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SYNTHESIS AND EVALUATION OF BIOLOGICAL ACTIVITY OF NEW ANTIMONY COMPOUNDS WITH METHOTREXATE

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ABSTRACT

The aim of the present work is to synthesize two new antimony compounds containing Methotrexate (MTX) as a ligand, in the mole ratio 1:1 and 1:2. Antimony complexes were characterized by FTIR, UV, CHNS analysis, Atomic Absorption and conductivity measurements.

The biological activity of MTX, SbCl3 and the two new compounds was evaluated against Hela and Rhabdomyosarcoma (RD) cell lines. The four compounds were effective, while compound (2) gave the best inhibition.

KEYWORDS: Antimony Complexes, Methotrexate, Cytotoxicity, HeLa cell, RD Cell

INTRODUCTION

Antimony comes from the Greek 'anti' and monos', which means a metal not found alone. It has been known since ancient times [1]. It forms trivalent organic derivatives like R3Sb, R2SbX and RSbX2 (where R is an organic group and X is a negative atom or group) [2].

Antimony was used in the Babylonian era for medical purposes (therapeutic) and some cosmetics as the oldest use of it was eyeliner [3]. Women of ancient Egypt used stibic stone, antimony sulfide (Sb2S3) to darken their eyes, and treat bacterial infections of the eyes [4].

Antimony (III) complexes have recently received a considerable attention, as they have been shown to exhibit antitumor properties [5]. In particular, antimony (III) complexes with aminopolycarboxy ligands or organoantimony (III) derivatives have shown a significant antitumor activity [6, 7].

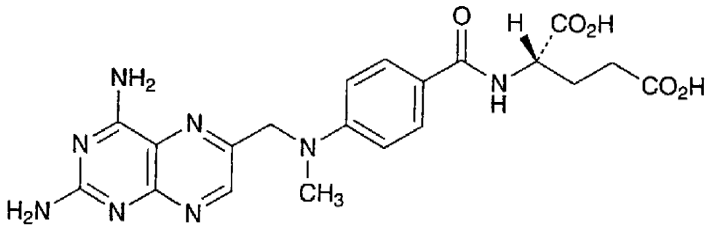
The antimony drugs are still the most effective treatment for a number of diseases. The reduction in mortality from 95% to less than 5% in the case of Kala-azar is due to antimony therapy [8]. A series of antimony (III) halide complexes of thione or thiolate ligands with consistent selective antiproliferative activity against human cervix carcinoma (HeLa) cells were described [9].

HeLa Cells: are the most widely used cancer cell lines in the world. These cells were taken from a lady called Henrietta Lacks from her cancerous cervical tumor in 1951 which today is known as the HeLa cells. These were the very first cell lines to survive outside the human body and grow [10].

Rhabdomyosarcoma (RMS): is a malignancy that arises from skeletal muscle precursors. It is the most common type of soft tissue sarcoma in children and adolescents less than 20 years old. There are two major subtypes of RMS, embryonal and alveolar, which differ markedly in their outcomes [11].

Embryonal RMS usually presents in children less than 10 years old and has a 5-year survival of close to 75%. [12].

Methotrexate: C20H22N8O5 2-[4-[(2, 4-diaminopteridin -6-ylmethyl) methylamino] benzoylamido] pentanedioic acid. [13].



Methotrexate is a folate antagonist first developed for the treatment of malignancies [14] , it is commonly used for the treatment of certain cancers including leukemia, Hodgkin's disease, head and neck cancers. In these illnesses, methotrexate is used in very large doses so that it interferes with the reproduction of the cancer cells. It is used in much smaller doses for the treatment of rheumatoid arthritis, Crohn's disease and psoriasis [15]. It is subsequently, used in non neoplastic diseases as an anti-inflammatory and/or immunosuppressive drug [16].

EXPERIMENTAL

Materials and Instruments

SbCl3 was supplied from BDH, purity 99 %; MTX was supplied from China, purity 98 %. The melting points were measured using (Stuart Scientific Co. LTD melting point-SMP1). C.H.N.S (Euro EA 3000) was used to find the percentages of the components of the prepared complexes. Atomic Absorption Flame Spectrophotometer- Nov AA 350 was used to find the percentage of the antimony in the prepared complexes.

FT-IR spectra were recorded using FT-IR 8000 Shimadzu in the range of (4000-200) cm^{-1}; samples were measured as (CsI disc). Shimadzu (UV-Vis)-160 spectro was used to record spectra of complexes. Also Elisa Reader-ASYS-Austria was used in the biological activity evaluation.

PREPARATION OF THE COMPLEXES

Antimony Complex [SbL] 1:1 Mole Ratio

In a round bottom flask (1.0 gm, 0.004 mole) of antimony(III) chloride dissolved in 5 ml of absolute ethanol was added drop wise to (2.0 gm, 0.004 mole) of the methotrexate dissolved in 15 ml of absolute ethanol. The mixture was heated to 30-35 ^{o}C with stirring for 3hrs. The resulting precipitate was filtered, washed with absolute ethanol, and then dried by using an oven at 50°C for 1h. The product was an orange powder, m. p. 170-172 ^{o}C. Yield 72 %.

Interaction Equation: $SbCl_3$ + MTX ⟶ MTX $SbCl_3$

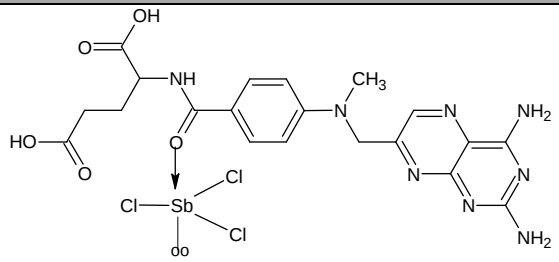
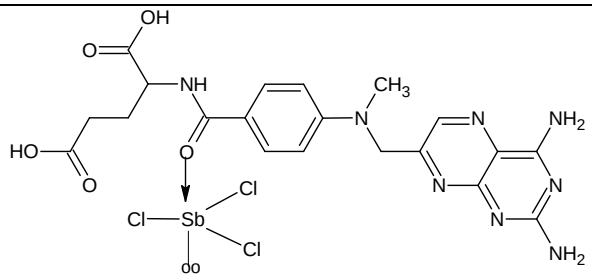
Antimony Complex [SbL] 1:2 Mole Ratio

The same procedure was used, except the mole ratio was (1:2) Sb: MTX. The product was an orange powder, m.p. 173-174 ^{o}C. Yield 54 %.

Interaction Equation: $SbCl_3$ + 2 MTX ⟶ MTX $SbCl_3$ + MTX

It seems that the reaction is going towards 1:1 mole ratio.

Table 1: The Structure and Physical Properties of Compound 1 and 2

Comp.	Structure	M.P. °C	Color	Mole Ratio	Yield %
1		170-172	orange	1:1	72
2		173- 174	orange	1:2	54

Cytotoxicity Assay

Four stock solutions were prepared from complex 1, complex 2, SbCl3 and MTX. Each stock was made by dissolving 0.002 g of the powder of each compound as well as a control negative, and sterilized by filtration using 0.22 µm Millipore filter, and then used in the cytotoxic assay to evaluate their biological activity against Hela and Rhabdomyosarcoma cell lines.

A set of 6 concentrations (10, 8, 6, 4, 2 and 1 µg/ml) was prepared for each compound, the cells were seeded at 10^4 cells per well and the exposure time of the assay was 48 hrs**.** Optical density of each well was measured by using ELISA reader at a transmitting wave length on (570 nm)**.**

RESULTS AND DISCUSSIONS

Characterization

The prepared compounds were characterized by the following techniques**:**

FTIR

The prepared compounds were characterized by the FTIR technique; the results showed the appearance of new peaks and disappearance of others found in the starting materials, these frequencies are listed in Table (**2**).

Peaks appeared at 416 and 406 cm^{-1}, are attributed to Sb-O bond which is not present in the basic materials, some peaks shifted to higher frequencies, this is an evidence of coordination correlation between Sb and O according to the HSAB theory [17]**.**

UV

Also the compounds were characterized by UV spectrophotometry, the results showed electronic transitions of the

type Charge Transferee (LMCT) at 272 nm assigned to the $t_{lu}(\pi) \longrightarrow \sigma_{lg}$ LMCT transition which also a characteristic for similar complexes**.**

CHNS Analysis

Elemental Analysis was performed for compounds 1 and 2. The listed results in Table (3), confirm their basic chemical structure, and reveal a good agreement with the calculated percentages. The percent deviation of the observed / calculated was found to be complied with the accurate analysis.

Table 2: The Most Diagnostic FTIR Bands of the Ligand and its Metal Complexes in (cm^{-1})

Compd. / Bands	[MTX]	[MTX $SbCl_3$] 1:1	[MTX $SbCl_3$] 1:2
v (O-H)	3564, 3529	3564, 3529	3564, 3535
v (N-H) of NH_2	3479, 3307	3450, 3299	3417, 3313
v (N-H) of amide	3348	3346	3346
v (C-H) arom.	3265, 3203 , 3163	3265, 3203, 3163	3265, 3203, 3163
v (C-H) aliph.	2389, 2343, 2129	2389, 2343, 2129	2389, 2343, 2129
v (C=N)	1544, 1506	1544, 1506	1544, 1506
v (C-N)	1367	1367	1367
v (C=O) of amide	1672	1724	1718
v (C=O) of COOH	1639, 1604	1639, 1604	1639, 1604
v (Sb-Cl)	----------	335	331
v (C=C)	1445, 1404	1445, 1402	1446, 1404
v (C-O)	1274, 1209	1274, 1205	1251, 1207
v (C-H) of CH_3	2636	2636	2636
v (Sb-O)	----------	416	406

The differences in the values between the two compounds is due to the presence of very little amount of MTX with compound (2)**.**

Atomic Absorption: was performed for complexes 1 and 2. The listed results in Table (3), confirm their basic chemical structure.

Table 3: Some Physical and Analytical Data of the Antimony Complexes with Methotrexate Ligand (L1)

Compound Formula Color	Yield %	M. P. °C	M. Wt. G. Mol^{-}	% Elemental analysis / Found (Calc.)				% Metal Found (Calc.)
				C	H	N	S	
C20H22N8O5SbCl3 Orange	72	170-172	682	36.1 (35.2)	3.3 (3.2)	16.1 (16.4)	----------	17.6 (17.8)
C20H22N8O5SbCl3 Orange	54	173-174	682	36.2 (42.2)	3.4 (3.8)	16.5 (19.7)	----------	17.5 (10.7)

The found values of CHNS analysis confirm that the product is (1:1) not (1:2) compared with the results obtained in 1:1 mole ratio reaction. The suggested molecular formula, Table (4) was supported by spectroscopic studies and molar conductivity measurements.

Table 4: Molecular Formula and Nomenclatures of Antimony Complexes with Ligand (MTX)

Molecular Formula	Nomenculture
C20H22N8O5SbCl3	Antimony(III) methotrexate
C20H22N8O5SbCl3	Antimony(III) methotrexate

Conductivity Measurement

Conductivity measurements were obtained using TRANS-BC3020 Instruments. These measurements were obtained in DMSO solvent as (10^{-3}M) concentration at 25 ^{O}C**.** The values were (35.3 and 36.5 µs) for compound **1** and **2** respectively.

Biological Activity Evaluation

The results showed that all these compounds are highly effective in inhibition of tumor cell type (Hela and RD). Results are presented in Tables (5) and (6). The highest values for inhibition were 68.6, 65, 59.6 and 53% for complex 1, 2, SbCl3 and MTX respectively on the HeLa cell line, and 70.6, 73.3, 68 and 58% for the same complexes and starting materials on the RD cell line**.** Comparing the results of the new compounds (1 and 2) with the starting materials (SbCl3 and MTX), it is very obvious that they are higher in their effectiveness due to the synergestic effect of Sb with MTX since they are themselves were effective against cancer [18]**.**

CYTOTOXIC EFFECT OF THE NEW COMPLEXES, SBCL3 AND MTX ON HELA CELL LINE

When the cancer cell line (Hela) was treated with the two starting materials and the new complexes, the results showed significant effects for all of these compounds, in all the concentrations used compared with the control negative which contains only cell line and the culture media. The toxic effect varied between the tested samples showing a significant cytotoxic effect started from 10 µg/ml to 1 µg/ml concentrations**.**

The cytotoxic study was done on HeLa cell line (passage number 23) isolated from human as an aggressive cervical adenocarcinoma, exposure time was 48 hrs**.** The inhibition rate percent (I.R. %) was calculated, and the results varied among starting materials and the new complexes as shown in Table (5). Figure (1 and 2)**.**

The results showed the cytotoxicity effect of these compounds in all concentrations and the highest inhibition rate (68.6%) recorded with the high concentration (10µg/ml) comparable to control negative**.** A decrease in inhibition rates (33% and 16%) happened in the lower concentrations (2 and 1 µg /ml), respectively**.**

The cytotoxic effect of complex **1** on Hela cell line, Figure (1 and 2) show that the high concentration (10 µg /ml) gave a significant high inhibition rate (68.6%) on cells, while the low concentration (1 µg /ml) gave the low inhibition rate (39.6%).

The higher the inhibition rate of the complexes is due to the synergistic effect produced from the coordination of Sb with MTX; the effectiveness of both antimony and MTX are overlapping, resulting a strengthening of bio-inhibition of the starting materials. The results are concentration dependent.

The inhibition rate follows the order:

Compound 1 > 2 > SbCl3 > MTX, e.g. I.R.% increased by 12-15% in case of MTX (10 µg /ml) due to the coordination with Sb.

Table 5: Initial Cytotoxic Effect on HeLa cell Line of Compounds 1, 2, SbCl3 And MTX by MTT Assay Method in Time of Exposure 48 hrs

Compound	Concentration µg /ml	Mean	(I.R. %)	Viability %
	10	0.094	68.6	31.4
1	8	0.108	64	36
	6	0.10	63.6	36.4
	4	0.141	53	47
	2	0.149	50.3	49.7
	1	0.181	39.6	60.4
	10	0.105	65	35
2	8	0.110	63.3	36.7
	6	0.121	59.6	40.4
	4	0.129	57	43
	2	0.152	49.3	50.7
	1	0.177	41	59
	10	0.121	59.6	40.4
	8	0.129	57	43
SbCl**3**	6	0.135	55	45
	4	0.156	48	52
	2	0.201	33	77
	1	0.252	16	84
	10	0.141	53	47
	8	0.150	50	50
MTX	6	0.156	48	52
	4	0.165	45	55
	2	0.192	36	64
	1	0.229	23.6	76.4

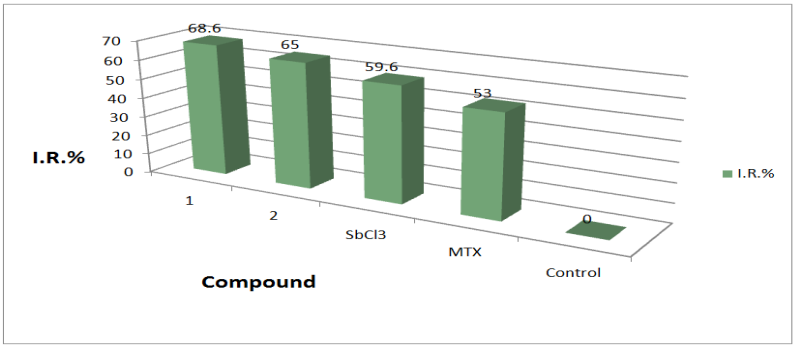


Figure 1: Over All Block Diagram of Cytotoxic Effect of Compound 1, 2 and the Starting Materials on HeLa cell Line

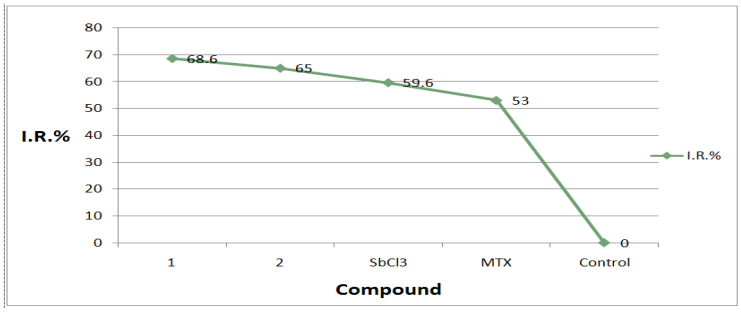


Figure 2: Over all Cytotoxic Effect of Compound 1, 2 and the Starting Materials on HeLa cell Line

CYTOTOXIC EFFECT OF THE NEW COMPLEXES, SBCL3 AND MTX ON RD CELL LINE

The results in Table (6) and Figures (3 and 4) showed the inhibition rate percent in 48 hrs exposure time for each cytotoxic effective concentration. The results explained the cytotoxic effect of the complexes 1, 2 and the starting materials SbCl3 and MTX on RD cell line (passage 45), revealed that the high concentration (10 µg/ml) gave the higher inhibition rates of cells (70.6, 73.3, 68 and 58 %) respectively.

The cytotoxic effect of complex 2 gave the highest inhibition rates (73.3%) at the high concentration (10 µg/ml) . SbCl3 showed high inhibition rate (68%) at the concentration (10µg/ml) and decreased as the concentration decreased too**.** The Methotrexate compound also showed higher inhibition rate (58%) at the concentration (10µg/ml) and a lower inhibition rate (31%) at the concentration (1 µg/ml) during 48 hrs of exposure, due to proliferation develop during low concentration.

The cytotoxic effect of reduced progressively from concentrations (8, 6, 4, 2 and 1 µg /ml) respectively**.** Unexpected reduction of the inhibition rates (24%) for the complex 1 is seen in the concentration (1 µg /ml)**.**

An explanation for this attitude, that in the cell culture experiment, it was important to be aware of growth state of the cell culture, as well as the quantitative characteristic of cell lines [19]**.**

The inhibition differences in Hela and RD cell lines responding to different treatment might indicate a presence or absence of cellular receptor in both types of cell line. Moreover the metabolic pathways to each treatment differed in response from one cell line to another [20, 21]**.** Also the differences are due to the difference between the two cell line types**.**

Table 6: Initial Cytotoxic Effect on RD Cell Line of Compounds 1, 2, SbCl3 and MTX by MTT Assay Method in Time of Exposure 48 hrs

Compound	Concentration µg/ml	Mean	(I.R. %)	Viability %
	10	0.088	70.6	29.4
	8	0.099	67	33
1	6	0.120	60	40
	4	0.123	59	41
	2	0.138	54	46
	1	0.228	24	76
	10	0.080	73.3	26.7
	8	0.081	73	27
	6	0.096	68	32
2	4	0.099	67	33
	2	0.114	62	38
	1	0.18	40	60
	10	0.096	68	32
	8	0.099	67	33
SbCl3	6	0.114	62	38
	4	0.126	58	42
	2	0.147	51	49
	1	0.159	47	53
	10	0.126	58	42
	8	0.135	55	45
	6	0.138	54	46
MTX	4	0.153	49	51
	2	0.174	42	58
	1	0.207	31	69

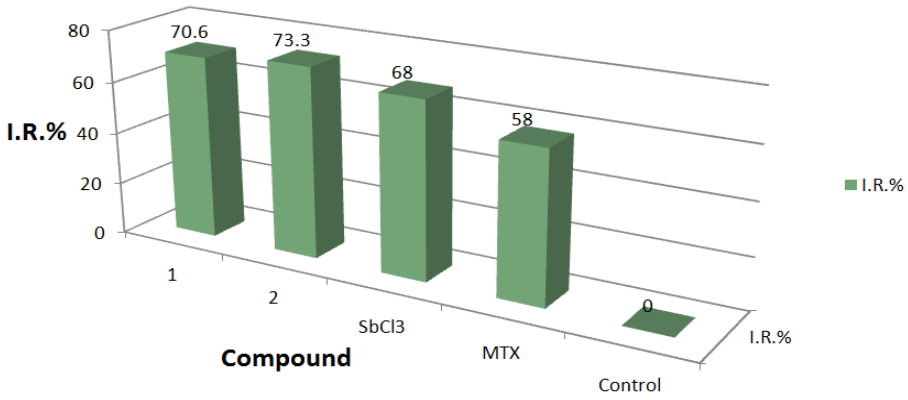


Figure 3: Over All Block Diagram of Cytotoxic Effect of Compound 1, 2 and the Starting Materials on RD Cell Line

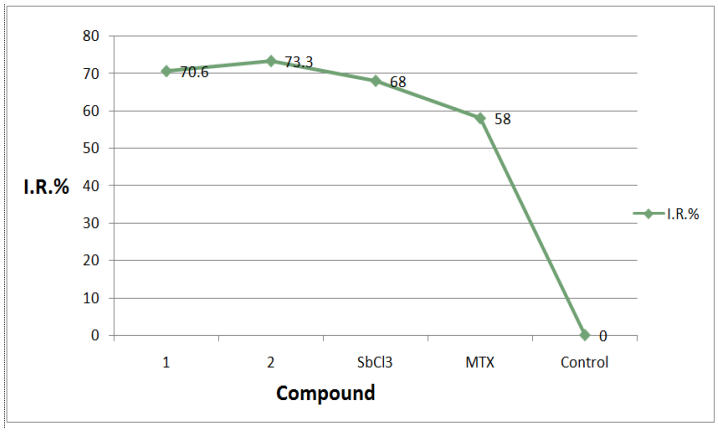


Figure 4: Over all Cytotoxic Effect of Compound 1, 2 and the Starting Materials on RD Cell Line

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