Antioxidant Properties of Organostibines Against Lipid Peroxidation in Homogenized Rat Brain

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The effect of six different synthesized organoantimony compounds viz. *tris*-(3-methyl-2-thienyl)stibine (**1**), *tris*-(5-chloro-2-thienyl)stibine (**2**), *tris*-(2-selenophenyl)stibine (**3**), chloro-*bis*(N,N-dimethyl-(1-ferrocenylethyl)amine)Sb(III) (**4**), *tris*-(2-dimethyl aminoethyl ferrocenyl)stibine (**5**), 1-(2-diphenylstibine-ferrocenyl)methyldimethylamine (**6**) on thiobarbituric acid reactive species (TBARS) production, free radical activity in the rat brain homogenate were determined. Their antioxidant activity were compared with the quercetin, Butylated hydroxytoluene (BHT; 2, [6]-di-*tert*-butyl-p-cresol) and α -tocopherol as reference antioxidants. All compounds showed a dose dependent inhibition on TBARS production. *Tris*-(5-chloro-2-thienyl)stibine (**2**) showed lipid peroxidation inhibition in homogenized rat's brain, double in magnitude, than shown by *tris*-(3-methyl-2-thienyl)stibine (**1**) activity. The compound (**2**) showed a reduction of > 80 % of DPPH radical reduction while the compound **1** did not show a significant antioxidant activity. Variety of simple organo antimony compounds display antioxidant activity *in vitro* against TBARS production, however detailed toxicological studies must be carried out with stibines in order to determine whether these compounds could be tested therapeutically as an antioxidant.

Key Words: Organostibines, Antioxidant, Thiobarbituric acid, Quercetin, α -Tocopherol lipid peroxidation.

INTRODUCTION

Antimony as a pure metal and metal alloys is extensively used in industry. When it is exposed to reducing acids or when batteries are overcharged, stibine, a highly toxic gas is produced¹⁻⁴. Antimony compounds exhibit antimicrobial, antibacterial, antifungal and antitumor activities⁵⁻⁸.

Antimony containing compounds produce cardiac functional alterations and toxicity in both experimental animals and humans. Potassium antimonyl tartarate (PAT) caused myocardium degeneration, increase in the connective and fibrous tissue of the heart, hepatic necrosis and kidney degeneration. Rat's injected with low doses of antimony, potassium antimonyl tartarate, antimony tri and penta sulfide, antimony tri or pentoxide exhibited cardiac toxicity and increased mortality^{9,10}. While sodium stibogluconate (SSG), in its short term treatment produced, increased white blood cells, serum aspartate aminotransferase, creatinine and blood urea and reduced blood hemoglobin (Hb) and glucose levels. Although antimony administration has been associated with cardiac toxicity virtually nothing is known about its mechanism of action. As such mammalian heart and brain are extremely vulnerable to oxidative stress through oxygen radical attack. The biochemical

activity of a few antimony complexes has been extensively investigated¹¹⁻¹³. Reports on the mechanism of biological action of antimony compounds is not very well documented and they are sometimes discrepant¹⁴⁻¹⁶. In many cases, antimony compounds toxicity is related to the formation of reactive oxygen species (ROS)¹⁷. There exists report on the increment in oxidative stress of cardiac myocytes (*in vitro*) by potassium antimony(III) tartrate (PAT), which is widely used as an anthelmintic drug⁵. The preliminary treatment of cells with vitamin E or the simultaneous introduction of antioxidants prevents the progress of lipid peroxidation (LP)¹⁸. Similarly intensification of lipid peroxidation and increment of product levels of thiobarbituric acid reactive substances (TBARS) were observed when plants were treated with PAT¹⁷. Antimony compound's toxicity depends on the oxidation state of antimony as well as on organic groups attached to the metal. Trivalent antimony derivatives are more toxic than pentavalent compounds^{18,19}.

We have previously reported the toxicity of four different trivalent antimony compounds on larvae of *Artemia salina* and cytotoxicity against different cancer cell line cultivates^{20,21}. The following study was conducted to assess the effects of a soluble

form of different synthesized antimony compounds on spontaneous and induced lipid peroxidation in homogenized rat's brain.

EXPERIMENTAL

Compound *tris*-(3-methyl-2-thienyl) stibine (**1**), *tris*-(5-chloro-2-thienyl)stibine (**2**), *tris*-(2-selenophenyl)stibine (**3**), chloro-*bis*-(2-N,N'-dimethylaminomethylferrocen-1-yl)stibine(III) (**4**), *tris*-(2-N,N'-dimethylaminomethylferrocen-1-yl)stibine (**5**), diphenyl (2-N,N'-dimethylaminomethylferrocen-1-yl) stibine (**6**) were synthesized by previously reported method²⁰⁻²³ and their chemical structures are shown in Fig. 1.

Fig. 1. Chemical structures of compound (1-6)

Reduction of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH): The basis of this method was described by Blois²⁴. The molecule of 1,1-diphenyl-2-picrylhydrazyl (DPPH[•]), is a stable free radical by virtue of the delocalization of the spare electron over the molecule gives rise to the deep violet colour characterized by an absorption band in ethanol solution at 517 nm. When a solution of DPPH[•] is mixed with any substance that can donate a hydrogen atom (antioxidant), DPPH[•] is reduced and change the colour of the solution from violet to pale yellow, with a concomitant diminution of absorption at 517 nm. DPPH free radical reacts with an antioxidant (AH) or a radical species (R[•]) according to equations as shown:

The test was carried out on 96 well microplates. 50 mL of the solution of the test compound was mixed with 150 mL of ethanolic solution of DPPH (final concentration 100 mM), then was incubated at 37 °C for 0.5 h and subsequently the absorbance was measured at 515 nm in a microplate reader ELx 808. The percentage of inhibition of each compound was determined by comparison with a 100 mM DPPH ethanolic blank solution.

Animals: Adult male Wistar rats (200-250 g) were provided by the Instituto de Fisiologia Celular, UNAM and approved by the Animal Care and Use Committee (Proy-Nom 087-ECOLSSA1-2000). They were maintained at 25 °C on a 12/12 h light-dark cycle with free access to food and water.

Rat brain homogenate: The animal's euthanasia was carried out avoiding unnecessary pain. Under mild diethyl ether anesthesia rats were euthanized by cervical dislocation and

cerebral tissue (whole brain) was rapidly dissected and homogenized as previously described²⁵, in phosphate buffered saline (PBS; 0.2 g KCl, 0.2 g KH₂PO₄, 8 g NaCl and 2.16 g NaHPO₄·7H₂O/L, pH 7.4) to produce a 1/10 homogenate, w/v. The homogenate was centrifuged for 10 min at 3400 rpm to yield a pellet that was discarded, protein content in the supernatant was measured using the Folin and Ciocalteu's phenol reagent²⁶ and adjusted to 2.5 mg protein/mL with PBS.

Lipid peroxidation: The supernatant (400 mL) was incubated at 37 °C for 0.5 h in presence of test sample (50 mL). Lipid peroxidation was started adding 50 mL of a freshly prepared 100 mM FeSO₄ solution (final concentration 10 mM) and incubated at 37 °C for 1 h²⁷. Spontaneous lipid peroxidation was carried out in absence of FeSO₄, the homogenate was incubated for 4 h at 37 °C with sample test. Thiobarbituric acid reactive substances (TBARS) were determined as described by Ohkawa *et al.*²⁸ with some modifications, adding 0.5 mL of the TBA reagent (1 % TBA in 0.05 N NaOH and 30 % trichloroacetic acid in 1:1 proportion). The final solution was cooled on ice for 10 min, then was centrifuged at 3000 g (10,000 rpm) for 5 min and finally heated at 95 °C in a boiling water bath for 0.5 h. After cooling on ice, the absorbance of respective supernatant was measured at 532 nm in a Spectronic Genesys 5 spectrophotometer. Butylated hydroxytoluene, quercetin and α-tocopherol were used as a positive standard. Concentration of TBARS was calculated by interpolation in a standard curve of tetramethoxypropane (TMP)²⁹. Final results were expressed as moles of TBARS per mg of protein. The inhibition ration (%) was calculated using the following formula:

$$\text{Inhibition ration (\%)} = \frac{(C - E)}{C} \times 100 \%$$

where C was the absorbance of control and E was the absorbance of the test sample.

Statistical analysis: All data were represented as mean ± standard error of mean (SEM). Data were analyzed by one-way ANOVA followed by Dunnet's test for comparisons against control. Values of $p \leq 0.05$ (*) and $p \leq 0.01$ (**) were considered statistically significant. The inhibitory concentration 50 (IC₅₀), were estimated by means of a linear regression equation.

RESULTS AND DISCUSSION

The chemical structures of the antimony compounds are shown in Fig. 1. A concentration dependent biological activity on spontaneous lipid peroxidation in homogenized rat's brain is shown by different compounds *viz.* *tris*-(3-methyl-2-thienyl)stibine (**1**), *tris*-(5-chloro-2-thienyl)stibine (**2**), *tris*-(2-selenophenyl)stibine (**3**), chloro-*bis*-(2-N,N'-dimethylaminomethylferrocen-1-yl)stibine(III) (**4**), *tris*-(2-N,N'-dimethylaminomethylferrocen-1-yl)stibine (**5**), diphenyl (2-N,N'-dimethylaminomethylferrocen-1-yl) stibine (**6**) are shown in Fig. 2A-B. Compound (**3**) has high antioxidant activity and it inhibits the major quantity of TBARS production with IC₅₀ of 3.5 mM. In comparison to compounds (**1**), (**2**) and (**3**) with reference antioxidant standards showed that none of these compounds were so active like BHT or quercetin (0.7 and 0.6 mM, respectively). But compounds (**2**) and (**3**) were found more

Fig. 2. Effect of compounds on TBARS production on (A) spontaneous lipid peroxidation and (B) induced lipid peroxidation. All compounds reduced TBARS in a dose-dependent manner. Data represent the mean $\pm$ SE of four independent experiments. * $p \leq 0.05$ and ** $p \leq 0.01$ indicate statistical differences from control; one way ANOVA followed by Dunnet's test

active when compared with α -tocopherol (15 mM), specially compound (3) was four times active while compound 2 was only 1.6 times and compound (1) was less active in comparison to α -tocopherol. If α -tocopherol is used as reference standard, compound (2) and (3) could protect rat's brain against spontaneous lipid peroxidation. Compound (3) shows good antioxidant effect on homogenized rat's brain. Tertiary ferrocenyl stibines also showed important antioxidant activity on concentration-dependant manner. Induced lipid peroxidation (Fig. 2B) and IC_{50} of studied ferrocenyl stibines were compared with quercetin, BHT and α -tocopherol as reference (IC_{50} 4.11 $\pm$ 0.26 mM, 12.86 $\pm$ 64 mM and 569.09 $\pm$ 24.54), respectively (Tables 1 and 2). These results show that these analyzed compounds at low concentrations inhibit have high percentage of TBARS formation than the reference antioxidants. But mechanism of their action is still unknown and this inhibition may be due to the oxidation-reduction of antimony/iron.

In case of DPPH radical trapping tendency compound (2), traps DPPH free radical more efficiently and presents IC_{50} , 24 mM which is double than of quercetin (IC_{50} 12 mM) and more or less of same magnitude as of α -tocopherol (IC_{50} 27 mM). On the other hand ferrocenyl stibines do not show any appreciable activity over DPPH radical. This is also important to mention that compounds (4) and (5) were dissolved in

TABLE-1
 IC_{50} VALUES OF COMPOUNDS ON TBARS PRODUCTION ON RAT BRAIN HOMOGENATE. BHT, QUERCETIN AND α -TOCOPHEROL WERE USED AS POSITIVE STANDARD

	Compound	IC_{50} (μ M) ^a
Spontaneous lipid peroxidation	1	17.59 $\pm$ 1.18
	2	9.22 $\pm$ 0.85
	3	3.51 $\pm$ 0.02
	BHT	0.70 $\pm$ 0.13
	Quercetin	0.57 $\pm$ 0.002
Induced lipid peroxidation	α -Tocopherol	14.94 $\pm$ 2.28
	4	2.28 $\pm$ 0.04
	5	2.19 $\pm$ 0.96
	6	1.10 $\pm$ 0.02
	BHT	12.86 $\pm$ 0.64
	Quercetin	4.11 $\pm$ 0.26
	α -Tocopherol	569.09 $\pm$ 24.54

^aEach value represent the mean $\pm$ SD of four independent experiment.

TABLE-2
EFFECT OF COMPOUNDS ON STABLE FREE RADICAL DPPH REDUCTION

Comp.	Concentration (μ M)	DPPH reduction (%)	IC_{50} (μ M)
1	100	6.63 ^{na}	—
	2	11.54 $\pm$ 2.69 *	23.96 $\pm$ 1.91
	10	19.96 $\pm$ 2.72 **	
	17.78	35.56 $\pm$ 3.02 **	
	31.62	64.14 $\pm$ 3.37 **	
	56.23	80.45 $\pm$ 4.49 **	
3	100	40.61 ^{na}	—
	4	42.81 ^{na}	—
	5	44.56 ^{na}	—
	6	36.38 ^{na}	—
	Quercetin	24.03 $\pm$ 2.59*	11.40 $\pm$ 0.69
	7.5	33.25 $\pm$ 1.74**	
	10	42.54 $\pm$ 2.56**	
	13.34	56.26 $\pm$ 2.82**	
	17.78	70.16 $\pm$ 3.32**	
	α -Tocopherol	29.52 $\pm$ 2.53**	
	23.71	41.86 $\pm$ 3.23**	
	31.62	53.86 $\pm$ 3.86**	26.39 $\pm$ 1.55
	42.17	72.43 $\pm$ 5.26**	
	56.23	87.94 $\pm$ 2.99**	

^{na}Percentages of inhibition less than 60 % at concentration of 100 μ M were considered no active. Data represent the mean $\pm$ SE of four independent experiments; * $p \leq 0.05$ and ** $p \leq 0.01$ significantly different compared with control group; one-way ANOVA followed by Dunnet's test.

ethanol while other compounds were dissolved in DMSO. Chloro substituted thienyl ring in compound (2) and the presence of selenophenyl ring in compound (3) made the Sb atom a little more electropositive which was confirmed by their X-ray crystal structure reported earlier by our group²¹ i.e., more active and inhibited TBARS production which was reflected by the IC_{50} values of compounds (2) and (3) (9.22 mM and 3.51 mM), respectively than methyl substituted thienyl ring as in compound (1). Free radical trapping capacity of compound (2), might be due to chloro group on its 5th position, which favours the resonance delocalization of radical and its interaction with DPPH (2,2-diphenyl-1-picryl hydrazyl) radical. On the other hand ferrocenyl compounds showed a low activity over DPPH radical, which might be because of reduction of this radical by these compounds.

In particular, toxicological data for antimony moieties are extremely limited. Sb(III) induces DNA strand lesions, but not DNA-protein crosslinks³⁰. Sb(III) also appears to interact with key sulphhydryl groups of leishmanial proteins, probably causing enzyme inhibition³¹. As reported by our group²⁰⁻²² that stibines (**1**) and (**2**) shows a significant selectivity (> 80 %) for carcinogenic leukemia cell K562 on growth inhibition 8 and shows less inhibition for colon HCT-15 (< 35 %). Compound (**2**) inhibits growth of all three of the cell lines (leukemia K562, colon HCT562 and U251). All these stibines are highly toxic for the growth of normal lymphocytes with 95 % lethality. But this study suggest that this toxicity may not be due to lipid peroxidation but may be due to other mechanisms besides peroxidation which contribute to cell death^{21,22}. Given that TBARS production is linked to lipid peroxidation and usually initiated by reactive oxygen species (ROS), this study confirms that the synthesized organoantimony compounds exhibits an antioxidant activity against lipid peroxidation may be by neutralization of free radicals.

Simple organoantimony compounds display antioxidant activity *in vitro*. It has been suggested that utilization of the redox activity of the antimony and, in particular, arsenic atoms of such substances could provide antioxidants of considerable potency, suitable as tools in free radical biology. Organoantimony and organoarsenic compounds are readily oxidized from the trivalent to the pentavalent state. This property makes them attractive as scavengers of reactive oxidizing agents such as hydrogen peroxide, hypochlorite and peroxy radicals and as inhibitors of lipid peroxidation in chemical and biological system^{32,33}.

Conclusion

The results of present investigation support the notion that a variety of organoantimony compounds are good antioxidants acting against TBARS production. However, since no detailed toxicological studies have been conducted for stibines, their therapeutic use as an anti cancer and anti leishmaniasis should be considered with interest. Based on the present results, we suggest that additional detailed toxicological studies must be carried out with stibines in order to determine whether these simple compounds could be tested therapeutically as an antioxidant.

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