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ACTIVITY OF CYCLOPHOSPHAMIDE AND 1-METHYLNITROSOUREA ON EHRLICH CARCINOMA TRANSPLANTED IN DIFFERENT SITES. CORRELATION BETWEEN DRUG LEVEL AND TUMOR INHIBITION

A. Bossi, T. Colombo, M. G. Donelli and S. Garattini

Istituto di Ricerche Farmacologiche "Mario Negri" via Eritrea, 62–20157 Milano, Italy

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Abstract—Previous findings show that experimental tumors of the mouse, such as Ehrlich carcinoma and Sarcoma 180, transplanted in different sites, elicit a different sensitivity to the same drug according to the site of tumor implantation. Cyclophosphamide is highly active on intraperitoneal or subcutaneous tumors, but is ineffective on intracerebral tumors. However, the opposite is true for drugs such as nitroso-derivatives, which are active only on intracerebral tumors. The studies presented in this report were performed in order to elucidate whether different concentrations of drugs at the three tumor sites are related to the different antitumoral effects. It was found that measurements of blood and tissue concentrations explain fairly well the different activities of the drugs being tested.

It is well known that primary cultures of human tumors, similar in origin and structure, growing in different individuals or in different sites may show a different sensitivity to antitumor agents [1–3]. In this respect previous findings from this Institute [4] showed that experimental tumors in the mouse, such as Ehrlich carcinoma and Sarcoma 180, transplanted intracerebrally were highly sensitive to a nitroso derivative, 1-methyl-1-nitrosourea (MNU), and insensitive to cyclophosphamide (CPA), whereas only CPA was effective against the same tumor transplanted intraperitoneally or subcutaneously. This selective pattern of activity of the two drugs was explained by postulating a preferential concentration of MNU in the brain, because of its liposolubility in contrast to CPA alkylating metabolites which were supposed to be unable to pass the blood–brain barrier [5]. This hypothesis is reinforced by the results obtained by measuring the amounts of MNU and CPA alkylating metabolites in the Ehrlich carcinoma transplanted intracerebrally, intraperitoneally or subcutaneously.

MATERIALS AND METHODS

Swiss Albino female mice (Charles River) with an average weight of 20 ± 2 g were used. The animals were kept in cages of Makrolon (20 × 26 × 13 cm), 10 in each cage, at a constant temperature of 22° and a relative humidity of 60 per cent. The animals had free access to water and food (Alal 56, obtained from Alal, Co., Milano).

Ehrlich carcinoma (Ehrlich ca.) was maintained in ascites form in this strain of mice for more than 300 serial passages; only ascitic fluids, obtained from mice inoculated 7–10 days before, were used in order to keep cell clumps and dead cells to a minimum. The intracerebral (i.c.) transplantation was performed by inoculating 10^5 cells suspended in 10 μl phosphate buffer (pH 6·9) into the thalamic area of the brain according to the technique described by Rosso *et al.* [6].

For subcutaneous (s.c.) injections, 5×10^6 cancer cells were suspended in 0·1 ml phosphate buffer (pH 6·9) and for intraperitoneal (i.p.) injection 10^6 cells were suspended in 0·2 ml phosphate buffer (pH 6·9).

The drugs employed were 1-methyl-1-nitrosourea (NSC 23909) and cyclophosphamide (NSC 26271), which are kindly supplied by Dr. H. Wood (Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. U.S.A.).

Evaluation of the antitumoral activity. The drugs were administered according to the schedule reported under Tables 1 and 2. The dose injected i.p. (CPA 85 mg/kg and MNU 20 mg/kg) represented about 1/3 of the LD_{50} after a 6 day treatment.

The antitumoral activity was evaluated by recording the survival time of mice with intraperitoneal or intracerebral tumor and by measuring the tumor weight 7 days after the last treatment for the subcutaneous tumor, i.e. 19 days after tumor implantation.

The analysis of variance was performed to assess the significance of differences between untreated and treated animals.

Evaluation of the drug concentration in blood and tissues. The rate of disappearance of MNU and CPA from blood and tissues was determined after intravenous injection in mice bearing tumors transplanted in different sites compared with normal animals. With the intracerebral localization of tumor, the drug concentration was measured in the whole brain because of the very small amount of tumor tissue. The biochemical determination of MNU in tissues was performed by modifying the spectrophotometric method described by Forist for nitroso compounds [7]. One ml of tissue homogenate (1:4 in KCl 1·15 per cent solution) was mixed with 0·8 ml acetate buffer (pH 4·0) and 0·2 ml 40% TCA. After centrifuging, 1 ml deproteinized supernatant was transferred to a glass stoppered test-tube to which was added the color reagent *N*(1-naphthyl)ethylenediamine dihydrochloride prepared as described by Forist [7] and 1 ml 6 N HCl.

After heating in a water bath at 60° for 45 min, the samples were cooled to room temperature and were read at a wavelength of 550 nm in a Beckman spectrophotometer. A tissue blank made according to this procedure gives an optical density ranging from 0·005 to 0·015 when the external blank is used for the zero setting. The optical densities are linear between 0·1 μg and 3 μg/ml external standard.

The recovery of MNU added to tissue homogenate in amounts from 0·25 to 40 μg/250 mg was 90 ± 4%. No drug was detectable in blood a few minutes after intravenous injection at least in the range of sensitivity of the method.

The alkylating metabolites of CPA were determined by measuring the chromogen developed by 4-(*p*-nitrobenzyl) pyridine with alkylating groups in the presence of NaOH [8]. CPA does not show cytotoxicity until metabolized by the hepatic microsomal enzymes [9, 10] into alkylating compounds, which are therefore considered the active form of the drug. 0·5 ml serum or 1 ml tissue homogenate (1:4 in KCl 1·15 per cent solution) were deproteinized with 0·2 and 0·3 ml TCA 20 per cent, respectively, and adjusted with water to a final volume of 1 ml for serum and 1·5 ml for tissue. 0·7 ml serum supernatant or 1 ml tissue supernatant was added to 1 ml of 0·2 M acetate buffer (pH 4·6) and adjusted to pH 4·7 with 1 N NaOH. After adding 1 ml of a 5 per cent solution of 4-(*p*-nitrobenzyl) pyridine in acetone, the tubes were placed in a boiling water bath for 20 min in order to perform the alkylation of the reagent (NBP). After boiling, 2 ml acetone, 5 ml ethylacetate and 1·5 ml 0·25 N NaOH were added to each tube, consecutively and mixed. After centrifugation the optical density of the organic phase was measured in a Beckman spectrophotometer at 540 nm within 5 min from the addition of NaOH.

The standards were prepared using known amounts of nitrogen mustard (HN_2) and the experimental samples were calculated as nmoles HN_2 equivalents. The blanks either for serum or for tissue did not exceed the absorbance of 0·005 when the external blank was used for the zero setting. The sensitivity of the procedure for HN_2 was linear between 0·1 and 5 μg/ml ethylacetate and acetone. The recovery of HN_2 added to 0·5 ml serum or 250 mg tissue homogenate in amounts from 1 μg to 30 μg was 91 ± 3%.

Calculations. The significance of the regression of the drug was assessed using Fisher's F ratio; the kinetic parameter, i.e. the apparent half-life, was calculated on the basis of the measurement of the drug in blood and tissues by using the method of peeling.

The Student's *t*-test was used to evaluate the significance of differences between the levels of the two drugs at each time. The area under the concentration–time curve ($c \times t$) was measured by trapezoidal integration employing only data within the limits of sensitivity of the method; the inferior limit of sensitivity was considered the zero value.

The analysis of variance allowed assessment of the mean significant difference between the $c \times t$ of the two drugs for each tumor.

RESULTS

The antitumoral activity of MNU and CPA in mice bearing Ehrlich carcinoma transplanted i.c., s.c. or i.p.

Table 1. Effect of intraperitoneal injection of MNU or CPA in mice bearing Ehrlich carinoma transplanted in different sites

Tumor site	Drug	Treatment (mg/kg, days)		Survival time (days ± S.E.)	Tumor wt as % body wt (g ± S.E.)	Significance analysis of variance
	—			14·7 ± 0·3		
Intracerebral	MNU	20	1–7	21·2 ± 1·4		P < 0·001
	CPA	85	1–7	16·5 ± 0·3		P < 0·01
	—			23·6 ± 0·5		
Intraperitoneal	MNU	20	3–9	25·5 ± 0·5		P > 0·05
	CPA	85	3–9	29·1 ± 1·8		P < 0·001
	—				2·25 ± 0·23	
Subcutaneous	MNU	20	8–11		1·91 ± 0·19	P > 0·05
	CPA	85	8–11		0·99 ± 0·17	P < 0·001

There were 20 animals per group. The weight of the s.c. tumor was recorded 7 days after the last treatment, i.e. 19 days after tumor implantation. The average tumor weights were 549 ± 57 mg for controls, 387 ± 44 mg for MNU treated mice and 165 ± 34 mg for CPA treated mice.

was evaluated after intraperitoneal or intravenous injection of these compounds.

Table 1 shows the results obtained after i.p. administration of MNU and CPA. It may be observed that MNU increased by 50% the survival time of mice bearing an intracerebral tumor but it appeared to be ineffective on the same tumor transplanted i.p. or s.c.

On the contrary CPA considerably reduced the growth of the s.c. tumor and it increased the survival time of mice transplanted i.p. but it had negligible activity on the intracerebral tumor. When MNU and CPA were injected intravenously, the same pattern of activity was evident (see Table 2).

In order to collect information on the concentrations of CPA and MNU in the body, drug levels were measured after intravenous injection in mice bearing Ehrlich carcinoma. Table 3 shows the alkylating activity present in the serum after injecting CPA into mice in the presence of established tumors transplanted in different sites. Although no great differences were evident between the areas under the curves ($c \times t$) for controls and tumor bearing animals, the apparent half-life of the NBP-positive metabolites in the serum was higher in the presence of a tumor transplanted i.p. compared with animals bearing an i.c. tumor or controls. In the presence of the subcutaneous tumor, however, an increase in the rate of disappearance of alkylating compounds was observed. The half-life was 12 min compared with 21 min in controls and the $c \times t$ was about 25 per cent ($P < 0.01$) less in tumor-bearing animals.

Table 4 shows the concentrations of CPA alkylating metabolites in the tumor. It may be seen that the peak level was higher in the intraperitoneal localization followed by the subcutaneous and the intracerebral. The $c \times t$ followed the same pattern and it is considerably lower for i.c. tumor ($< P$ 0·01 relative to i.p. or s.c. tumor). A similar trend was present also for the apparent half-life although the difference was not significant.

Table 5 shows the levels of MNU injected intravenously in the tumor of mice bearing Ehrlich carcinoma growing in different sites. The peak level was much higher in the cerebral tumor than in any other localization. The $c \times t$ was also higher in the intracerebral tumor and very low in the s.c. Ehrlich carcinoma ($P < 0.01$ relative to i.c. or i.p. tumor).

DISCUSSION

The results reported in this paper confirm previous observations [4, 11, 12] that the same tumor transplanted in different sites may show a different sensitivity to the same chemotherapeutic agent. A typical example is the case of the Ehrlich carcinoma which is sensitive to cyclophosphamide (CPA) but insensitive to l-methyl-l-nitroso-urea (MNU) in the subcutaneous or intraperitoneal transplantation while it is more sensitive to MNU than to CPA in the intracerebral localization (see Table 6).

CPA has been shown previously to be relatively inactive on experimental brain tumors [13] as well as on the leukemia L-1210 transplanted intracerebrally [5]. On the contrary compounds belonging to the group of nitrosoureas are known to accumulate in the nervous tissue [5, 14–18] where they may exert either a carcinogenic [19, 20] or a chemotherapeutic effect on tumors transplanted in the brain [5, 13, 21–23].

The explanation of these findings is given by the present observation that the two drugs, CPA and MNU, are able to reach the tumor in the various sites in a different degree. As summarized in Table 6, it is evident that the peak level and the $c \times t$ of CPA alkylating metabolites are lower in the intracerebral than in the i.p. or s.c. localization. Conversely the peak level and $c \times t$ of MNU are higher in the intracerebral than in the i.p. or s.c. transplanted tumor.

As the NBP reaction does not distinguish between the active and inactive metabolites of CPA it should be added that more precise correlation could probably be obtained when more specific and sensitive methods for measuring the active metabolite(s) of CPA and MNU

Table 2. Effect of intravenous injection of MNU or CPA in mice bearing Ehrlich carcinoma transplanted in different sites

Tumor site	Drug	Treatment (mg/kg, days)		Survival time (days ± S.E.)	Tumor wt as % body wt (g ± S.E.)	Significance analysis of variance
	—			14·2 ± 0·2		
Intracerebral	MNU	20	1,3,5	18·3 ± 0·7		$P < 0.001$
	CPA	85	1,3,5	16·7 ± 0·4		$P < 0.01$
	—			22·4 ± 0·5		
Intraperitoneal	MNU	20	3,5,7	22·6 ± 0·6		$P > 0.05$
	CPA	85	3,5,7	31·2 ± 1·5		$P < 0.001$
	—				2·54 ± 0·28	
Subcutaneous	MNU	20	5,7,9		3·06 ± 0·36	$P > 0.05$
	CPA	85	5,7,9		1·76 ± 0·28	$P < 0.05$

There were 20 animals per group. The weight of the s.c. tumor was recorded 8 days after the last treatment, i.e. 20 days after tumor implantation. The average tumor weights were 699 ± 73 mg for controls, 736 ± 100 mg for MNU treated mice and 462 ± 76 for CPA treated mice.

Table 3. Levels of CPA (85 mg/kg i.v.) alkylating metabolites in serum of mice bearing Ehrlich carcinoma transplanted in different sites

		nmoles equiv. HN_2/ml after minutes								Apparent $t_{1/2}$ (min) 95% fid. lim.	$c \times t$ (μg/ml × min)
		5	10	15	20	40	60	120	150		
A	Controls	198·7 ±10·0	292·6 ±11·1	301·8 ±26·4	263·2 ±10·3	155·8 ±18·7	70·5 ±5·5	10·3 ±·09	<10	21′ 17″ (19′58″–22′48″)	11,056·60 ± 602·08
B	Intracerebral tumor	142·4 ±0	262·6 ±13·3	186·9 ±17·8	258·2 ±8·9	158·0 ±2·2	71·2 ±8·9	15·5 ±2·2	<10	24′ 21″ (21′35″–27′55″)	11,464·95 ± 199·60
C	Intraperitoneal tumor	154·3 ±5·9	160·2 ±10·3	210·7 ±7·8	246·3 ±10·7	145·4 ±2·9	94·9 ±11·9	32·6 ±2·9	<10	34′ 25″ (31′57″–37′19″)	12,210·92 ± 317·32
D	Subcutaneous tumor	238·0 ±11·1	307·4 ±24·1	298·0 ±12·2	248·0 ±10·0	93·4 ±4·9	27·0 ±2·0	<10	<10	12′ 27″ (11′29″–13′34″)	9,355·80 ± 208·86

The experiment was performed 12 days after i.c. tumor transplantation (100,000 cells/0·01 ml/mouse); 16 days after s.c. tumor transplantation (5,000,000 cells/0·1 ml/mouse); 13 days after i.p. tumor transplantation (1,000,000 cells/0·2 ml/mouse).
A–D: $P < 0{\cdot}01$. If not indicated, the differences are not statistically different.

Table 4. Levels of CPA (85 mg/kg i.v.) alkylating metabolites in the tumor of mice bearing Ehrlich carcinoma transplanted in different sites

		nmoles HN_2 equiv./g or ml after minutes									Apparent $t_{1/2}$ (min) 95% fid. lim.	$c \times t$ (μg/ml × min)
		5	10	15	20	40	60	120	180	240		
A	Intracerebral tumor	16·0 ±3·1	21·4 ±0	19·6 ±1·8	19·6 ±1·8	24·9 ±1·8	23·1 ±4·7	16·0 ±3·1	<10	<10	n.d.*	1,250·79 ±265·21
B	Intraperitoneal tumor	18·6 ±0	39·2 ±0·8	48·0 ±2·7	59·6 ±2·3	85·02 ±1·9	70·3 ±3·6	30·7 ±0·8	18·7 ±2·7	12·02 ±0·8	68′ 57″ (62′ 56″–76′ 14″)	5,523·29 ±194·92
C	Subcutaneous tumor	48·9 ±4·8	51·1 ±4·1	n.d.	66·4 ±7·8	56·7 ±4·1	37·4 ±2·7	12·8 ±2·9	<10	<10	50′ 24″ (33′ 16″–103′ 58″)	3,568·13 ±184·25

The experiment was performed 12 days after i.c. tumor transplantation (100,000 cells/0·01 ml/mouse); 16 days after s.c. tumor transplantation (5,000,000/0·1 ml/mouse); 13 days after i.p. transplantation (1,000,000 cells/0·2 ml/mouse).

A–B: $P < 0{\cdot}01$; A–C: $P < 0{\cdot}01$. If not indicated, the differences are not statistically different.

* The data available were insufficient to calculate a significant regression line.

Table 5. Levels of MNU (20 mg/kg i.v.) in the tumor of mice bearing Ehrlich carcinoma transplanted in different sites

		μg/g or ml ± S.E. after minutes						Apparent $t_{1/2}$ (min) 95% fid. lim.	$c \times t$ (μg/g × min)
		1	5	10	20	40	60		
A	Intracerebral tumor	24·6 ± 1·0	11·2 ± 0·3	7·7 ± 0·3	3·9 ± 0·6	3·3 ± 0·2	<1	9′49″ (7′45″–11′55″)	195·66 ± 10·10
B	Intraperitoneal tumor	5·1 ± 0·4	8·8 ± 0·4	8·2 ± 0·4	4·3 ± 0·8	1·4 ± 0·2	<1	13′22″ (10′48″–15′56″)	161·94 ± 9·93
C	Subcutaneous tumor	1·3 ± 0·3	2·7 ± 0·4	2·7 ± 0·2	1·9 ± 0·5	<1	<1	—	30·98 ± 3·44

The experiment was performed 12 days after i.c. tumor transplantation (100,000 cells/0·01 ml/mouse); 16 days after s.c. tumor transplantation (5,000,000 cells/0·1 ml/mouse); 13 days after i.p. tumor transplantation (1,000,000 cells/0·2 ml/mouse).

In the case of the s.c. tumor, the data available did not allow to calculate the half-life.

A–C: $P < 0{\cdot}01$. If not indicated, the differences are not statistically different.

Table 6. Summary of the effect and drug concentrations after administration of CPA or MNU in mice bearing tumors in different sites

Tumor localization	CPA Effect	CPA Peak level (μg/g)	CPA $c \times t$ (μg/g × min)	MNU Effect	MNU Peak level (μg/g)	MNU $c \times t$ (μg/g × min)
i.c.	+	24·9	1250	+ + +	24·6	195
i.p.	+ + +	85·0	5523	—	8·8	161
s.c.	+ + +	66·7	3568	—	2·7	30

become available. These results, however show the importance of the knowledge of distribution and metabolism of antitumoral drugs for a better use of these agents in the treatment of experimental and hopefully clinical cancers.

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