μM) and preincubation for 5 min. Reactions were started by the addition of ethanol (1.16 mM) since this has shown the potential to quench the inhibition. Rates of reaction were expressed relative to BIT and CMIT in the absence of 'neutraliser' concentration. Results are presented in Fig. 3 for the thiol-containing neutralisers.

Both thiol-containing agents neutralised the activity of CMIT and BIT when present at relatively high concentrations. At thiol concentrations less than 10% of that for the inhibitors little effect was observed in either instance. For CMIT, however, activity was significantly enhanced at and above equimolar thiol concentrations and DTT was the more effective enhancing agent. This possibly relates to the additional presence of amine groups on GSH. Neither histidine nor valine affected the activities of either isothiazolone towards ADH. The previously reported quenching of CMIT activity in bacterial growth systems, by valine, probably relate therefore to quenching of its mercaptoacrylamide.

The results of these studies indicate that the isothiazolones have two modes of action as inhibitors of ADH activity. These results are consistent with the findings of our previous investigations (Collier et al., 1990a,b,c) which suggest that there is a basic dichotomy in the activity of the chlorinated and non-chlorinated isothiazolones. The presence of a chlorine atom on the isothiazolone ring enables the thio-acyl chloride tautomerisation which, in turn, results in a greatly enhanced anti-microbial activity.

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