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Biologic Effects of Recent Antimitotic Compounds
A comparative evaluation

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Screening of antiproliferative agents has been a major field of investigation in the recent years. Widely different methods were devised in order to detect and possibly characterize the activity of thousands of compounds, with the hope that they might prove useful tools for the clinician, who has to carry the ultimate and most delicate trial. One may wonder whether the preliminary screening procedures make it now possible to reach the stage of the clinical trial with a sufficient amount of knowledge about the mechanism of action and the main (both favorable and toxic) effects of the compounds.

This should be allowed by the amazing variety of tests through which any new agent can be investigated: in a very concise list we might begin with microrganisms (particularly the so called " resistant lines "), which have been employed with success in cancer chemoterapy research programs (Foley *et al.*, 1958). Spontaneous and experimental tumors in animals give however the most valuable approach to this goal: a combination of in vivo and in vitro studies of such tumors has proved sometimes very useful, provided the variability of cell lines in culture is taken into account.

Tissue cultures have been set up also with human neoplastic cell lines, and used for chemotherapy screening. Hormone-responsive tumors may also be very valuable in assaying steroids and other hormons for anti-neoplastic activity.

Human tumors in heterologous hosts have been shown to respond to some potential anticancer agents: the relationship between inhibition of the host's and tumor's growth can yield profitable data on the specificity of the compound.

In all the above mentioned systems biological features like survival, inhibition of growth, and cytotoxicity, were mainly investigated, while biochemical studies received less attention. Only in more suitable systems, like cell homogenates, metabolic pathways were studied in order to evaluate the influence of antitumor agents; peculiar biochemical reactions were identified, which may be altered by such compounds as, e. g., the folic acid antagonists (Osborn *et al.*, 1958).

However, this could not be achieved for many compounds: and the result was that many antineoplastic drugs, when admitted to the clinical trial, showed unpredicted patterns of activity with regard both to the indication, clinical action and side effects.

It is a common experience for chemotherapists, for instance, to use so called " antimitotic " compounds and find out, on the basis of the clinical observation, that many phenomena are not easily explainable in terms of simple mitosis-inhibiting effect but are apparently in relationship with peculiar antimetabolic effects.

This is not of course merely a question of semantics, but it can be easily suggested that very few anticancer agents can be properly called antimitotic compounds, and that the majority of them are, in fact, antimetabolites.

On this ground the present investigation was started.

The design of the experiment was to set up firstly a specific test for antimitotic activity: to this aim the study of survival and proliferative activity of chick embryo blood cells was chosen.

As a test for basic metabolic activity, the approach to the study of the pathway of nucleic acid synthesis seemed appropriate, since most of the antimetabolic agents influence, at one step or another, the sequence of biochemical reactions involved in DNA or RNA duplication. The incorporation of nucleic acid precursors in human leukocytes, cultivated in vitro, was therefore chosen. Both the effects of antiproliferative compounds, added to the culture medium, and the action of a previously in vivo administered dose on leukocytes cultivated in vitro were studied. As the white cell cultures were set up with the addition of phytohemoagglutinins, the blastic transformation of leukocytes in the experimental conditions described was also investigated.

All these experimental procedures had been already tried in our Institute for the evaluation of single antiproliferative compounds; in this investigation, however, all the agents were tested ex novo in strictly identical conditions, specially in the " mitotic test ", so that the results could be statistically evaluated.

In the present paper a first set of data is presented, concerning both the mitotic test and the human blood cell cultures: a comparative evaluation was performed on the influence of the compounds tested upon the proliferative activity of chick embryo blood cells. Many data are still lacking about the in vitro incorporation studies: the investigation is still in progress.

By the mitotic test, the following compounds were assayed:

1. *Desacethyl-methyl-colchicine* (Colcemid): a typical spindle poison, as all colchicine derivatives, and may thus be considered a paradigmatic mitosis-inhibiting agent. It has been clinically used in the treatment of chronic myeloid leukemia.

2. *Vinblastine*: an alkaloid from Vinca Rosea Lynn., which has been successfully employed in the therapy of lymphogranuloma. Both antimitotic and antimetabolic actions have been suggested.

3. *Vincristine*: another alkaloid from the same source as above, now employed in the treatment of some forms of acute leukemia.

4. *Mechloretamine* (Nitrogen mustard, HN_2): the prototype of the nitrogen mustard compounds, which act by alkylation of reactive atoms of pyrimidine bases of nucleic

acids; N-7-guanine and N-3 adenine atoms seem to be particularly involved in these reactions, which result in intramolecular cross-linkage between the DNA strands (Lawley, 1964). Mechlorethamine is still widely employed in the treatment of Hodgkin's disease and other lymphomas.

5. *Cyclophosphamide*: a cyclic molecule containing the chloroethylaminic group. The final product of the biological activation of this drug seems to be a stable compound, N-beta-chloroethylazaridine, which operates many alkylation reactions (Rauen *et al.*, 1964).

Cyclophosphamide is widely used in the treatment of lymphomas and some forms of carcinomas too.

6. *Tris-ethylenimino-benzoquinone* (Trenimon): formed by conjugation of an alkilating agent with a well-known cytostatic agent as benzoquinone; it has also been employed in the treatment of lymphomas and epithelial tumors as well.

7. *L-phenylalanine mustard* (melphalan): the alkylating agent is here coupled with an essential aminoacid, with the aim to carry the antiproliferative activity in protein-synthetizing tissues. The main field of activity of this compound is therefore the whole range of paraproteinemic diseases, like multiple myeloma, Waldenstrom's disease, and related disorders.

8. *The methyl-hydrazine derivative* known as Natulan, is a recent cytostatic drug with different ways of action; one is the liberation of H_2O_2 (as ionizing radiations do) and the decrease of DNA viscosity; the formation of formaldeyde (cytostatic by itself) and the formation of azametine and N-hydroximethyl derivatives, which have alkylating properties, have also been suggested (Berneis *et al.*, 1963).

The drug is mainly employed in the treatment of Hodgkin's disease.

9. *5-Fluoro-uracil*: a pyrimidine analog, acting in DNA synthesis at the step in which deoxyuridilic acid is formylated in the process of formation of thymidylic acid. Effects also on RNA synthesis have been reported (Cohen *et al.*, 1958).

The drug is clinically employed in the treatment of some tumors of epithelial origin.

10. *Prednisolone*: one of the many steroids which showed some results in the treatment of myelo-proliferative disorders; their mechanism of action, manifold in nature, includes possibly a cytostatic effect, mainly on the lymphoid line.

The mitotic test on chick embryo blood cells

The experimental procedure for the mitotic test is presented in Tab. 1: the different compounds, with saline as control, were injected into a total amount of 1260 eggs (63 experimental series).

For each series 20 embryos were treated and for each compound two different dosages were used, corresponding respectively to 1 : 10 000 and 1 : 100 000 of the

**Tab. 1. Chick embryo survival and mitotic activity
Experimental design**

Compound		Lower dose mcg	Higher dose mcg
Desacethylmethylcolchicine	(DMC)	0.05	0.5
Vinblastine	(VLB)	0.2	2
Vincristine	(VCR)	0.05	0.5
Mechlorethamine	(HN2)	0.2	2
Cyclophosphamide		10	100
Tris-ethylenimino-benzoquinone	(trenimon)	0.01	0.1
l-phenylalanine mustard	(melphalan)	0.2	2
Methyl-hydrazine	(natulan)	10	100
5-fluouracil	(5-FU)	10	100
Prednisolone		2	20

Time of administration: 80th hour.
Time of examination: 5th-24th and 48th hour after injection.

Doses are expressed in micrograms.

single maximum dose clinically employed in an adult patient. Such clinical doses are in our experience of the following magnitude:

Demecolcine: 5 mg
Vinblastine: 20 mg
Vincristine: 5 mg
Mechloretamine: 20 mg
Cyclophosphamide: 1 g
Tris-ethyleniminobenzoquinone: 1 mg
L-phenylalanine mustard: 20 mg
Methyl-hydrazine: 1 g
5-fluoro uracil: 1 g
Prednisolone: 200 mg

Dosages of this magnitude were thereafter employed in the " *in vivo* " experiment (see later).

After some preliminary observations, the time of administration was decided to be at the 80th hour of the embryo incubation, as the most suitable to find a high rate of mytotic activity at an early stage of differentiation. The embryos were examined 5, 24 and 48 hours after the injection; the survival rate was first assessed and the mytotic activity at these periods was then determinated on blood smears treated with Giemsa-stain.

The results concerning the embryo lethality rate are presented in Tab. 2: against a very slow death rate of the controls and of some experimental series too (e. g. with natulan), high or very high values are seen for demecolcine and for vinblastine, while the other Vinca alkaloid, vincristine, does not show such a pronunced effect. With the higher doses of demecolcine and vinblastine the lethality index approaches

Tab. 2. Lethality (%)

		Time		
		5th hr	24th hr	48th hr
Saline		0	5	5
DMC	0.05	35	40	40
DMC	0.5	100	90	90
VLB	0.2	20	50	75
VLB	2	40	100	100
VCR	0.05	10	10	15
VCR	0.5	10	20	20
HN2	0.2	15	20	20
HN2	2	15	30	30
Cyclophosphamide	10	0	30	40
Cyclophosphamide	100	20	45	100
Trenimon	0.01	20	30	30
Trenimon	0.1	25	30	35
Melphalan	0.2	0	10	10
Melphalan	2	5	10	5
Natulan	10	0	5	5
Natulan	100	0	10	5
5-FU	10	0	5	5
5-FU	100	0	80	85
Prednisolone	2	0	25	45
Prednisolone	20	20	25	65

Doses are expressed in micrograms.

or equals the 100%. Among the other compounds, only cyclophosphamide gives comparable values of death rate: and this, of course, at the higher dose, and in the last observation time (48 hours). No other alkylating agent seems to show a comparable effect; 5-FU, on the other hand, gives a high death rate with the larger dose, while the smaller one does not show any appreciable effect.

The mitotic index[1] and the differential mitotic count are presented in Tab. 3: as for the mitotic count the most remarkable data are those showing a high number of blocked mitoses with both doses of Vinblastine at the 5th hour after the injection: subsequently such values decrease considerably with the increase of the death rate, indicating that the few surviving embryos do escape the mitotic inhibition and maintain a fair growth rate.

Such behaviour is in contrast with what happens in the series treated with other agents which induce a high death rate, as cyclophosphamide and 5-FU; the difference can be presumably ascribed to the different mechanism of action of the latter compounds.

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[1] For each series of 20 units, the mitotic index was determined on 5 randomly selected smears from different living embryos. When the total surviving units were lower than 5, an identical member of readings was performed on the available smears.

Tab. 3. Differential mitotic count

	Time	Proph.	Metaph.	Teloph.	Blocked	Mitotic index
Saline	5th hr	3.6	8.2	17.6	1.2	29.4 ± 8.91
	24th hr	4.0	12.4	11.0	0.4	27.4 ± 7.20
	48th hr	5.0	10.8	12.6	1.0	28.4 ± 9.56
DMC 0.05	5th hr	4.0	8.0	16.0	15.6	28.0 ± 2.35
	24th hr	3.8	12.0	14.6	5.6	30.4 ± 6.80
	48th hr	0.2	4.4	14.0	4.2	18.6 ± 7.80
DMC 0.5	5th hr	—	—	—	—	—
	24th hr	2.4	9.2	11.6	4.8	23.2 ± 2.86
	48th hr	2.4	6.6	10.4	3.4	19.4 ± 6.19
VLB 0.2	5th hr	2.0	0.4	3.6	156.8	6.0 ± 9.05
	24th hr	3.0	11.8	11.6	10.0	26.4 ± 4.61
	48th hr	0.6	6.0	9.4	12.0	16.0 ± 2.64
VLB 2	5th hr	1.2	0	0.4	167.0	1.6 ± 1.67
	24th hr	—	—	—	—	—
	48th hr	—	—	—	—	—
VCR 0.05	5th hr	4.8	9.4	19.6	3.2	33.8 ± 5.67
	24th hr	3.6	7.8	11.0	2.2	22.4 ± 10.10
	48th hr	4.2	8.2	15.8	8.6	28.2 ± 5.45
VCR 0.5	5th hr	4.6	8.8	15.2	2.0	28.6 ± 6.46
	24th hr	2.0	6.8	11.0	7.2	19.8 ± 5.80
	48th hr	2.4	4.4	13.6	7.2	20.4 ± 9.07
HN2 0.2	5th hr	1.6	7.6	10.4	2.0	19.6 ± 5.94
	24th hr	2.6	8.8	13.6	2.6	25.0 ± 10.12
	48th hr	0	4.8	7.6	2.2	12.4 ± 7.23
HN2 2	5th hr	1.0	2.2	7.0	0.2	10.2 ± 6.42
	24th hr	3.4	10.8	12.6	10.6	26.8 ± 10.56
	48th hr	0.8	2.2	11.6	2.4	14.6 ± 2.70
Cyclophosphamide 10	5th hr	3.0	6.2	16.4	2.8	25.6 ± 8.20
	24th hr	2.4	5.2	7.2	2.0	14.8 ± 8.67
	48th hr	2.0	6.6	11.8	2.8	20.4 ± 8.32
Cyclophosphamide 100	5th hr	0.4	2.6	7.0	2.4	10.0 ± 2.55
	24th hr	0.8	4.4	6.2	5.0	11.4 ± 5.81
	48th hr	—	—	—	—	—
Trenimon 0.01	5th hr	1.4	5.0	11.8	5.8	18.2 ± 11.03
	24th hr	4.2	7.0	10.6	8.4	21.8 ± 6.26
	48th hr	1.2	2.4	12.2	4.0	15.8 ± 1.48
Trenimon 0.1	5th hr	1.8	2.4	5.6	2.0	9.8 ± 4.44
	25th hr	1.8	4.8	9.8	6.6	16.4 ± 9.55
	48th hr	0.2	3.2	11.6	3.0	15.0 ± 5.66

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Tab. 3 *(contd.)*

	Time	Proph.	Metaph.	Teloph.	Blocked	Mitotic index
Melphalan 0.2	5thhr	2.0	4.4	14.8	2.6	21.2 $\pm$ 4.92
	24thhr	0.6	5.2	10.6	1.4	16.4 $\pm$ 6.58
	48thhr	3.4	10.6	13.6	0.4	27.6 $\pm$ 8.65
Melphalan 2	5thhr	1.0	7.2	11.6	4.4	19.8 $\pm$ 3.92
	24thhr	2.6	3.4	13.2	0.6	19.2 $\pm$ 6.87
	48thhr	1.2	4.0	16.6	1.4	21.8 $\pm$ 5.27
Natulan 10	5thhr	2.4	7.6	14.4	2.0	24.4 $\pm$ 5.68
	24thhr	2.4	9.8	11.2	5.4	23.4 $\pm$ 10.69
	48thhr	0.6	3.2	11.0	2.4	14.8 $\pm$ 6.18
Natulan 100	5thhr	2.0	7.6	14.4	0.2	24.0 $\pm$ 8.94
	24thhr	1.8	11.0	16.4	4.4	29.2 $\pm$ 4.89
	48thhr	1.4	4.8	13.6	3.4	19.8 $\pm$ 9.98
5 - FU 10	5thhr	1.0	18.8	10.6	2.0	30.4 $\pm$ 10.05
	24thhr	2.8	3.4	9.8	2.2	16.0 $\pm$ 7.38
	48thhr	2.4	4.6	12.2	5.2	19.2 $\pm$ 4.60
5 - FU 100	5thhr	0.6	15.6	10.2	0.4	26.4 $\pm$ 17.93
	24thhr	0.2	1.2	2.2	3.6	3.6 $\pm$ 1.52
	48thhr	1.2	8.2	13.2	3.0	22.6 $\pm$ 10.83
Prednisolone 2	5thhr	6.0	11.8	10.4	2.0	28.2 $\pm$ 7.29
	24thhr	4.2	11.2	17.4	3.0	32.8 $\pm$ 6.68
	48thhr	1.4	2.0	11.6	2.0	15.0 $\pm$ 6.29
Prednisolone 20	5thhr	0.8	10.0	13.4	5.6	24.2 $\pm$ 7.22
	24thhr	3.8	5.0	18.4	2.4	27.2 $\pm$ 11.73
	48thhr	1.4	4.6	9.6	2.6	15.6 $\pm$ 8.02

Doses are expressed in micrograms.

A statistical evaluation, based on Tuckey's test, on the mitotic index values, is presented in Tabs. 4, 5, 6: continuous lines for each observation time (5, 24, 48 hours) represent homogenous values, not differing in significant degree ($p > 0.05$) within that range. The group of values including saline differs significantly, e. g., in Tab. 4, only from the last 5 values in the column, in Tab. 5, only from the last value, in Tab. 6, from no recorded value.

A few series for each table (high dosage-demecolcine in Tab. 4, high dosage-vinblastine in Tab. 5, high dosage-vinblastine and cyclophosphamide in Tab. 6), are excluded from the comparison, as all injected embryos were dead at the time of observation.

An overall comment about these data should take in account the fact that, at prolonged time intervals, the significant differences among the experimental series tend to decrease: this is presumably due to the fact that surviving embryos, having passed a stage of mitotic inhibition, resume a normal proliferative activity.

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Tab. 4. Mitotic activity: Statistical evaluation (Tuckey's test)
After 5 hrs

33.8	VCR	0.05
30.4	5-FU	10
29.4	Saline	
28.6	VCR	0.5
28.2	Prednisolone	2
28.0	DMC	0.05
26.4	5-FU	100
25.6	Cyclophosphamide	10
24.4	Natulan	10
24.2	Prednisolone	20
24.0	Natulan	100
21.2	Melphalan	0.2
19.8	Melphalan	2
19.6	HN2	0.2
18.2	Trenimon	0.01
10.2	HN2	2
10.0	Cyclophosphamide	100
9.8	Trenimon	0.1
6.0	VLB	0.2
1.6	VLB	2
* DMC 0.5		

* No surviving embryo in these series.

Doses are expressed in micrograms.

Tab. 5. Mitotic activity: Statistical evaluation (Tuckey's test)
After 24 hrs

32.8	Prednisolone	2
30.4	DMC	0.05
29.2	Natulan	100
27.4	Saline	
27.2	Prednisolone	20
26.8	HN2	2
26.4	VLB	0.2
25.0	HN2	0.2
23.4	Natulan	10
23.2	DMC	0.5
22.4	VCR	0.05
21.8	Trenimon	0.01
19.8	VCR	0.5
19.2	Melphalan	2
16.4	Trenimon	0.1
16.4	Melphalan	0.2
16.0	5-FU	10
14.8	Cyclophosphamide	10
11.4	Cyclophosphamide	100
3.6	5-FU	100
* VLB 2		

* No surviving embryo in these series.

Doses are expressed in micrograms.

**Tab. 6. Mitotic activity: Statistical evaluation (Tuckey's test)
After 48 hrs**

28.4	Saline	
28.2	VCR	0.05
27.6	Melphalan	0.2
22.6	5-FU	100
21.8	Melphalan	2
20.4	VCR	0.5
20.4	Cyclophosphamide	10
19.4	DMC	0.5
19.4	Natulan	100
19.2	5-FU	10
18.6	DMC	0.05
16.0	VLB	0.2
15.8	Trenimon	0.01
15.6	Prednisolone	20
15.0	Trenimon	0.1
15.0	Prednisolone	2
14.8	Natulan	10
14.6	HN2	2
12.4	HN2	0.2
	* VLB	2
	* Cyclophosphamide	100

* No surviving embryo in these series.

Doses are expressed in micrograms.

Experiments on human leukocytes cultivated in vitro

This part of the experiment was designed to study the influence of antiproliferative agents on processes taking place in human leukocytes surviving in vitro in a medium containing phytohemoagglutinins (P.H.A.): one is the so called " blastic transformation ", which has been extensively studied by many Authors, and consists in the appearance of large reticular or " immature " cells (hence the name of blasts) which are still capable of mitosis, but show no farther differentiation. The origin of these cells (presumably from lymphocytes) and the mechanism of their formation by P.H.A. (probably on immunological ground) have also been discussed (Robbins, 1964). Much is known about the degree of blastic transformation in different haematological disorders and also about factors which inhibit this phenomenon in some disease like chronic lymphocytic leukemia (Astaldi et al., 1966); little is known, however, about the possible interference of antiproliferative agents on this process: some data only are available, concerning a limited degree of inhibition shown by cortisone or prednisolone added to the culture medium, and strictly depending upon the temporal relationship between P.H.A. and hormone addition to the culture medium (Astaldi and Sauli, 1963; Elves et al., 1964).

In the same P.H.A. stimulated blastoids cells, the study of cellular proliferation

by the autoradiographic technique, using tritium-labelled precursor of nucleic acids, like uridine and thymidine was performed: this is based on some assumptions which have been thoroughly discussed in the literature (Cronkite *et al.*, 1962). With particular regard to thymidine the assumptions are the following:

1. Thymidine, although not on the normal pathway of DNA synthesis, is a specific precursor for DNA, being available for only a short period of time after the administration.

2. Thymidine is incorporated into DNA only at the time of synthesis, during the preparation for cell division; the radioactivity is then distributed to the daughter cells at mitosis.

3. In a nondividing cell, formed from a labelled cell, the activity is stable for the cell life.

4. A further assumption is that the dosage of radioactivity used does not affect in a significant extent the biological potentiality of a labelled cell.

In the most recent investigations emphasis was also placed on the importance of thymidine phosphorilating enzymes, which perform the conversion of thymidine to TMP (the compound actually incorporated into the cell) and to TTP. A strict relationship was in fact demonstrated by Bianchi *et al.* (1964) between the rate of cell proliferation and the activity of the thymidine kinases.

In the present experiment both the incorporation of H^3-thymidine and H^3-uridine (which is considered a common precursor of both DNA and RNA) were studied, determining the percentage of labelled cells at different time intervals in culture and the grain count above the cells.

The whole investigation was carried on two different features:

1. The *in vitro* effect of cytostatic agents, added directly to the culture medium.

2. The *in vivo* effect of a massive dose of the same compound (usually the maximum tolerated single dose in a human adult subject) on leukocytes removed 48 hrs later and cultivated in the usual manner.

Both effects were always assayed on leukocytes of the same patient (for each compound). As control data for the *in vivo* experiment, the basal values of the in vitro experiments were recorded.

The compounds were added to the medium at two concentrations, the higher being the lower dose employed in the " mitotic test " (1 : 100 000) and the lower being ten times less (1 : 1 000 000). At different time intervals (24, 48, 72 hrs) samples were taken from the culture tubes and incubated for I hour at 37° with the labelled nucleosides.

Smears were then prepared and exposed for 1 week at 4° with AR-10 Kodak stripping film. The slides were then stained with Giemsa-solution; the degree of blastic transformation and precursor incorporation was then determined.

The investigation is still in progress and only data referring to some of the antiproliferative agents studied in the previous mitotic test are so far available.

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The in vitro effect has been studied on vinblastine, vincristine, cyclophosphamide, mechloretamine, the methylhydrazine derivative, 5-fluoro-uracil and prednisolone.

Considering first the *blastic transformation*, it may be noted that it is scarcely affected by vinblastine, cyclophosphamide, and mechloretamine, while natulan and prednisolone do not show any effect at all (Figs. 1, 2, 3, 4, 5). The most striking inhibition on phytoblast formation is exerted by 5-fluoro-uracil, the presence of which stops already after 48 hrs (and more after 72 hrs) the increase of phytoblasts in culture, as observed in the control series (Fig. 6).

The percentage of incorporation is roughly proportional to the degree of blastic transformation; this is an additional proof of the metabolic activities of blastoid cells, already demonstrated in many experiments, by other Authors and ourselves (Cooper *et al.*, 1963; Hayhoe and Quaglino, 1965; Taglioretti *et al.*, 1965).

It may be noted that vinblastine induces a more pronounced inhibition of H_3-uridine than of H_3-thymidine uptake (Tab. 7); this seems to confirm data by Creasey and Markiw, indicating a rather peculiar effect of Vinca alkaloids on RNA synthesis (Creasey and Markiw, 1964).

A complete inhibition of both thymidine and uridine labelling is observed with 5-fluoro-uracil, quite consistently with the block of blast cell formation.

Prednisolone, on the other hand, does not seem to inhibit either the blastic transformation or the uptake of nucleic acid precursors. This finding will be discussed later.

The *in vivo effect* of a single dose on the behaviour of human leukocytes in culture was studied, as before mentioned, administering the compounds by intravenous injection to patients with epithelial neoplastic diseases (mainly bronchogenic tumors) in good haematological conditions.

The maximum single dose usually administered in the clinical treatment of neoplastic disorders, or a slightly lower one, were given.

They were, for the agents so far tested, as follows:

1. Vinblastine: 20 mg
2. Mechloretamine: 15 mg
3. Cyclophosphamide: 1 g
4. The methyl-hydrazine derivative(Natulan): 750 mg
5. 5-fluorouracil (5-FU): 750 mg
6. Prednisolone: 200 mg

A blood sample was taken 48 hrs after the administration; leukocytes were separated and cultures were set up according to the previously described method. At the usual time intervals the degree of blast cell formation and the uptake of labelled thymidine and uridine were determined.

In the present paper only data referring to the blast cell transformation are presented, and not for all compounds tested for the in vitro effect; data concerning the incorporation of H_3-thymidine and H_3-uridine are still in course of determination and will be soon reported.
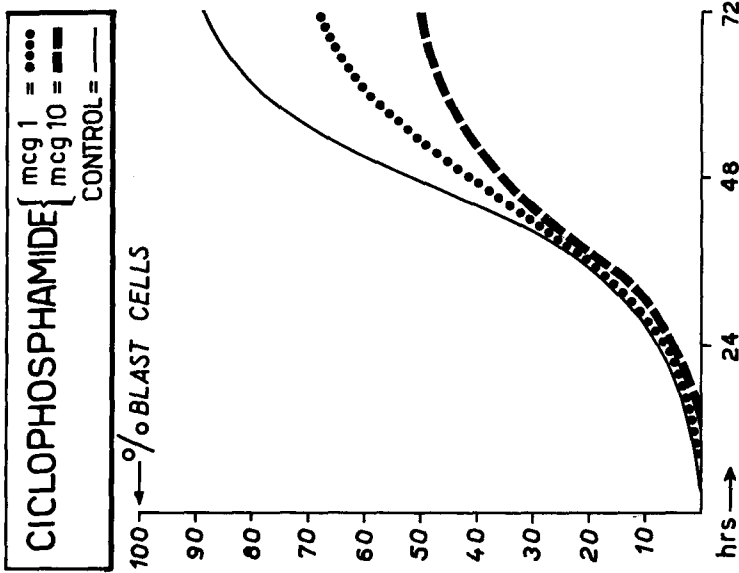
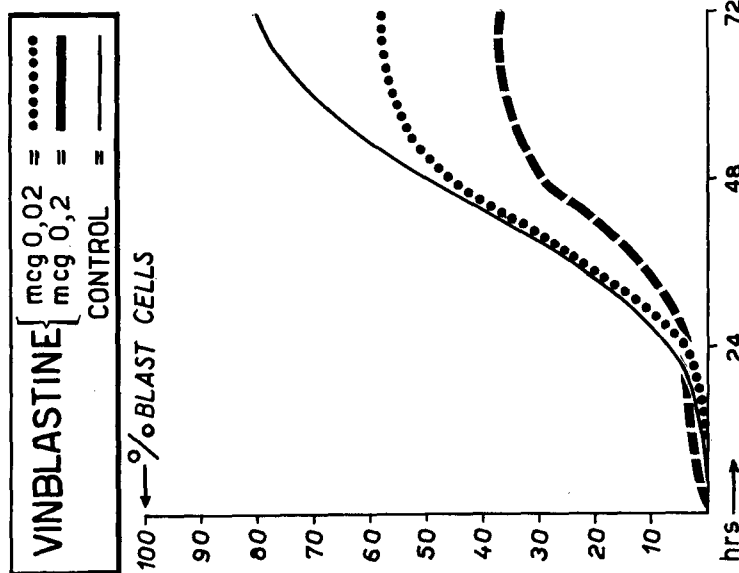Fig. 2. Blastic transformation *in vitro* of human leukocytes in the presence of ciclophosphamide

Fig. 1. Blastic transformation *in vitro* of human leukocytes in the presence of vinblastine

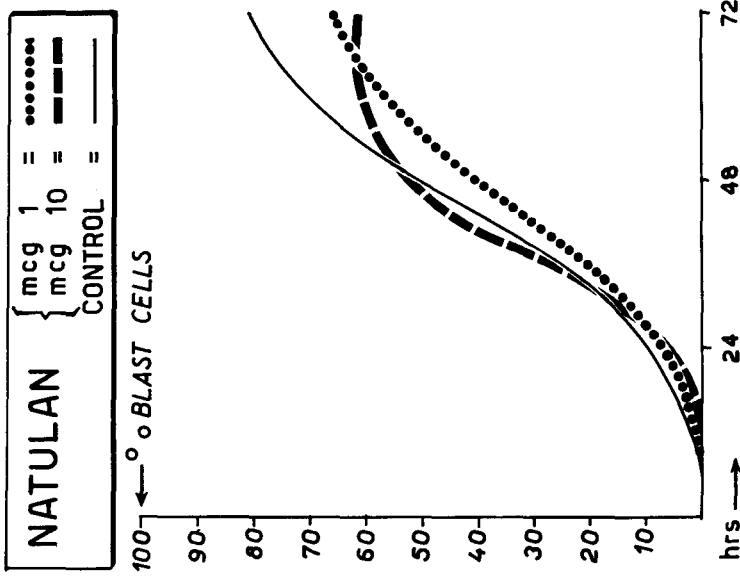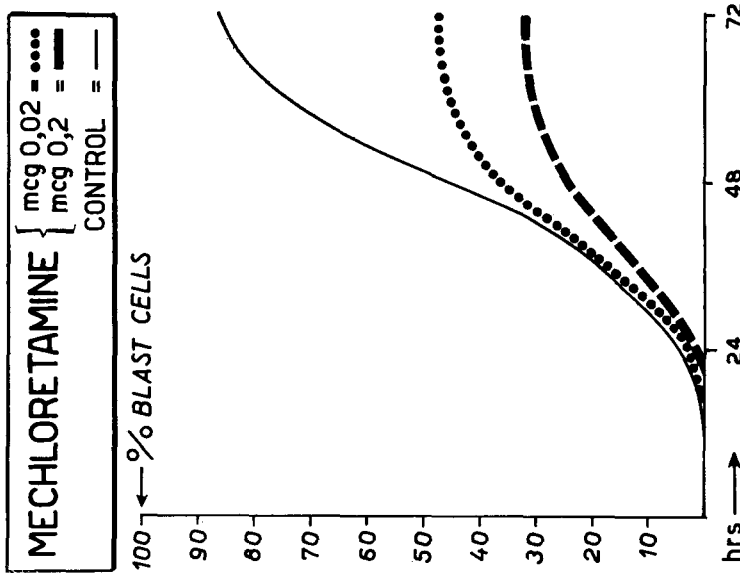Fig. 3. Blastic transformation *in vitro* of human leukocytes in the presence of mechloretamine
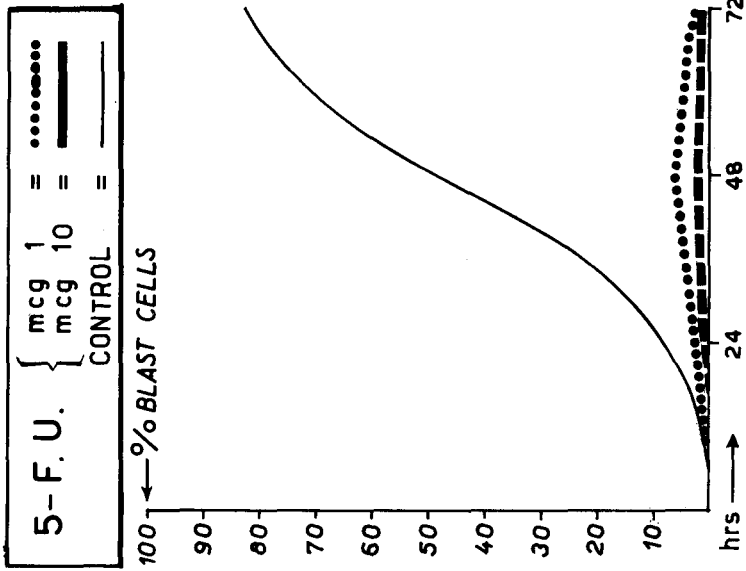
Fig. 4. Blastic transformation *in vitro* of human leukocytes in the presence of natulan

Fig. 6. Blastic transformation *in vitro* of human leukocytes in the presence of 5-fluorouracil
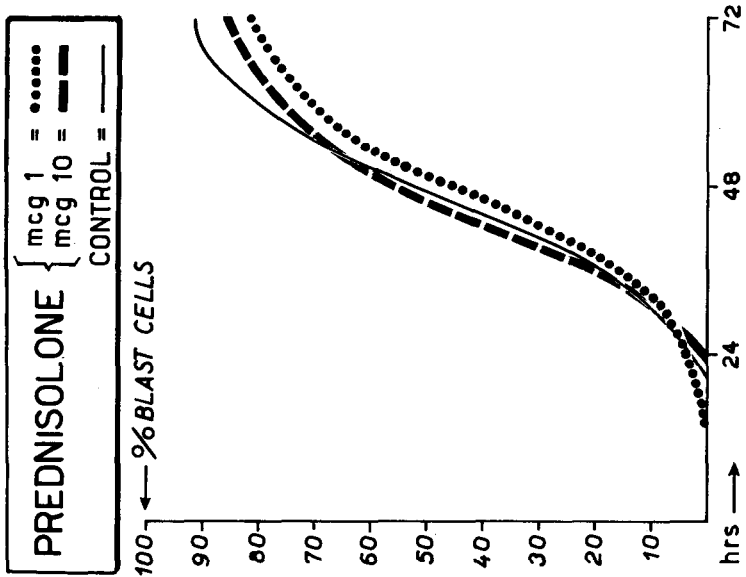

Fig. 5. Blastic transformation *in vitro* of human leukocytes in the presence of prednisolone

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Tab. 7. % of labeled cells and grain count
(*in vitro* experiment)

		24 hrs			48 hrs			72 hrs		
		Uridine %		Thymidine %	Uridine %		Thymidine %	Uridine %		Thymidine %
		Grain count			Grain count			Grain count		
Cyclophosphamide	Control	7	4	4	50	12	30	70	15	42
	1	7	4	6	34	8	27	60	15	37
	10	6	3	7	21	10.5	20	27	17	32
Vinblastine	Control	25	9	13	50	7.5	27.5	70	13	37
	0.02	13	6.5	8	50	8.5	20	46	12.5	25
	0.2	18	7	8	30	9	12.5	27	9	22
5-F.U.	Control	10	8.5	4	42	11	37	75	14.5	68
	1	12	7	2	4	7	2	1	4	0
	10	18	8	2	0	0	0	0	0	0
Natulan	Control	17	9.5	11	46	12	35	70	11	60
	1	16	10	2	40	13.5	38	53	9.3	39
	10	16	7.5	2	28	9	30	22	10	27
Prednisolone	Control	9	13	11	50	11.5	27	76	9	47
	0.2	13	9.5	12	48	12	30	75	11	47
	2	11	11	7	48	10	23	68	7	38
Mechloretamine	Control	21	12	23	40	11	27.5	80	16	37
	0.02	29	12.5	17	30	7.5	20	50	13.5	21
	0.2	18	13	8	28	9	11	28	14	24

Doses are expressed in micrograms.

The behaviour of human leukocytes in culture after the cytostatic treatment *in vivo* is presented in Fig. 7.

It can be seen that in prednisolone treated subjects no inhibition on blastic transformation is present; the slope of the curve is practically identical to that observed for human leukocytes in basal conditions, namely with no previous treatment. A slight decrease of blast cell formation is induced by cyclophosphamide and the methylhydrazine (Natulan).

A remarkable inhibition is caused by the treatment with mechloretamine and 5-fluorouracil (the latter more pronounced at 72 hrs of incubation). The most striking effect is however evident in leukocytes from vinblastine-treated patients, which do not show even the slightest degree of blast cell formation.

This is partially in contrast with the in vitro effect of the same compound, which allowed a moderate degree of blastic transformation, when added to the culture medium.

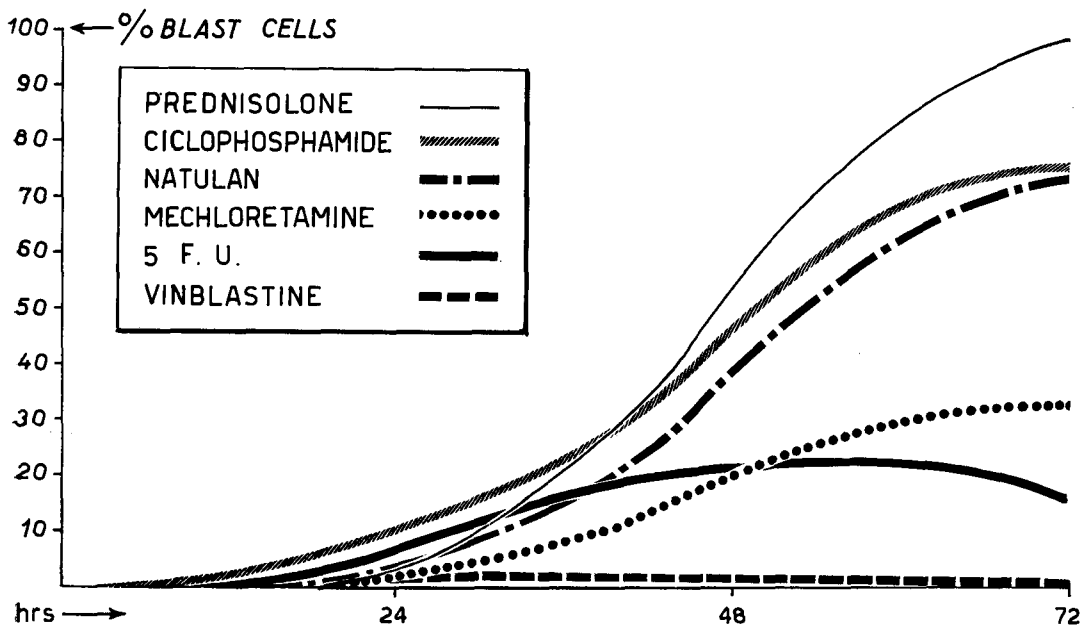BLASTIC TRANFORMATION IN VITRO
AFTER A SINGLE DOSE IN VIVO

Fig. 7.

Conclusions

The aim of the present experiment was a reevaluation of the antiproliferative effects of a group of drugs, which have already a more or less important place in the therapy of neoplastic diseases. It is therefore to be considered as a " retrospective " investigation, designed to verify, in a single planned experiment, a remarkable amount of data obtained separately during the past in our laboratory and published in the last decade (Carrara and Eridani, 1958; Eridani et al., 1961; Valentini et al., 1964). Many compounds which were tested some time ago have not been re-examined because not admitted, for different reason, to the clinical routine; very recent compounds were, on the contrary, assayed for the first time.

The so called antimitotic properties were assayed on a system which has been throughly exploited for the study of proliferative activity of blood cells, specially by the use of the " stathmokinetic index ".

In the present case it could be seen that, beside colchicine, only very few of the tested compounds exhibit an anti-mitotic activity: vinblastine e. g., which for many aspects has been compared to the colchicine alkaloids and, among alkylating agents, only cyclophosphamide at high dosage.

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Another interesting remark concerns the capacity by the embryo to resume a normal proliferative activity after a certain period of time, if an antimitotic does not actually affect the majority of dividing cells: this seems to indicate the existence of a critical point, or, better, of a critical amount of inhibition, below which the embryo can resume a normal proliferation and development. This was also true for other compounds previously tested as thalidomide (Villa *et al.*, 1963), which do not have a typical antiproliferative effect, but simply slow down the mitotic activity: the degree of this " ralentissement " is the determining factor for the embryo death or survival.

The *in vitro* experiment on the incorporation of labelled nucleic acid precursor into normal human leukocytes involves more complicated considerations: first of all the limiting factors about the interpretation of the results should be taken in account, as it has been fully rewieved by many Authors (Polli and Bianchi, 1965; Rubini *et al.*, 1962): most important seem to be the differences observed according to the change of concentration of the labelled compounds and to the time of exposure. The fact that intermediate steps cannot be followed between the addition of the precursor and the appearance of the label on autoradiographic preparations should also be considered.

However, in standard conditions of concentration and exposure, it is generally agreed that the amount of labelling can be assumed as a fair indication of DNA or RNA synthetizing activity by the cells.

It is therefore legitimate to consider the degree of inhibition of labelling as a good index of an antimetabolic effect along the pathway of nucleic acid synthesis. In the present case the synthetic process takes place in a peculiar cell line, the so called " blast cell " growing in P.H.A. stimulated cultures.

In this respect it is interesting to observe that a fairly constant correlation seems to exist between the degree of blastic transformation and the uptake of labelled thymidine and uridine, indicating the high biological potentiality of phytoblasts. The nature and the significance of such cells is still obscure, but the present results seem to indicate that the immunological phenomena are not the only factors involved in their formation, as it is generally assumed: prednisolone, indeed, does not show to exert the least inhibitory effect on their growth and activity. Other compounds, like some alkylating agents and, above all, 5-fluorouracil, show, on the other hand, a very pronounced effect both on phytoblast appearance and their synthetizing activity.

The *in vivo* effect of the antiproliferative agents is also being investigated, with the above described procedure on both phenomena, blastic transformation and labelled precursors uptake.

This research is still in progress and only few data are available. It is however worth to be pointed out that the influence of some compounds is quite different from that observed by their addition to the culture medium: one example is given by vinblastine, which shows a more marked effect *in vivo* than other compounds, like 5-fluoro-uracil, which have a striking inhibitory effect *in vitro*; for this agent the action *in vivo* is anyway still remarkable.

In summary it may be seen from the available data, with all the limitations which have been stressed, how complex is the task to define the exact mechanism of action of antiproliferative agents. It is suggested that the widely heterogeneous group of these drugs could be divided in the following classes, according to the present results:

1. Agents with specific anti-mitotic activity and antimetabolic effect also (at least as far as DNA and RNA synthesis are concerned).

To this group should belong in the first place the alkaloid Vinblastine, which shows a complex pattern of activity.

2. Agents with no antimitotic activity " sensu stricto " but with pronounced antimetabolic effects.

An example of this class is, according to our results, 5-fluoro-uracil.

3. Agents with antimitotic activity only at high dosage, and fairly constant antimetabolic effects: this is the case of most alkylating compounds.

4. Agents with very poor antimitotic and antimetabolic effect, which probably exert their clinical action by more complicated mechanism which cannot be elucidated by the simple tests performed in this experiment. This is, e. g. a, the case of corticosteroids like prednisolone, which have antianabolic effects mainly on protein synthesis and other targets (Polli *et al.*, 1965). The inhibition of blastic transformation *in vitro* after a massive dosage of a cytostatic agent given *in vivo* seems to point to the immuno-competent potentiality of lymphocytes as a target for the action of some antimitotics, provided an immunological reaction is at the basis of such transformation.

As a final consideration, it should be clear that any attempt to label antineoplastic drugs with one eponym or another may be painstakingly elusive at the present time, and all classifications (including our own) are an over-simplification of reality.

Summary

An evaluation of the antimitotic activity of a group of antitumoral agents was performed. Their action was first assayed on chick embryo blood forming tissue, determining its survival, mitotic index and mitotic differential count: the statistically most active compounds were demecolcin, vinblastine and cyclophosphamide.

The same compounds were also tested on the *in vitro* blastic transformation of human leukocytes and their incorporation of tritiated DNA and RNA precursors.

The most active compounds in this assay was 5 fluoro-uracil; a lesser degree of inhibition was caused by vinblastine, cyclophosphamide and mechloretamine.

The effect of a single massive dose of the same compounds *in vivo* was also studied determining the *in vitro* activity of leukocytes of such patients, removed 48 hrs after the administration.

Preliminary results show that such leukocytes lose in variable degree their phyto-responsiveness *in vitro*.

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RIASSUNTO

È stata compiuta una valutazione sull'attività antimitotica di un gruppo di farmaci antitumorali entrati da tempo o di recente nell'uso clinico.

La loro attività è stata dapprima saggiata sul tessuto emopoietico dell'embrione di pollo, studiandone la sopravvivenza, l'indice mitotico e la formula mitotica: i composti più attivi in tal senso, dopo una valutazione statistica, sono risultati la demecolcina, la vinblastina e la ciclofosfamide.

È stata poi studiata l'azione degli stessi composti sulla trasformazione blastica di leucociti umani coltivati *in vitro* con aggiunta di fitoemoagglutinine e sulla incorporazione da parte di queste cellule di precursori degli acidi nucleici marcati con tritio.

Il composto più attivo in questo senso si è dimostrato il 5-fluorouracile; fra gli altri la vinblastina, la ciclofosfamide e la mecloretamina esercitarono un più modesto grado di inibizione.

È stato infine studiato l'effetto di una singola dose massiva di tali composti, iniettata in soggetti neoplastici, sui leucociti prelevati 48 ore dopo e coltivati *in vitro* nelle stesse condizioni. Dalle prime risultanze di tale ricerca, che è tuttora in corso, sembra emergere la possibilità che nei leucociti di soggetti così trattati venga meno, in varia misura, la capacità di rispondere alla stimolazione con fitoemoagglutinine in cultura.

RÉSUMÉ

Une évaluation sur l'activité antimitotique d'un groupe de produits pharmaceutiques antitumoraux a été effectuée.

Leur activité a été observée auparavant sur le tissu hématopoïétique de l'embryon de la poule, en examinant la survie, l'index mitotique et la formule mitotique: statistiquement les composés les plus actifs dans ce sens sont la demecolcine, la vinblastine et la cyclophosphamide.

On a étudié ensuite l'action des mêmes composés sur la transformation blastique des leucocytes humains cultivés *in vitro* avec administration de phytohémoagglutinines et sur l'incorporation de la part de ces cellules des précurseurs des acides nucléiques marqués.

Le composé le plus actif dans ce sens est le 5-fluoruracil; parmi les autres, la vinblastine, la cyclophosphamide et la mechloréthamine exercent un degré d'inhibition plus modeste.

Finalement, on a étudié l'effet d'une seule dose massive de ces composés, injectée chez des sujets avec néoplasie, sur les leucocytes prélevés 48 heures après et cultivés *in vitro* dans les mêmes conditions.

Les premiers résultats de cette recherche, qui est encore en cours, semblent indiquer que dans les leucocytes des sujets ainsi traités, il y ait une moindre capacité de répondre à la stimulation avec phytohémoagglutinine *in vitro*.

ZUSAMMENFASSUNG

Eine Gruppe antitumoraler Arzneimittel wurden nach ihrer antimitotischen Wirkung bewertet.

Ihre Aktivität wurde vorerst auf dem Haematopoësegewebe des Hühnerembryons verfolgt, unter Beobachtung des Überlebens, des Mitoseindex und der Mitoseformel. Die statistisch gesehen aktivsten Präparate waren: Demecolcin, Vinblastin und Cyclophosphamid.

Anschliessend studierte man die Wirkung dieser Präparate einerseits auf die blastische Umwandlung der *in vitro* kultivierten menschlichen Leukozyten unter Verabreichung von Phytohaemoagglutininen und andererseits auf die Inkorporation von Vorgängern der mit Tritium markierten Nucleinsäuren seitens dieser Zellen.

Das in diesem Sinn aktivste Präparat war 5 – Fluoruracil; von den andern bewirkten Vinblastin, Cyclophosphamid und Mechlorethamin einen geringeren Hemmungsgrad.

Zum Schluss studierte man bei Neoplasiepatienten die Wirkung einer einzelnen massiven Injektionsdosis dieser Präparate auf die Leukozyten, welche 48 Stunden nachher entnommen und in vitro kultiviert wurden. Die ersten Ergebnisse dieser Forschung, welche gegenwärtig noch im Gange ist, scheinen anzudeuten, dass in den Leukozyten der auf diese Art behandelten Patienten die Fähigkeit, auf die Stimulation mit Phytohaemoagglutinin *in vitro* zu reagieren, vermindert ist.