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A New Antitumour Substance, 7-Oxabicyclo (2.2.1)-5-heptene-2,3-dicarboxylic Anhydride

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Abstract—*7-Oxabicyclo*(2.2.1)-5-*heptene*-2,3-*dicarboxylic anhydride has been found to possess antitumour activity against Ehrlich ascites carcinoma cells. The tumour cells incubated with the drug showed a decrease in the viable counts and cell proliferation. These effects were confirmed by* in vivo *studies in Swiss albino mice. The compound has a direct cytotoxic effect on the tumour cells. Vacuolization and disruption of the cytoplasm accompanied by unequal nuclear division and scattered chromosomes were recorded. In addition,* 250 *and* 10 mg/kg *were found to be the MTD and MED respectively. A dose of* 25 mg/kg *injected i.p. for* 5 *consecutive days in the tumour-transplanted animals caused a significant increase in their survival period. The compound has been shown to have a significant inhibitory effect on the DNA and RNA biosynthesis of EAC cells after* 3 hr *of administration; the protein biosynthesis was less affected. Meanwhile, the cellular contents of these metabolites were significantly reduced.*

INTRODUCTION

AMONG various chemical compounds randomly chosen and tested for their antitumour activity, 7-oxabicyclo(2.2.1)-5-heptene-2,3-dicarboxylic anhydride (OHD) was found to possess the most potent antitumour activity against experimental Ehrlich ascites carcinoma cells. In 1970, Riley and Perham[1] found that the amino groups of lysozyme can be completely blocked with total loss of enzymic activity. Moreover, Tishler and Bell [2] reported that OHD possess defoliating properties as well as a herbicidal effect.

Since the structure of OHD is new to the known chemical categories of antitumour agents (Fig. 1), it was of interest to investigate its effect on experimental tumour system both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Animals

Adult female Swiss albino mice, weighing 20–23 g, were used for maintenance of the tumour line and the subsequent experiments. Animals were maintained on laboratory chow and water *ad libitum*.

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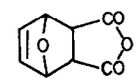


Fig. 1.

Transplantation of tumour cells

Transplanation of Ehrlich ascites carcinoma ('EAC') was carried out by collecting ascites from donor mice bearing 7-day-old Ehrlich ascites tumour. A 0.2-ml portion of the ascitic fluid containing 2.5×10^6 cells with 95% viability, as determined by the trypan blue dye exclusion methods [3], was injected into recipient mice.

In vitro *effect of OHD on EAC cells*

Aliquots (1.5 ml) of Hanks' medium containing 7.5×10^4 Ehrlich ascites tumour cells obtained from 7-day-old donors were mixed with different concentrations of OHD and were then incubated at 37°C aseptically. Samples were taken at 24, 48 and 72 hr. The concentration that kills 50% of the cells (IC_{50}) was determined by the trypan blue exclusion method [3].

In vivo *effect of OHD on EAC cells*

The maximum tolerated dose (MTD) was determined as described by Basil [4] and the minimum effective dose (MED) was reached

after the method of Ishidate *et al.* [5]. According to Goldin [6], the ratio of MTD to MED is defined as the chemotherapeutic index.

The life-span prolongation effect of OHD on the experimental animals was also determined. Mice bearing 24-hr-old EAC were divided into 6 groups, each of 10 mice. The tested compound was administered via i.p. injection either once daily or every second day for a total of 5 injections. Controls receiving only saline were included in each experiment. The mice were observed for mortality and weighed from the onset to termination of experiment. The percentage body weight change was used as an indication of toxicity.

Effect of OHD *on the biosynthesis of* EAC *macromolecules*

Animals bearing 7-day-old EAC were given a single i.p. dose of the tested compound OHD. A group of animals bearing tumour received saline and was used as a control. At selected time intervals (3, 6, 24 and 48 hr) EAC cells were withdrawn on heparin-saline, washed twice and suspended in Hanks' medium at a final concentration of 10^7 cells/ml. The cells were then incubated at 37°C for 5 min, after which 10 μCi/ml [^{3}H]-methyl thymidine* (28 Ci/mmole), [^{3}H]-5-uridine (21.7 Ci/mmole) or [^{3}H]-4,5-L-leucine (1 Ci/mmole) were added and allowed to react for 30 min. The rest of the experimental steps were carried out as described by Fujimoto *et al.* [7]. Labelled cells were collected on glass fibre paper circles (2.4 cm diameter) and the radioactivity was measured as described by Mans and Novelli [8].

*The labelled substances were obtained from Radiochemical Centre, Amersham, U.K.

Effects of OHD on EAC contents of lipid, protein, DNA and RNA

EAC cells taken from treated and untreated animals at the pre-indicated time intervals were used.

Fractionation of EAC cells was carried out as described by Schneider *et al.* [9]. Total lipids were determined as described by Knight *et al.* [10]. Proteins were measured by the method of Daughaday *et al.* [11], contents of RNA were assessed by the orcinol reagent [12], while those of DNA were determined following the method of Dische and Schwarz [13].

RESULTS

During the *in vitro* evaluation of OHD against EAC cells the following results were concluded: (1) a concentration of 25 μg/ml of OHD was maximal, above which no more destruction of EAC cells was observed (Table 1); (2) the IC_{50} of OHD was found to be 10 μg/ml (Table 1); (3) the concentration of OHD that causes a decrease in the growth rate of EAC cells by 50% after 24 hr incubation was expressed as the minimum effective concentration (MEC) and was found to be 3.75 μg/ml.

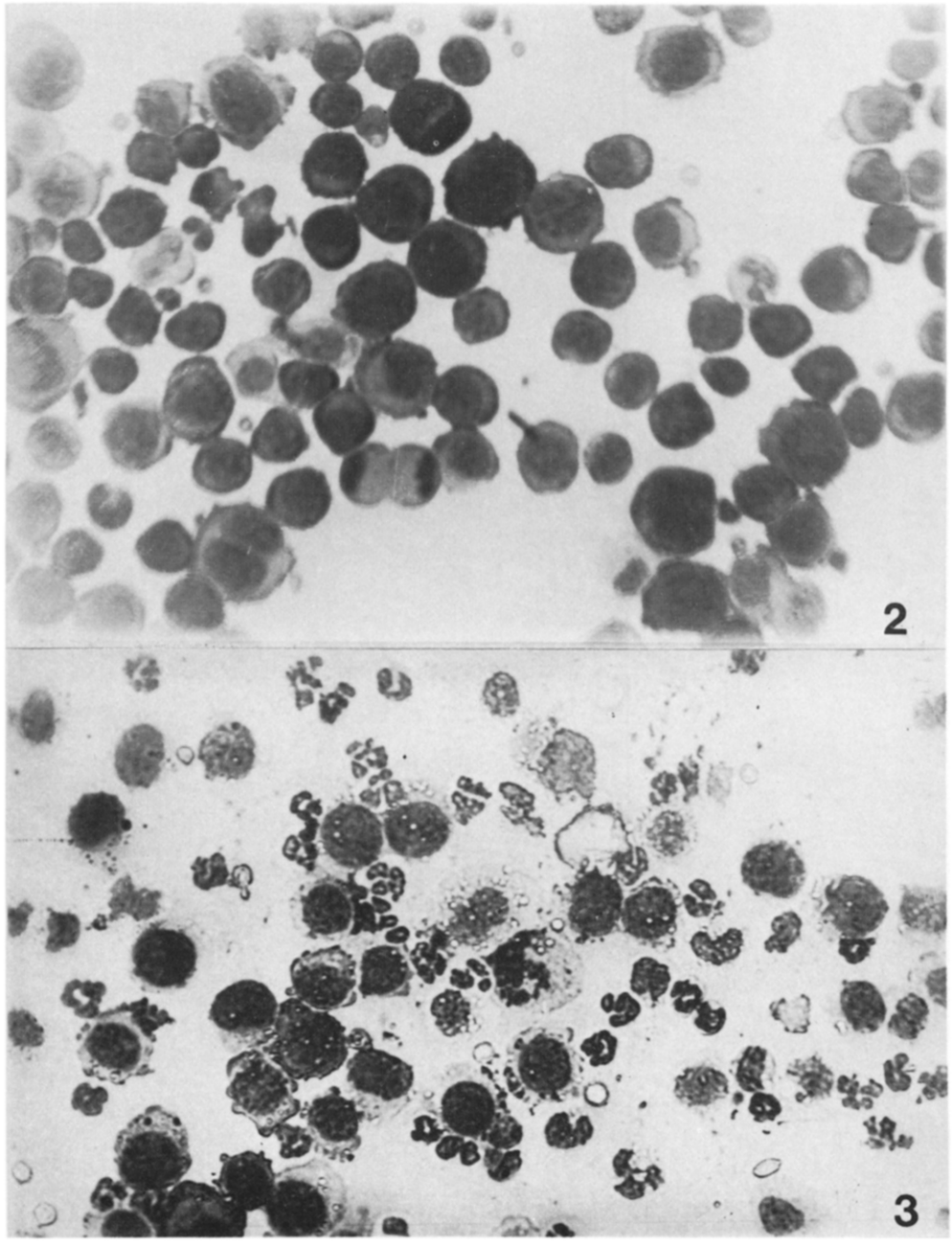
The substantial *in vitro* effects of OHD on EAC cells suggested that it would be worthwhile to evaluate its *in vivo* effects.

The LD_{50} of OHD was 350 mg/kg of body weight when injected via the i.p. route in Swiss albino mice (20 g ± 1.0 g). When 250 mg/kg of body weight was injected in animals bearing EAC cells (Table 2), more than 50% of the neoplastic cells showed disruption of the cytoplasm accompanied with rupture in the cytoplasmic membrane (Figs. 2–5). Moreover, vacuolization in the cytoplasm and/or irregularities in the nuclear membrane and uneven pattern of nuclear division were also observed.

Table 1. In vitro *effect of OHD on the viability and growth rate of EAC cells*

Concentration of OHD μg/ml	Percentage viability at incubation time: 24 hr	48 hr	72 hr	Percentage growth rate at incubation time: 24 hr	48 hr	72 hr
0.00	100	100	100	100	100	100
0.5	87.5	58.5	57.7	67.1	66.2	61.6
1.0	84.5	56.7	54.4	63.9	64.7	57.9
2.5	79.8	54.9	47.8	54.3	56.3	47.3
5.0	64.6	46.4	42.5	48.9	45.9	46.6
10	49.5	38.6	41.7	45.5	42.8	43.5
25	34.9	35.1	40.3	41.1	39.9	39.2
50	34.1	33.3	35.1	37.2	35.8	35.2
100	34.7	33.9	35.8	35.7	34.7	34.6

The rate of control was taken as 100 for growth rate and viability. Hank's medium was used.



Figs. 2–5: *Ehrlich ascites carcinoma cells after the treatment with different doses of OHD given after* 3 *days of tumour transplantation* (*Table* 2).

Fig. 2. *Untreated Ehrlich ascites carcinoma cells.*

Fig. 3. *Treated Ehrlich ascites carcinoma cells showing vacuolization and disruption of the cytoplasmic mass accompanied with rupture in the cytoplasmic membrane.*

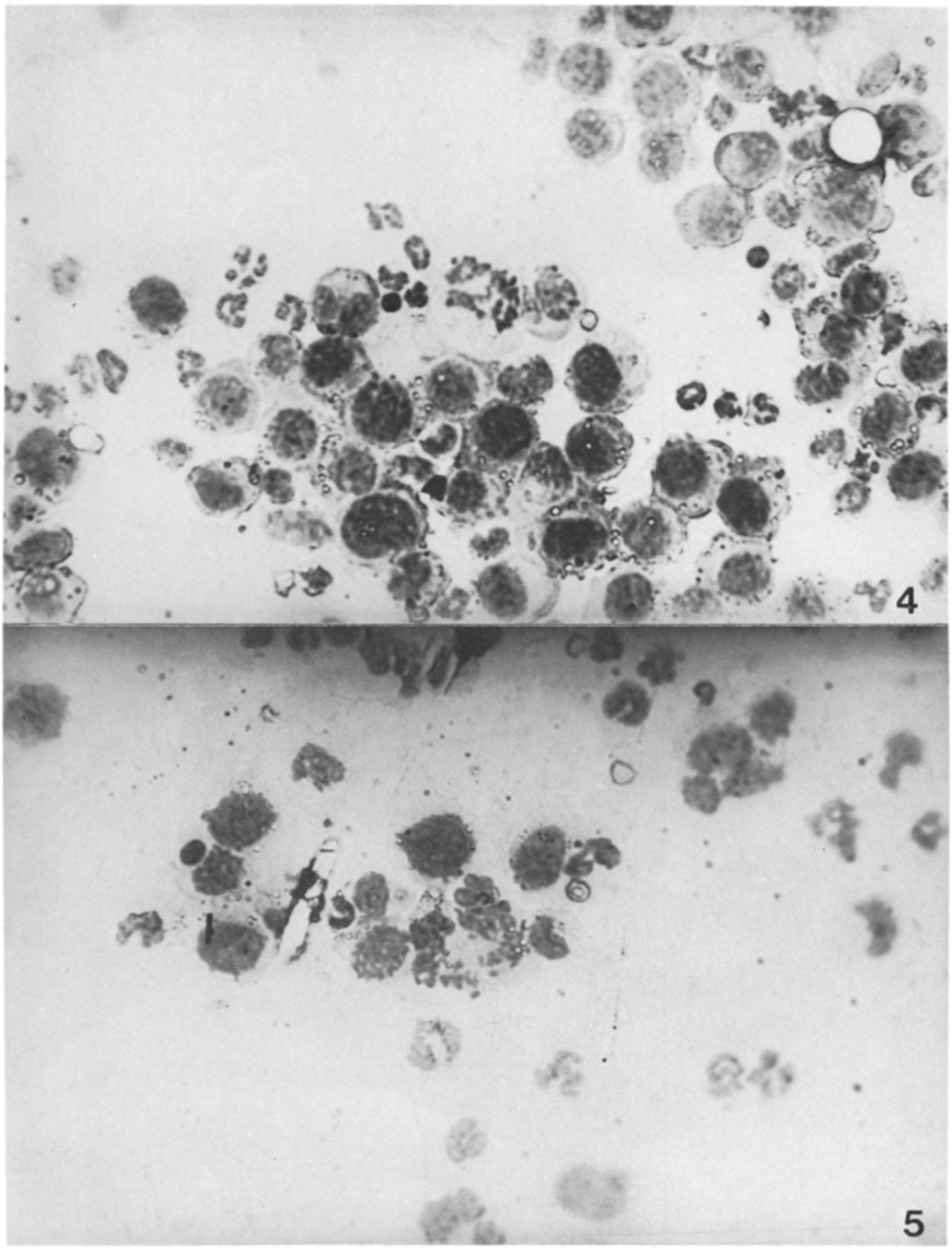


Fig. 4. Treated cells showing unequal nuclear division and scattered chromosomes.

Fig. 5. Treated cells showing complete cytolysis.

This effect lasted for 48 hr, after which (by 72 hr) cytolysis occurs. In addition, 250 mg/kg was found to be the highest dose that does not cause any mortalities (MTD). On the other hand, the MED was recorded at 10 mg/kg (Table 2), and consequently the chemotherapeutic index (MTD/MED) was 25.

Next, the effect of OHD on the prolongation of the life span of mice bearing tumour is given in Fig. 6 (a and b), while the percentage change in body weight is given in Table 3. These results show that a dose of 25 mg/kg injected via the i.p. route for 5 consecutive days was the most effective.

A single dose of 100 mg/kg of OHD decreased the DNA and RNA contents of EAC cells during the first 24 hr (Table 4). However, the EAC cells could overcome the dramatic

Table 2. Determination of the MTD, MED and CI of OHD

*Concentration (mg/kg)	Percentage of abnormal cells at incubation time: 24 hr	48 hr	72 hr
250	+ +	+ +	cytolysis
100	+ +	+	+ +
50	+	+	+ +
10	±	+	+ +
5	±	±	−
0.0		no abnormal cells	

*A single i.p. dose was given 3 days after tumour transplanation.
− Less than 20%.
± 20–30%.
+ 30–50%.
+ + 50–75%.
+ + + More than 75%.

Table 3. Effect of different doses of OHD on the average change in weight (%) of mice bearing EAC

Survival time (days)	Control	Daily dose (mg/kg) 10	25	50	EOD* dose (mg/kg) 10	25	50
1	0	0	0	0	0	0	0
2	+ 2.68	− 1.15	− 1.59	− 1.81	− 0.57	− 2.26	− 1.55
3	+ 4.19	+ 1.46	− 1.27	− 0.33	− 0.44	+ 2.26	− 3.47
4	+ 2.58	+ 0.9	+ 0.18	− 0.14	+ 1.62	+ 2.11	− 1.50
5	+ 3.12	+ 5.12	+ 1.9	+ 2.58	+ 0.44	+ 4.14	− 3.47
6	+ 7.25	+ 3.17	+ 5.54	+ 4.82	+ 2.24	+ 5.17	− 0.66
7	+ 9.30	+ 4.97	+ 6.95	+ 6.59	+ 3.16	+10.39	+ 3.23
9	+11.34	+10.84	+11.22	+14.46	+10.31	+12.03	+12.65
10	+14.46	+10.94	+11.67	+15.47	+10.36	+11.65	+15.97
11	+19.28	+10.19	+12.49	+17.37	+16.15	+13.02	+16.25
13	+20.25	+ 7.13	+18.93	+28.40	+24.18	+21.76	+19.11
14	+23.17	+ 9.04	+23.57	+27.06	+27.03	+22.84	+39.53
15	+28.48	+ 8.38	+21.48	+43.20	+27.42	+23.45	+39.48
16	+39.92	+11.30	+11.76	+52.74	+26.81	+35.95	+63.93
17	+41.09	+20.58	+11.49	+55.56	+31.46	+36.18	+66.46
18	+43.33	+22.49	+13.2	+60.04	+33.00	+49.81	+73.72
20	+67.62	+24.75	+16.4	+62.77	+42.00	+70.21	—
22	+69.91	+48.64	+21.71	+65.58	+42.07	+70.68	
24	+100.4	+45.58	+18.66	—	+41.25	+69.74	
27	—	+52.21	+29.97		+27.25	+88.44	
28		+57.48	+31.12		—	+89.95	
29		+57.53	+31.24			+90.65	
31		+62.19	+36.33			+93.72	
32		+62.15	+37.42			—	
33		+62.05	+38.54				
35		—	—				

*Every other day.
—Death of the last animal.

action of OHD and return to almost their normal values 48 hr after treatment. Meanwhile, the protein contents of EAC cells were significantly decreased throughout the period of treatment. However, those reported after 24 and 48 hr are less in magnitude (Table 5). Moreover, the same dose was found to be optimal for the effect of OHD on the percentage reduction of lipid content of EAC cells at a period of 24 hr (Table 6).

The effects exerted by OHD on the *de novo* synthesis of EAC macromolecules were studied (Fig. 7). At 3 hr after drug administration the inhibition of the incorporation of labelled

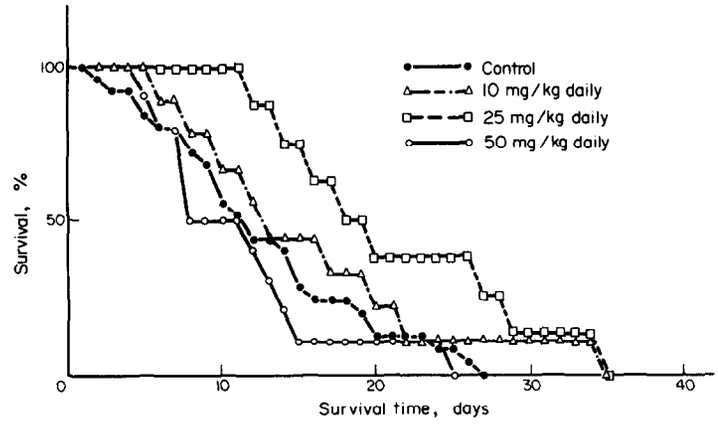


Fig. 6a. Effect of different doses of OHD on the survival time of mice bearing EAC.

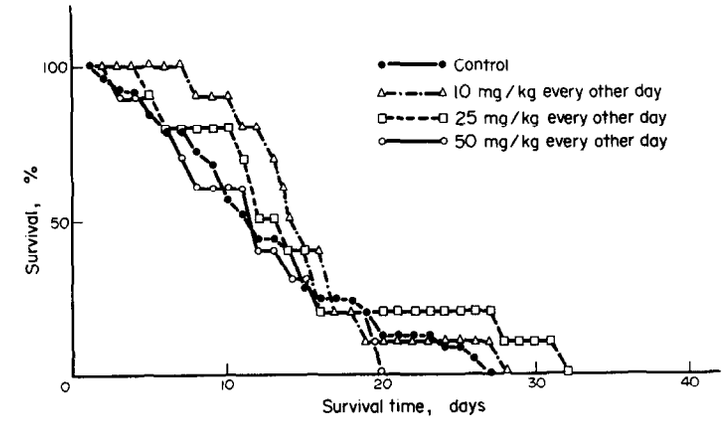


Fig. 6b. Effect of different doses of OHD on the survival time of mice bearing EAC.

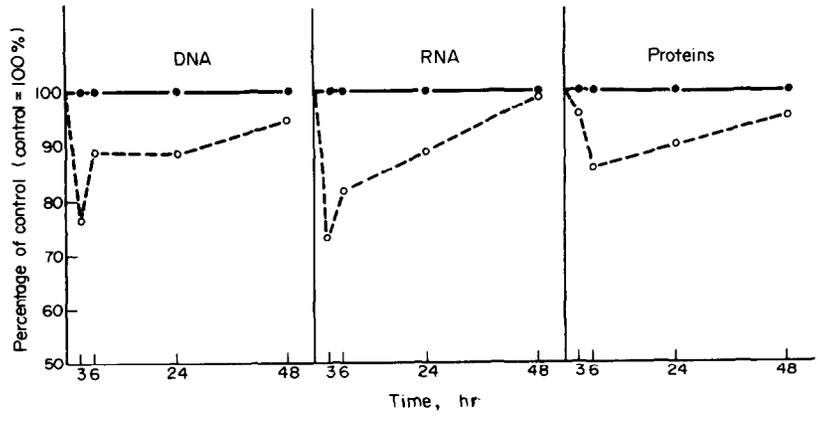


Fig. 7. Effect of a single i.p. injection (100 mg/kg) of OHD on DNA, RNA and protein biosynthesis in EAC cells.
●——● *Control,* O---O *treated.*

thymidine and uridine into DNA and RNA respectively was optimal, the percentage inhibition being 23.3 for DNA and 26.4 for RNA, while the only significant inhibition of the rate of protein biosynthesis (13.8%) was observed 6 hr after drug administration.

DISCUSSION

In vitro studies proved that a concentration of 1.0 μg/ml of OHD could decrease the viability of EAC cells. Moreover, the growth rate of neoplastic cells indicates that the inhibitory effect of OHD on cell proliferation is directly proportional to its concentration. A concentration-dependent effect had been also reported by Sakurai [14], working with cultured Yoshida sarcoma cells using different alkylating agents.

It was of interest, therefore, to evaluate the *in vivo* activities of OHD. The compound possesses an MTD of 250 mg/kg, an MED of 10 mg/kg and a chemotherapeutic index of 25. According to the measures given by Goldin [6], who stated that a chemotherapeutic index of 16 is considered a high one, OHD could be safely regarded as a potential anticancer agent.

Microscopic examination of treated cells showed, as with most anticancer agents, disruption of the cytoplasm and cytoplasmic membrane [15, 16]. Moreover, unequal nuclear division and scattered chromosomes were also observed. When the effect of OHD on the life-span of mice bearing EAC cells was investigated, the maximum significant effects were attained at 10 and 25 mg/kg (daily), whereas 50 mg/kg exerted toxic effects. Meanwhile, the tumor cells were classified into three compartments, A, B and C [17–21]. According to the results stated herein it is clear that OHD localizes its effect at compartment A only. This could be a possible reasoning for the reduction of drug activity which was further proved by tracing the effect of OHD on the biosynthesis of EAC macromolecules.

A single dose of OHD (100 mg/kg) significantly reduced the cellular contents of nucleic acids, proteins and lipids. This effect lasted for 24 hr, after which the cellular contents returned to normal values. The preceding results lead us to trace the effects of OHD on the rate of synthesis of EAC macromolecules using the appropriate radioactive precursors. The effect of OHD is directed primarily towards the *de novo* synthesis of DNA and RNA, while the effect on protein synthesis was less sensitive. These results are in line with those reported for anthramycin [22], mithramycin, olivomycin and chromomycin [23].

Table 4. Effect of a single i.p. injection of OHD on the contents of EAC DNA and RNA

Condition	Amount of DNA (μg/10^6 E.A. cells) at:				Amount of RNA (μg/10^6 E.A cells) at:			
	3 hr	6 hr	24 hr	48 hr	3 hr	6 hr	24 hr	48 hr
Untreated	6.27 ± 0.28	6.42 ± 0.24	6.69 ± 0.33	6.39 ± 0.21	7.84 ± 0.38	7.73 ± 0.32	9.59 ± 0.42	8.64 ± 0.32
Range	(5.67–6.93)	(5.42–6.81)	(5.86–7.43)	(5.94–6.90)	(6.83–8.46)	(6.43–8.67)	(8.76–11.41)	(7.87–9.30)
Treated (100 mg/kg)	3.88 ± 0.26	3.90 ± 0.22	4.17 ± 0.34	5.59 ± 0.30	5.46 ± 0.37	5.60 ± 0.33	5.92 ± 0.34	7.76 ± 0.42
Range	(3.32–4.50)	(3.42–4.47)	(3.53–5.16)	(4.99–5.90)	(4.79–6.52)	(4.95–6.43)	(4.96–6.87)	(6.41–8.75)
P	< 0.001	< 0.001	< 0.01	N.S	< 0.01	< 0.01	< 0.001	N.S

N.S = Not significant.

Table 5. Effect of a single i.p. injection of OHD on the contents of EAC proteins

Condition	Amount of proteins (μg/10^6 EA cells) at: 3 hr	6 hr	24 hr	48 hr
Untreated	159.62 ± 6.48	1.55.62 ± 5.84	94.41 ± 4.21	138.35 ± 3.29
Range	(141.87–172.42)	(146.87–168.42)	(84.81–106.4)	(127.64–147.25)
Treated (100 mg/kg)	112.44 ± 8.68	111.80 ± 5.19	76.01 ± 4.97	114.84 ± 2.89
Range	(95.31–128.46)	(101.10–123.08)	(67.00–86.41)	(109.89–120.88)
P	< 0.01	< 0.01	< 0.05	< 0.01

Table 6. Effect of a single i.p. injection of OHD on the contents of EAC lipids

Condition	Amount of lipids (μg/10^6 E.A. cells) at: 3 hr	6 hr	24 hr	48 hr
Untreated	21.39 ± 1.10	21.56 ± 1.10	12.76 ± 0.52	26.51 ± 0.43
Range	(18.42–24.84)	(18.63–24.97)	(11.59–15.0)	(25.55–28.08)
Treated (100 mg/kg)	19.59 ± 0.88	19.86 ± 1.14	10.50 ± 0.46	25.4 ± 0.70
Range	(17.83–20.50)	(17.73–21.64)	(9.33–11.35)	(23.11–26.94)
P	N.S	N.S	< 0.02	N.S

N.S = Not significant.

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