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# Interpretation of the Cytostatic Properties of Sodium Morpholyldithiocarbamate, a Chelating Agent

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**Summary.** The antioxidant properties of sodium morpholyldithiocarbamate (*MorDTC*) were studied in order to contribute to the interpretation of its antitumor activity and synergetic effect over *cis*-platinum. *MorDTC* inhibits pyrogallol autooxidation better than vitamin C but does not dismutate the superoxide radical generated by the xanthine – xanthine oxidase system. Nevertheless, the complexes formed *in situ* between *MorDTC* and Mn(II), Co(II), Ni(II), and Cu(II) do dismutate the superoxide radical with a SOD-like activity (SOD = superoxide dismutase), expressed in terms of $IC_{50}$ values within the range of 3.2 to 62 $\mu M$. The highest activity corresponds to Mn(II) and Cu(II) complexes. The possible inhibitory action of *MorDTC* on erythrocyte SOD was also studied *in vitro*; however, the results were negative. Therefore, *MorDTC* should dismulate the superoxide radical after chelating metals without inhibiting SOD, the enzyme playing this role *in vivo*.

**Keywords.** Dithiocarbamates; SOD inhibition; SOD mimic.

**Interpretation der cytostatischen Eigenschaften von Natriummorpholyldithiocarbamat, einem komplexbildenden Reagens**

**Zusammenfassung.** Die antioxidativen Eigenschaften von Natriummorpholyldithiocarbamat (*MorDTC*) wurden im Hinblick auf seine Wirkung gegen Tumore und seinem synergistischen Effekt im Zusammenhang mit *cis*-Platin untersucht. *MorDTC* hemmt die Autoxidation von Pyrogallol besser als Vitamin C, greift aber das vom System Xanthin–Xanthinoxidase gebildete Superoxidradikal nicht an. Dieses wird jedoch von dem *in situ* aus *MorDTC* und Mn(II), Co(II), Ni(II) und Cu(II) gebildeten Komplexen mit einer SOD-vergleichbaren Aktivität umgesetzt (SOD = Superoxiddismutase; $IC_{50}$ im Bereich von 3.2 bis 62 $\mu M$). Die höchste Aktivität wurde für Mn(II)- und Cu(II)-Komplexe gefunden. Die hemmende Wirkung von *MorDTC* auf erythrocytische SOD wurde *in vitro* untersucht. Die Ergebnisse waren negativ. *MorDTC* ist daher nach der Komplexierung mit Metallen in der Lage, das Superoxidradikal anzugreifen, ohne SOD, das Enzym, das diese Aufgabe *in vivo* übernimmt, zu hemmen.

## Introduction

Dithiocarbamates (*DTC*) constitute a type of compounds with a very wide spectrum of antifungal [1], antimicrobial [2], cytostatic [3], antiviral [4], immunoregulatory

[5, 6], *etc.* properties. These studies have been almost exclusively limited to sodium diethyldithiocarbamate (*DDTC*), one of the smallest and most stable *DTC*s.

Reports of cytostatic properties of *DTC* have been constrained to *in vitro* studies of *DDTC* [3]. *DTC* have also been tested *in vivo* on rodents to control nephrotoxic effects of *cis*-platinum (*cis*-diamminodichloroplatinum(II)) [7, 8], and it has been established that other *DTC*s than *DDTC* are more effective, without observing any cytostatic effects of DTC itself.

The biological properties of *DTC* are closely related to their strong chelating ability that involves the formation of very stable neutral complexes with all transition metal ions and also many representative metal ions. Such complexes show high hydrophobic characteristics and, therefore, very low water solubility.

It has been thought that the stability of *DDTC* makes it a preferential *DTC* for biological studies [6]. When a *DTC* is administered *in vivo*, it must be by injection since the acidity of the gastric system decomposes it almost immediately. Once administered, *DTC* will form very stable neutral chelate complexes with biometals. Therefore, the stability of *DTC* salts must not be a determining factor of its biological properties. That is the reason why steric and electronic, but not stability factors, must be considered in the prediction and interpretation of the biological properties of *DTC*.

Sodium morpholyldithiocarbamate (*MorDTC*) is of moderate toxicity ($LD_{50} = 410$ mg/kg in mice and 660 mg/kg in rats) [9]. *MorDTC* shows a moderate antitumor activity (ILS = 28–60%) on P-388 Leukemia, *Lewis* carcinoma, and *Acaton* tumor [9]. The most interesting aspect of the antitumor activity of *MorDTC* is that it does not only inhibit the toxicity of *cis*-platinum in mice inoculated with the experimental tumor Leukemia L-1210 as *DDTC* does, but also enhances two- to threefold the antitumor activity of the former [9]. Therefore, the antitumor activity of *MorDTC*, when administered at the same time or after *cis*-platinum, shows a synergetic effect over the antitumor activity of *cis*-platinum. The purpose of the present paper is to contribute to the interpretation of the antitumor activity of *MorDTC* and its synergetic effect over *cis*-platinum from the point of view of the antioxidant and chelating properties of this compound.

## Results and Discussion

### *Inhibition of pyrogallol autooxidation*

*MorDTC* behaves as an antioxidant when tested against pyrogallol at *pH* values over 7.0. This antioxidant property of *MorDTC* is similar to that of *DDTC* and greater than that of vitamin C (ascorbic acid) as can be seen from Fig. 1. This activity is observed even for pyrogallol:DTC molar ratios as high as 40:1 (Fig. 1). A similar study, but in presence of divalent transition metals, could not be carried out because these complexes absorb intensively at 440 nm ($\log \varepsilon = 2$–3), the selected wavelength [10].

*MorDTC* inhibits the autooxidation of pyrogallol in a catalytic way. This was confirmed by gravimetric determinations of the content of *MorDTC*, as its copper(II) complex, after several hours of interaction with pyrogallol, with $99.0 \pm 0.5\%$ of recovery.

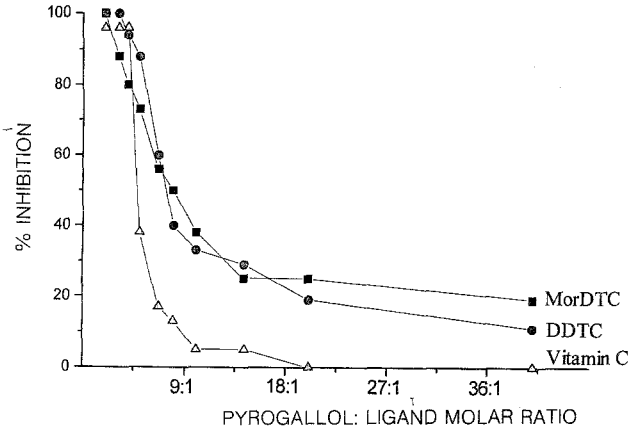


**Fig. 1.** Inhibition of pyrrogalol autoxidation at *pH* 7.4 by *MorDTC* (■), *DDTC* (●), and Vitamin C (△) for different molar ratios

*SOD-like activity*

Considering the antioxidant activity of *MorDTC* against pyrogallol, it was expected that this compound should also behave as an antioxidant against $O_2^{\cdot -}$ since SOD also inhibits the autooxidation of pyrogallol [10]. This hypothesis was not confirmed; *MorDTC* does not vary at all the OD *vs.* time curve, even at concentrations up to $10^{-3}$ *M*. Therefore, *MorDTC* is not capable, as its sodium salt, to dismutate the superoxide radical. This negative result could be due to the fact that the anionic *DTC* must repel electrostatically the anionic superoxide radical. When *MorDTC* forms neutral complexes with transition metal ions, SOD-like activity takes place.

The dismutation of superoxide radical involves two stages:

i) oxidation: $$O_2^{\cdot -} = O_2 + 1e^-$$

i) reduction: $$O_2^{\cdot -} + 1e^- + 2H^+ = H_2O_2$$

In the oxidative stage, the metal ion involved is reduced, whereas in the following stage the oxidation of the reduced form of the metal ion takes place. This way, the metal can participate cyclically in the dismutation of $O_2^{\cdot -}$ as confirmed by EPR of *bis*-(salicylate)-copper(II) interaction with the superoxide radical [13].

For Mn(II), the first stage would be the formation of Mn(III) and $H_2O_2$, whereas in the following stage, Mn(II) and $O_2$ are formed. For Cu(II), the dismutation of $O_2^{\cdot -}$ should start from the oxidation of $O_2^{\cdot -}$. This explains why Mn(II) and Cu(II), both salts and complexes, were the most active metals in the dismutation of $O_2^{\cdot -}$. The redox properties of Co(II) and Ni(II), especially in dithiocarbamate complexes, are very constrained and, therefore, must be less active. These considerations were confirmed experimentally by the $IC_{50}$ values reported in Table 1. The latter two metal complexes with MorDTC are of magnitude one order less active than the Mn(II) and Cu(II) complexes. Nevertheless, SOD presents an $IC_{50}$ value of $2.0 \times 10^{-9}$ *M*, three orders more active than the Cu(II) complex.

In all cases, except for Cu(II), the dithiocarbamate complexes are one order more active than the corresponding salt. This exception for Cu(II) can be explained by different points of view. Considering that dithiocarbamates are $\pi$-acceptors, they should favor Cu(II) reduction, but, at the same time, they stabilize a square planar geometry, and, therefore, sterically restrict the reduction to Cu(I) with a tetrahedral

**Table 1.** Concentrations for 50% inhibition ($IC_{50}$) of the reduction of *NBT* by divalent metal ions and their complexes with *MorDTC* formed *in situ*

| Metal ion | $IC_{50}$ ($\mu M$) | |
|---|---|---|
| | Uncoordinated metal | $M(MorDTC)_2$ |
| Mn(II) | 21 | 7.0 |
| Co(II) | 140 | 62 |
| Ni(II) | 480 | 36 |
| Cu(II) | 2.3 | 3.2 |

distribution. Finally, the hydrophobic surroundings that provide the dithiocarbamates coordinated to Cu(II) must affect the proton diffusion required for superoxide dismutation.

The divalent metal salts selected for these experiments were sulfates. It should not be interpreted that the divalent metal ion exists, under our experimental conditions, also as sulfates; even more, others would be the forms of presentation *in vivo* [14]. According to *Costanzo* [15], Cu(II) ions in phosphate buffer solutions exist preferentially as $Cu(HPO_4)$. *DTC*s form very stable complexes with most transition metal ions. In the case of the divalent metals studied, *MorDTC* forms neutral complexes of the same order of stability as *DDTC* with $\log \beta_2$ values of 9.10, 13.60, 14.70, and 26.05 for Mn(II), Co(II), Ni(II), and Cu(II), respectively [16]. Therefore, the total coordination of the metal ion can be considered without introducing any significant error. That is why *MorDTC* has been used to determine Cu(II) in biological systems [17].

The corresponding *DDTC* complexes could not be studied because of their lower solubility. Nevertheless, this was possible for the Mn(II) complex for which an $IC_{50}$ value of 11 $\mu M$ was achieved, almost an order of magnitude higher than for the corresponding *MorDTC* complex.

*Inhibition of the SOD activity*

The maximum inhibition of SOD by both *DDTC* and *MorDTC* was attained at 110 min (Fig. 2) by inhibition determinations every 20–30 min.

*MorDTC* does not inhibit the enzymatic activity of SOD, with only a very slight variation in the % of inhibition (Fig. 2). *DDTC*, on the contrary, inhibits to 100% the SOD activity [18]. This difference between the inhibition capacity of *MorDTC* and *DDTC* could be explained by their sterical differences and, for that reason, the interatomic distances in these molecules were calculated.

For the geometry of SOD, of both human and bovine erythrocytes, the active-site channel has been determined [19]. This channel presents a wide access entrance of 24 Å, followed by a narrower section of 10 Å that is 5 Å deep. The final access to Cu(II), at the active site, is less than 4 Å wide. The geometrical characteristics of this active-site channel constrains the access to Cu(II) to only very small molecules, such

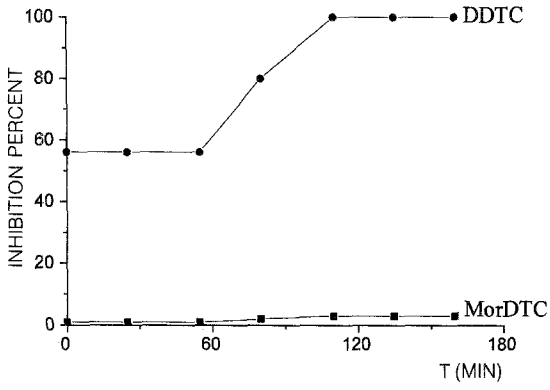


**Fig. 2.** Inhibition of the enzymatic activity of SOD (for $IC_{50}$) by 2 m*M* concentrations of *MorDTC* (■) and *DDTC* (●)

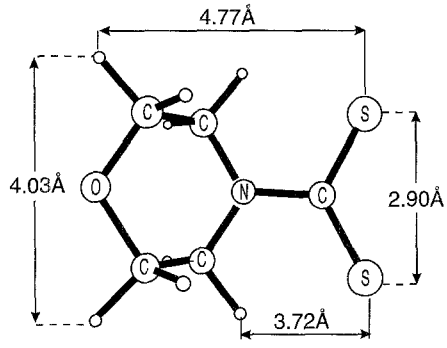


**Fig. 3.** Interatomic distances calculated for *MorDTC*

as $CN^-$, $N_3^-$, and *DDTC* [20]. The fact that these inhibitors are anions results from the fact that at the entrance of the active-site channel Arg-141, Lys-120, and Lys-134 protonated amino acid residues are located that serve to attract the anionic superoxide radical [21].

The mean S–S distance for most of the *DTC* sodium salts, structurally characterized by X-ray diffraction, is of about 3.0 Å [22] and, therefore, it should be expected that any *DTC* could inhibit SOD. The fact that *MorDTC* does not inhibit SOD is that the aliphatic moiety, in this compound, is cyclic and, therefore, has a certain rigidity. The calculated H–H distance (Fig. 3) of the $OC_4H_8N$ cyclic moiety is 4.03 Å, slightly greater than the width limit of the final section of the active-site channel, and also greater than the corresponding distance calculated for the narrowest conformation of *DDTC* (3.89 Å) (Fig. 4). This difference, plus the fact that the latter *DTC* has a very flexible structure, could serve to explain why only *MorDTC* is sterically impeded to chelate to Cu(II) at the active site of SOD. This consideration is also

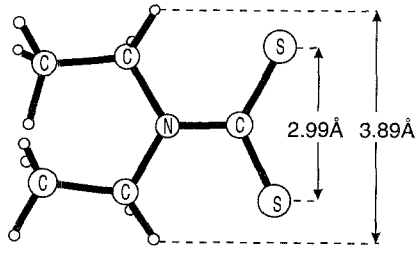


**Fig. 4.** Interatomic distances calculated for the narrowest conformation of *DDTC*

supported by the fact that dimethyldithiocarbamate inhibits SOD even more efficiently than *DDTC* [23].

The fact that *MorDTC* does not inhibit the enzymatic activity of SOD, whereas *DDTC* does, could serve to explain their different antitumor behavior. When *MorDTC* is administered to a biological system, it can dismutate superoxide radicals, previous to complex formation with the surrounding metal ions. In this case, the excess of *MorDTC* can not inhibit SOD, and neither can this second event compete with the complexation process. In the case of *DDTC*, this does not happen since it can inhibit SOD, a negative action to antitumor activity. This negative side-effect of *DDTC* should be considered in further biological studies.

The antitumor activity of *MorDTC*, when administered with *cis*-platinum [9], could also be interpreted considering the antitumor activity of the potential *in vivo* formation of Pt(*MorDTC*)$_2$. The *in vitro* cytotoxicity of this complex has been determined on KB cells with a reported $IC_{50}$ value of 16.63 $\mu M$ [24]; this is equal to 9.41 μg/ml, a value higher than that reported, also on KB cells, for *MorDTC*, for the sodium salt (< 1 μg/ml) [9].

## Experimental

### *Materials*

All reagents used were of analytical grade. NaOH, $CS_2$, ascorbic acid, and $Et_2O$ were purchased from Riedel-de Häen, sodium diethyldithiocarbamate, *DMSO*, pyrogallol, and divalent metal sulfates (Mn, Co, Ni, Cu) from British Drug House, nitroblue tetrazolium chloride (*NBT*), xanthine oxidase, and superoxide dismutase (SOD; from bovine erythrocyte) from Boehringer Mannhein and xanthine and morpholine from Fluka. Freshly distilled water ($CO_2$-free) was used in all cases.

### *MorDTC*

An ethanolic solution of morpholine was added dropwise to an ethanolic solution of $CS_2$ at 0–5 °C (molar ratio: morpholine:$CS_2$ = 1:1). The resulting mixture was treated with $Et_2O$, and an aqueous solution of NaOH for a $CS_2$:NaOH molar ratio of 1:1. The product was filtered, washed, and recrystallized from ethanol. M.p.: > 300 °C; IR: $\nu$ ($cm^{-1}$) = 1460 ($\nu_{C=N}$), 981 ($\nu_{C-S}$), 542 ($\nu_{C-S} + \delta_{SCS}$); UV: $\lambda_{max}$ (nm) = 263 ($\log \varepsilon = 4.18$; CSS $\pi - \pi^*$); 284 ($\log \varepsilon = 4.18$; NCS $\pi - \pi^*$); $^1$H NMR ($D_2O$): $\delta$ (ppm) = 4.38 (t, 4H, $-OCH_2-$, $J_{H-H}$ = 5.1 Hz); 3.77 (t, 4H, $-NCH_2-$, $J_{H-H}$ = 4.9 Hz).

### *Inhibition of pyrogallol autooxidation*

The inhibition of pyrogallol autooxidation was performed as reported by *Puget* [10] on solutions $4 \times 10^{-4}$ *M* of pyrogallol and producing slopes of ~0.020 ΔOD/min throughout 10 min at 440 nm.

### *SOD-like activity*

The SOD-like activity was studied using $O_2^{\cdot -}$ generated by the xanthine–xanthine oxidase system under conditions similar to those described by *Fridovich* [11]: phosphate buffer *pH* 7.8 (50 m*M*), *NBT* (25 $\mu M$), xanthine (100 $\mu M$), and the amount of xanthine oxidase required for slopes of ~0.025 ΔOD/min. *EDTA* was not used in order to avoid competition with the chelating agents submitted to study. *NBT* reduction by the superoxide radical was spectrophotometrically monitored at 560 nm. Other details of SOD-like activity are as previously reported [12].

*Inhibition of SOD*

The *in vitro* inhibition of SOD was performed by incubating SOD at 37 °C, mixed with the chelating agent at concentrations of $2 \times 10^{-3}$ *M*. The optimum incubation time was checked for each system.

*Kinetic determinations*

The kinetic measurements were performed on an Ultrospec III (Pharmacia-LKB) spectrophotometer using its *Enzyme Kinetics* (*EK*) software. The assay time was generally fixed to not less than 10 min. The slope (ΔOD/min) obtained showed in all cases a linearity greater than 0.993. Each series of experiments was carried out with 4 cuvettes, synchronized in time by the *EK* program. The first cuvette was always the xanthine oxidase system without the assay. In the determinations of the inhibition of SOD the first cuvette also contained this enzyme at $IC_{50}$ values. The % of inhibition were calculated related to the slope of this first cuvette (ref. slope) using the following equation:

$$\% \text{ inhibition} = (\text{assay slope} - \text{ref. slope}) \times 100/\text{ref. slope}$$

The concentration of the compound that inhibits the reduction of *NBT* to 50% ($IC_{50}$), was determined by regression analysis and interpolation of the % inhibition *vs.* assay concentration curve for not less than five experimental points for each system with inhibition values within the range of 10 to 75%. In all cases, a linearity greater than 0.994 was achieved, except for the Ni(II)-*MorDTC* system where a linearity of 0.947 was obtained. Other details are as previously reported [12].

*Molecular modeling*

Molecular modeling calculations were performed using the AM1 semiempirical method supported in the MOPAC (6.0 version) software.

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