REPORT NO. IAEA-R-1691-F

TITLE

Enhancement of the radiation-lethal effect of hypoxic cancer cells by some nitroheterocyclic compounds, (part of a coordinated programme on the improvement of radiotherapy of cancer using modifiers of radiosensitivity of cells)

FINAL REPORT FOR THE PERIOD

1975-11-01 - 1981-11-30

AUTHOR(S)

I. Chiricuta

INSTITUTE

Institute of Oncology
Cluj-Napoca
Romania

INTERNATIONAL ATOMIC ENERGY AGENCY

DATE December 1981

ENHANCEMENT OF THE RADIATION LETHAL EFFECT ON HYPOXIC CANCER CELLS BY NITROHETEROCYCLIC COMPOUNDS

IAEA Research Contract No.1691/RB as part of the coordinated research programme on "Improvement in radiotherapy of cancer using modifiers of radiosensitivity of cells".

Chief scientific investigator : Prof.Dr.Ion Kirioutz, M.D., director of the Oncological Institute, Cluj-Napoca, Romania ; contributors : I.Mustea, Adela Bara, I.D.Postescu, Rodica Gomes and C.D.Olinici.

The investigations carried out in our Institute were focused on the following subjects :

I Syntheses, physico-chemical and pharmacological characterizations as well as testing as radiosensitizers of some nitroheterocyclic derivatives ;

II "In vivo" evaluation of misonidazole effect on the micronuclei incidence in irradiated Ehrlich ascites tumour cells ;

III Interferences of hypoxic cells radiosensitizers with the energy generating pathways.

I. Syntheses, physico-chemical and pharmacological characterizations, testing of radiosensitizing effect

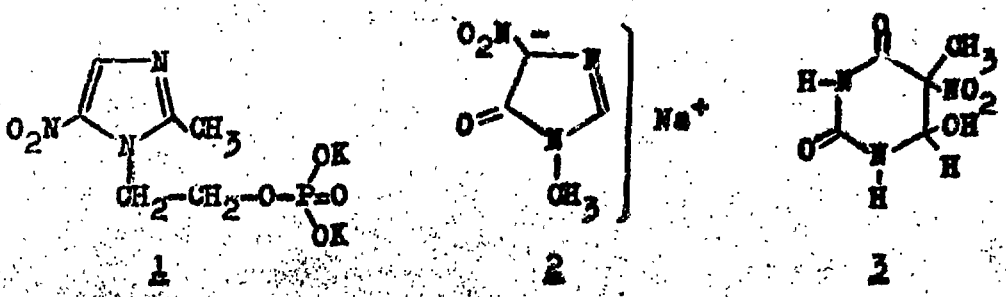
The possibilities to enhance the ionizing radiations lethal effect on hypoxic cells by chemicals gain more and more interest. This explains the increased number of investigations concerning a wide variety of compounds expected as being potential radiosensitizers.

Aiming to find new radiosensitizing agents with low toxicity and good solubility in water we obtained and characterized the following compounds :

1-(hydroxyethyl-2'-phosphate)-2-methyl-5-nitroimidazole, dipotassium salt, (MNP), (1) ;

1-methyl-4-nitroimidazole-5-one, sodium salt, (MNI), (2) ;

4-hydroxy-5-nitro-4,5-dihydrothymine, (HNT), (3).



1 2 3

The syntheses of MNP and HNT were subjects of two romanian patents, and MNI was obtained as described in literature. The main physico-chemical and pharmacological properties of these compounds are listed in Table I.

All three compounds are soluble in water and have low toxicities (LD_{50} 1950-3565 mg/kg). The one electron reduction potentials (E_7^1)(determinations effected at the Gray Laboratory, England, by courtesy of Dr.P.Wardman) indicated for MNI and HNT values more positive (-452 and -456 mV, respectively) than that of metronidazole, suggesting from this point of view that these two compounds might be considered as potential radiosensitizing agents.

In order to extend the study of the influence of various heteronuclei and different chemical neighbourhoods as well on the radiosensitizing properties a series of ten other nitroheteroaromatic compounds (4 - 13) were sent to Dr.Shenoy at Bhabha Atomic Research Center, Trombay, Bombay, India, they following to be tested on bacteria for eventual radiosensitizing effects :

5-nitrouracil (4)

4,6-dihydroxy-5-nitropyrimidine (5)

TABLE I - Physico-chemical and pharmacological data of compounds MNP, MNI and HNT (1 - 3)

COMP.	Melting point °C	IR absorptions cm^{-1}	UV absorptions		One electron red. potential (E_7^1) mV	Acute toxicity "in vivo" (LD_{50}) mg/kg	Part. coef. octanol/ water (pH 7,20°C)
			Abs.max.(λ) nm	Mol.abs. coef.($\log\varepsilon$)			
MNP	160-165 (ethanol)	1539 and 1375 (ν_{as} and $\nu_s NO_2$) 1123, 1110 (ν_{P-O-C})	320	3.94	-509	1950	< 0.01
MNI	293-295 (ethanol/ water)	1637 (ν_{CO}) 1553 and 1382 (ν_{as} and $\nu_s NO_2$)	370	4.13	-452	3565	< 0.01
HNT	182-184 (abs. ethanol)	3400-3000 (ν_{OH}, ν_{NH}) 1733,1691 (ν_{CO}) 1554,1340 (ν_{NO_2})	265 330	3.41 3.18	-456	2800 (at pH 5)	0.017

2-amino-4-hydroxy-5-nitro-6-methylpyridine (6)
2-acetylamino-4-hydroxy-5-nitro-6-methylpyridine (7)
4-nitropyridine-N-oxide (8)
2-hydroxy-5-nitropyridine (9)
2-amino-5-nitropyridine (10)
4,6-dimethoxy-5-nitropyridine (11)
2-nitro-3-hydroxypyridine (12)
5-nitro-6-hydroxythymine (13).

Compounds 4 - 7, 10, 11 and 13 were synthetized according with literature ; compounds 8, 9 and 12 were purchased from Aldrich Co. (Europe).

Dr.Shenoy probably will be informing us about the results of these investigations.

Refering again to compounds 1 - 3 , the experiments on hypoxic cells V 79 (effected at Dr.Révész Laboratory, Radiobiology Unit, Institute for Tumour Biology, Karolinska Institutet, Stockholm, Sweden) showed that MNI and HNT have no radiosensitizing effect whereas MNP displayed an enhancement ratio (E.R) of 1.17 (at 8 mM) but lower-than in case of parent compound, metronidazole (E.R = 1.53)[1]. The decreased sensitization was related to a decreased one electron reduction potential and octanol/water partition coefficient.

II. "In vivo" evaluation of misonidazole effect on the micronuclei incidence in irradiated Ehrlich ascites tumour cells[2].

The micronucleus assay was adapted for the estimation of the "in vivo" effect of misonidazole on the incidence of micronuclei in irradiated Ehrlich ascites tumour cells.

1) I.D.Postescu et al., Strahlentherapie, 1979, 155, 1979.

2) Olinici,C.D., Mustea,I. - Int.J.Radiat.Biol., 1978,34,589-593.

Our results showed that pretreatment with misonidazole 0.5 mg/g body weight, 30 min. prior to irradiation in Ehrlich ascite bearing mice, induced an increase in the incidence of micronuclei as compared with the group containing animal treated only by irradiation (Fig.1). The difference between irradiated controls and misonidazole pretreated and irradiated mice was more pronounced at higher doses of radiation (400 R) a fact that is in agreement with the data of Révész[3] regarding the dependence of the radiosensitizing effect of some electron-affinic compound on the radiation dose.

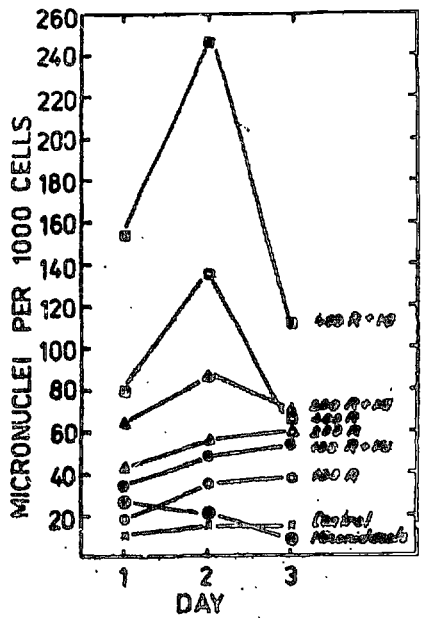


FIG.1. Effect of misonidazole on the incidence of micronuclei in Ehrlich ascites tumour-cells irradiated with different doses of radiation.

The micronucleus test, rather simple and quickly performed may be an useful tool in the "in vivo" testing of electron-affinic compounds as potential radiosensitizers.

---

3) Révész,L., 1975 - Advances in Chemical Radiosensitization (Vienna ; International Atomic Energy Agency), p.55.

III. Interferences of hypoxic cells radiosensitizers with the energy generating pathways

A particular interest we afforded to the knowledge of the effects of hypoxic cells radiosensitizers on the cellular energetic metabolism, this aiming to a better understanding of the possible mechanisms involved in aerobic toxicity induced by these compounds.

The aerobic toxicity seems as being the major limiting factor in the using of large doses of radiosensitizers required for obtaining significant therapeutic efficiency.

The following aspects of energy metabolism alterations in the presence of radiosensitizers were approached :

a) "in vitro" oxygen utilization

b) oxygen partial tension in muscle tissues

c) uncoupling effect of oxydative phosphorilation

d) bypass effect in electron-transport chain.

Our results conducted to the following conclusions :

a) "In vitro" oxygen utilisation

Both metronidazole and misonidazole at concentrations of 10^{-2} - 10^{-3} M showed an inhibitory effect on the oxygen utilisation ratio in mice tissue homogenates (Table II). The inhibition was higher in liver and heart.

b) Oxygen partial tension in muscle tissue

Metronidazole and misonidazole, both at concentrations of 0.3 or 0.6 mg/g body weight caused an increased of the oxygen partial tension in leg muscle of mice with a factor of 2 - 2.5 and 4.5 - 5 , respectively (Fig.2)[4]. These factors were scored from polarographical measurements of the oxygen diffusion current.

4) Bara Adela, Mustea,I. - Radiobiol.Radiother.,1980,21,453.

TABLE II - Effects of MET, MIS and DNP on the relative oxygen utilization ratio (OUR) of mice tissue homogenates.

TISSUE	O U R				
	METRONIDAZOLE		MISONIDAZOLE		DNP
	10^{-3} M (6)	10^{-2} M (10)	10^{-3} M (6)	10^{-2} M (10)	10^{-4} M (6)
BRAIN	0.83±0.032	0.59-0.043	0.86-0.050	0.68±0.051	0.63±0.028
HEART	0.79±0.069	0.50±0.090	0.71±0.046	0.51±0.056	0.51±0.037
LIVER	0.90±0.034	0.48±0.047	0.90±0.023	0.54±0.069	0.64±0.017
SPLEEN	0.99±0.090	0.76-0.089	0.87±0.015	0.65±0.061	0.66±0.016
MUSCLE	0.94-0.027	0.61-0.041	0.97-0.017	0.56±0.050	0.70±0.027

The number in brackets = no.of determinations ;

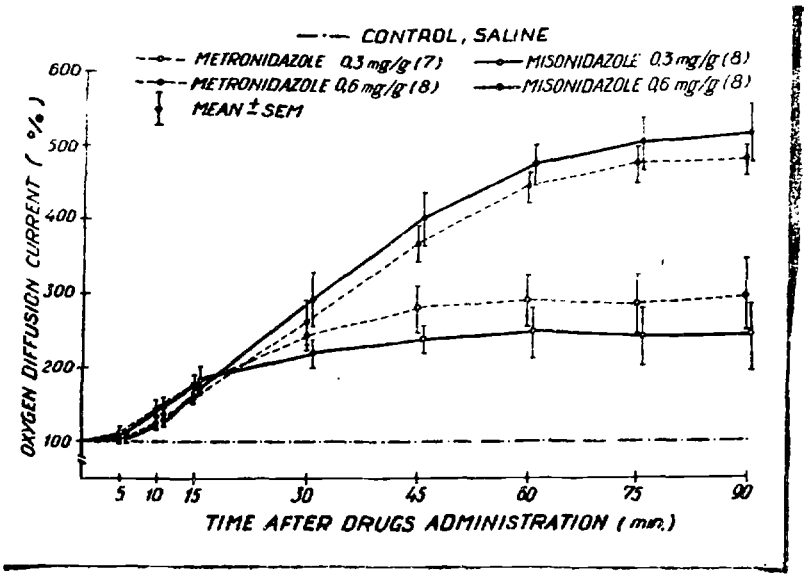


FIG.2. Effects of metronidazole and misonidazole on oxygen diffusion current in hind leg muscle of NMRI mice breathing air. The mean value of the current before drugs administration was arbitrarly considered 100 %. Numbers in brackets = no.of animals used.

c) Uncoupling effect of oxydative phosphorylation

Hypoxic cells radiosensitizers with electron-affinity, E_7^1 comparable or more positive than misonidazole induced effects similar to the uncouplers of oxydative phosphorylation as the decrease of respiratory control of liver mitochondria in guinea pig (Table III)[5], the release from the Crabtree effect induced by addition of glucose in Ehrlich ascites tumour cells (Fig.3, Table IV,V)[6,7] and the stimulation of ATP-ase activity in Ehrlich ascites tumour cells rendered permeable by sonication or treatment with dextran (Table VI)[7].

---

5) Mustea,I., Bara Adela, Petrescu, I. and Révész,L. - Br.J.Cancer, 1978, 37, Suppl.III, 159.

6) Mustea,I., Bara Adela, - Br.J.Cancer, 1979, 40, 295.

7) Bara Adela, Mustea,I., Postescu,I.D. - Int.J.Radiat.Biol., in press.

TABLE III - The effect of metronidazole and misonidazole on the oxygen consumption and respiratory control (R_c) of liver mitochondria with α-ketoglutarate substrate.

SENSITIZER	nAtO_2/min/mg protein		R_c ($\frac{\text{state 3}}{\text{state 4}}$)
	With ADP added (resp. state 3)	Without ADP (resp. state 4)	
Control	29.96 ± 0.99	8.40 ± 0.33	3.57 ± 0.12
Misonidazole :			
- 10 mM	29.17 ± 1.76	10.28 ± 0.60 x)	2.84 ± 0.14 x)
- 20 mM	25.70 ± 1.07 x)	12.58 ± 1.12 +)	2.04 ± 0.13 x)
Metronidazole:			
- 10 mM	33.78 ± 2.17	10.89 ± 0.82 +)	3.10 ± 0.25
- 20 mM	34.63 ± 1.10 x)	11.83 ± 0.75 +)	2.93 ± 0.25

Composition of the reaction medium : saccharose 175 mM, KCl 50 mM, Tris.HCl buffer 20 mM pH 7.4, phosphate buffer 5 mM, EDTA-K 0.5mM, $MgCl_2$ 2.5 mM, bovine serum albumin 1-2 mg/sample, α-ketoglutarate substrate 5 mM. When added, ADP 2 mM.

Mean ± s.e. are indicated from 4 separate experiments in each of which 4-5 replicate measurements were made.

x) Significant $p < 0.05$; +) Significant $p < 0.01$.-

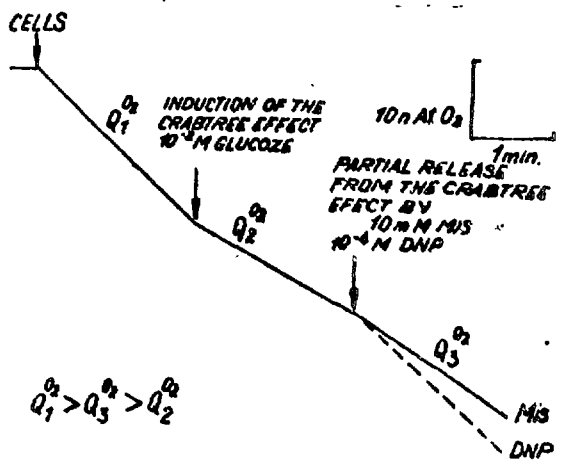


FIG. 3.

Polarographic registration of O_2 consumption in Ehrlich ascites tumour cells suspended in glucose-free Tyrode medium. The Crabtree effect was induced by addition of 10^{-2}M glucose. Partial release from the Crabtree effect was obtained by adding 10 mM misonidazole or 10^{-4} mM DNP. Ehrlich tumour cell density was 5 x 10^6/ml.

d) Bypass effect in the electron-transport chain

Electron-affinic radiosensitizers of hypoxic cells interfere with cellular electron transport carriers by providing an electron shunt directly to oxygen without yielding to a corresponding ATP

TABLE IV - The effect of misonidazole, NDPP and DNP on relative oxygen utilization ratio (OUR) of Ehrlich ascites tumour cells (5×10^6/ml).-

COMP.	mM	OUR			
		Tyrode medium		Krebs-Ringer medium	
		- glucose (x)	+ glucose (xx)	- glucose (x)	+ glucose (xx)
MIS	5		1.08±0.022 (8)		
	10	0.92±0.03 (10)	1.19±0.047 (9)	0.81±0.086 (7)	1.20±0.037 (4)
	20		1.28±0.061 (10)		
NDPP	0.5	0.96±0.023 (4)	1.26±0.082 (4)	0.88±0.043 (6)	1.25±0.095 (4)
DNP	0.1	1.18±0.106 (6)	1.68±0.078 (9)	0.85±0.05 (7)	1.13±0.046 (5)

(x) $\frac{O_2 \text{ consumpt.rate with drug added}}{\text{Endogenous } O_2 \text{ consumption rate}}$ (xx) $\frac{O_2 \text{ consumpt.rate with drug and glucose added}}{O_2 \text{ consumption rate with glucose added}}$

TABLE V - Release from the Crabtree effect (RCE) in the presence of MIS, NDPP and DNP

COMPOUND	mM	Tyrode medium		Krebs-Ringer medium	
		OUR (x)	RCE	OUR (x)	RCE
MIS	5	0.59	8.88		
	10	0.65	22.22	0.74	31.57
	20	0.70	33.33		
NDPP	0.5	0.69	31.11	0.77	39.47
DNP	0.1	0.92	82.00	0.70	21.05

$\frac{O_2 \text{ consumpt.rate with drug and glucose added}}{O_2 \text{ consumption}}$

TABLE VI - ATPase activity in the Ehrlich ascites tumour cells (nmol Pi/min/mg protein)

COMPOUND	E_7^1/mV [a]	Concentration mM	ATPase activity in cell made permeable			
			With Dextran		By sonication	
			Mean value ± SE	Percentage activity [b]	Mean value ± SE	Percentage activity
MET	-486	5	16.14 ± 3.85 (5) [c]	96	-	-
		10	13.90 ± 1.43 (6)	82	19.58 ± 1.76 (9)	116
MIS	-389	1	16.31 ± 0.70 (5)	97	-	-
		5	22.59 ± 1.81 (5)	134	-	-
		10	29.59 ± 2.16 (6) x)	176	45.02 ± 2.67 (9) x)	267
PNAP	-355	1	24.95 ± 1.33 (5) x)	148	-	-
NDPP	-315	1	27.04 ± 1.81 (6) x)	160	-	-
DNP	-	0.1	44.33 ± 1.56 (6) x)	263	65.27 ± 6.49 (9) x)	387
Mg^{2+}	-	5	-	-	25.64 ± 3.48 (9) x)	152

a) E_7^1/mV = one-electron reduction potential (Wardman and Clarke, 1976) ;

b) Percentage activity was calculated considering the ATPase activity in basal medium as 100 %. Basal medium : 50 mM Tris-HCl pH 7.4 ; 0.5 mM EDTA ; 100 mM KCl ; 5 mM ATP. Mean value of basal activity was 16.86 ± 1.83 ;

c) Numbers in brackets represent number of experiments ;

x) Statistically significant values calculated by Student's t test for $p < 0.05$

amount (Fig.4)[8,9]. This effect could be rendered evident by an increase of oxygen consumption caused by a radiosensitizer in cells, mitochondria or microsomes whose oxygen consumption was previously inhibited in sites I-III with specific inhibitors.

Aiming to achieve test conditions in the screening of the hypoxic cells radiosensitizers for their biological effects important in predicting the level of aerobic toxicity, we investigated the bypass effect comparatively in normal tissue homogenates and tumour cells inhibited with different inhibitors : Amital (site I), Antimycin A (site II) and KCN (site III). In this purpose EATC have been prouved as a more adequate material than normal tissue, rendering evident the bypass effect even for radiosensitizers with low electron-affinity (metronidazole, tinidazole)(Fig.5). The efficiency of specific inhibitors for evaluating the interference of hypoxic cell radiosensitizers with the electron transport chain decreases in order : KCN $\geq$ Amital $>$ Antimycin A (Table VII).

A quantitative relationship between the electron-affinity of MET, TIN, MIS, PNAP and NDPP and their bypass effect could be established working on EATC whose oxygen consumption inhibited with 1-2 mM KCN.

Furthermore, a satisfactory correlation was found between the electron-affinity of above mentioned radiosensitizers and both their bypass effect we determined and the chronic aerobic cytotoxicity data reported by Adams et al.[10](Fig.6).

---

8) Ainsworth,P.J. et al. - Can.J.Biochem., 1978, 56, 457.

9) Biaglow,J.E. - Pharmac.Ther., 1980, 10, 283.

10) Adams,G.E. et al. - Int.J.Radiat.Biol., 1979, 35, 151.

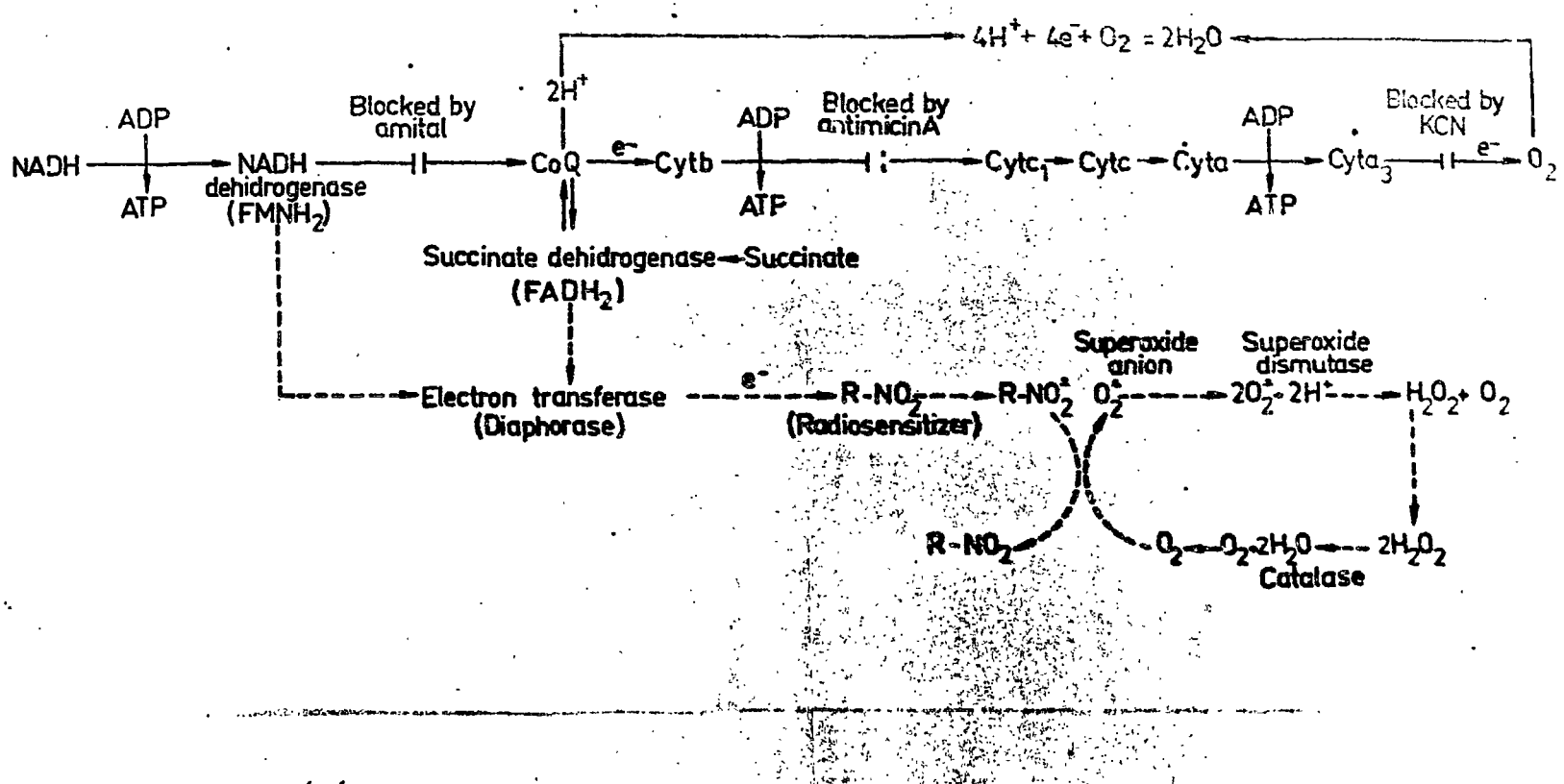


FIG.4. The interference of electron-affinic radiosensitizers with the electron transport chain.-

——— normal pathway

- - - - - possible bypass effect in the presence of electron-affinic radiosensitizers.

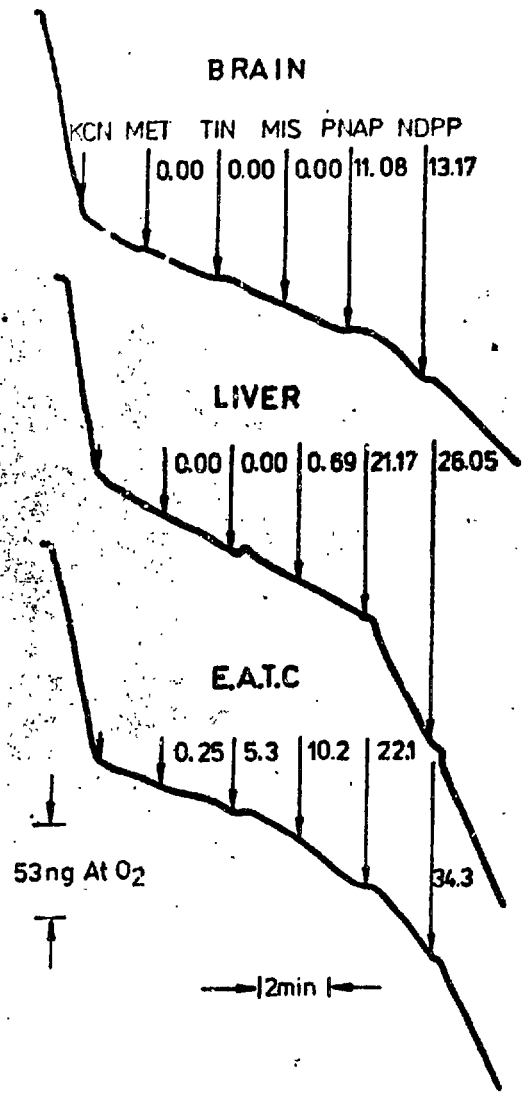


FIG.5. Release from the inhibition of oxygen consumption in 2 mM KCN inhibited liver, brain homogenates and EATC by various radiosensitizers added successively in order of their increased electron-affinity.

- Effects of MIS and MET on the release from inhibition of oxygen consumption caused by specific inhibitors.

Concentration specific inhibitor (mM) Anti-myein A			Inhibition of oxygen consumpt.%	Concentration of radiosensitizer (mM) MIS	MET	Release from the inhibition %
-	-		82.00(20)	-	-	-
-	-		80.81	1	-	5.99±1.02 (4)
-	-		82.64	10	-	19.03±0.67* (6)
-	-		83.38	-	1	0.65±0.40 (5)
-	-		80.82	-	10	3.09±0.73 (5)
0.05	-		79.51(18)	-	-	-
0.05	-		80.06	1	-	3.97±0.84 (4)
0.05	-		84.48	10	-	4.38±0.35 (5)
05	-		77,49	-	1	N.R. (5)
05	-		75,28	-	10	N.R. (4)
	1		95,26(23)	-	-	-
	1		95.21	1	-	9.24±0.76* (5)
	1		95,17	10	-	19,69±1.30* (6)
	1		94.94	-	1	3.17±0.71 (5)
	1		95.60	-	10	6.63±0.35 (7)
-	-		81.12(11)	-	-	-
-	-		82.93	10	-	5.89±0.49 (7)
-	-		77.95	-	10	N.R. (4)
0.05	-		59.21(13)	-	-	-
0.05	-		59.43	1	-	N.R. (4)
0.05	-		65.40	10	-	8.32±0.29 (4)
0.05	-		61.30	-	10	N.R. (5)
	2		92.66(19)	-	-	-
	2		3.67	1	-	1.46±0.09 (4)
	2		91.78	10	-	10.39±2.83* (5)
	2		91.89	-	1	N.R. (4)
	2		93.24	-	10	1.92±0.75 (6)

In brackets represent the number of experiments ;

* ally significant values for $p < 0.05$;

release

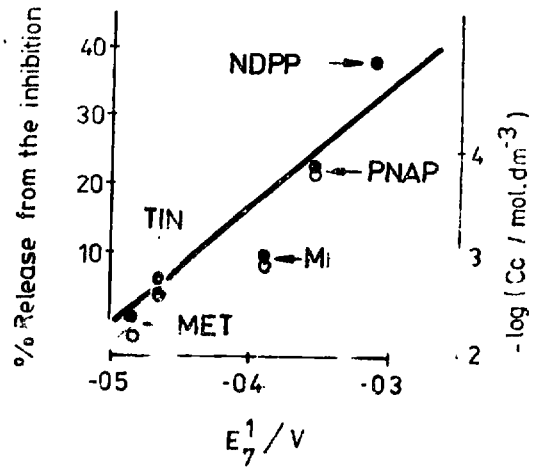


FIG.6. Dependence of the bypass ef ct in EA') determined in the present (r (.) a i chronic aerobic cytotc icit (o) repo ed by Adams et al. /10/ on one lectron reduction potential values of Wardman and Clarke. Our results represent mean values of three separate determinations.

The knowledge of hypoxic cells radiosensitizers effects on the energy metabolism has been prouved particularly useful in understanding some undesirable side effects that limit the extention of these products in human therapy. Though compounds inhibiting the oxygen consumption are suitable for the radiosensibilization of hypoxic cells in tumours and they enhance the response to radiation by increasing the tumour oxygenation, our results showed that these compounds also produce a supplementary oxygenation of normal tissues thus leading to possible increase of their sensitivity to radiations.

On the other side, our investigations briefly presented in this report are in accord with the oppinions stating the metabolic origin of aerobic cytotoxicity. Thus, besides the bypass effect in electron transport chain, the oxydative phos-

phorylation uncoupling effect and the stimulation of ATPase activity, must be taken into consideration. These interactions lead to an alteration of cellular energy balance explaining thus the phenomena of aerobic toxicity observed in experimental studies and clinical trials, as well.

We have shown that MIS displays biochemical properties specific to the uncouplers of oxydative phosphorylation compounds known to develop neurotoxicity and damages in the nervous system[11, 12, 13]. Such effects were also observed in mice following chronic administration of MIS[14].

In order to diminish the toxic effects in patients treated with MIS a total dose not exceeding 12-15 g/m^2 is now recommended [15, 16], or an association with hepatic enzyme-inducing agents (phenytoin, phenobarbitone) able to cause a rapid clearance of the drug[17, 18, 19].

Our preliminary experimental data point-out that the aerobic toxicity of electron-affinic radiosensitizers might be reduced by a metabolic control consisting in the stimulation of the cell energy metabolism and the tissue redox equilibrium restoration and we obtained uncouraging results.

---

11) Reiss,D.S., Leer,M.B. and Sapirstein,V.S. - Abstr.int.Soc. Neurochem., 197 , 7, 118.

12) Spencer,P.S., et al. - Science, 1979, 204, 633.

13) Cammer,W. - Biochem.Pharmac., , 29, 15.

14) Conroy,P.J. et al., Int.J.Radiation Oncology Biol.Phys. 1979, 5, 983.

15) Wiltshire,C. et al. - Br.J.Cancer, 1978, 37, Suppl I, 286.

16) Wasserman,T.H. - Int.J.Radiation Oncology Biol.Phys., 1979, 5, 775.

17) Workman,P. - Br.J.Cancer, 1979, 40, 335.

18) Bleehen,N.M., Cancer Clin.Trials, 1980, 3, 267.

19) Workman,P., Bleehen,N.M., Wiltshire,C.R. - Br.J.Cancer, 1980, 41, 302.