Indian J Physiol Pharmacol 1998; 42 (2) : 223–230

ROLE OF BIO-METAL Fe(III) IN ANTICANCER EFFECT OF DACARBAZINE

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(Received on May 3, 1997)

Abstract : Physicochemical, Microbial and Pharmacological studies on Fe(III)–Dacarbazine complex have been done in solid and aqueous phase. On the basis of elemental analysis, polarographic studies, amperometric titrations and IR spectral studies the probable formula for the complex has been worked out to be 1 : 1, Fe(III)–Dacarbazine. The metal ligand interaction has been studied using polarographic method at 25±1°C and at ionic strength of μ=1.0 (KCl).

Microbial studies on the complex was done against various pathogenic bacteria viz. *Pseudomonas mangiferae*, *Staphylococus aureus*, *Salmonella typhi* and *Vibreo colarae* and fungi i.e. *Trichothesium* and *Chrysosporium* sp. using Raper`s method. Mouse sarcoma cell line 180 and Balb/C mice were used for the anticancer screening of solid complex *in vitro* and *in vivo* respectively.

The observed polarographic data, on lingane treatment revealed the formation of single (1 : 1) (M : L) complex with Fe(III) and dacarbazine ligands. The results of amperometric titrations of Fe(III) with dacarbazine in 1M KCl supporting electrolyte pH 7.0±0.1 supported the above findings the IR data speaks of the complex formation between the metal and the dacarbazine ligand through the two nitrogen one each of primary amide and trizo groups. The results of microbial and pharmacological studies with the M:Drug complex revealed that the anticancer activity of the drug metal complex is nearly doubled as compared to the pure drug. As such Fe(III) dacarbazine complex may be recommended to the therapeutic experts for its possible use as more potent anticancer drug.

Key words : biometal anticancer effect dacarbazine

INTRODUCTION

The biochemical, pharmacological and medicinal importance of metal-drug complexes is very well established (1, 2). In continuation of the work done in our laboratory on the study of electrochemical, bio-inorganic, microbial and pharmacological behaviour of some metal-drug complex (3–6), the present paper deals with the said studies on the Fe(III)–Dacarbazine [5–(3, 3–dimethyltriazeno)–imidazole–4–carboxamide] (anticancer drug) complex.

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Experimental

Chemicals and reagents – All the chemicals used were of Anala-R/BDH grade. The drug Dacarbazine (C_6H_{10} N_6O) was procured from Sigma Chemical Company, USA. Double distilled water and absolute ethanol were used a solvents.

Polarographic measurements were made on an Elico (Hyderabad) pulse polarograph model CL–90, coupled with a X–Y polarocard model LR–108. The electrode system consisted of a dropping mercury electrode (DME) as a working electrode, a coiled platinum wire as an auxillary electrode and saturated calomal electrode (SCE) as a reference electrode.

Experimental sets were prepared by keeping overall iron (metal ion) and potassium chloride (supporting electrolyte) concentration fixed at 1.0 mM and 1.0 M respectively. The ligand concentration was varied from 0.0 mM to 5 mM. The pH of the test solution was adjusted to 7.0 ± 0.02 using HCl/NaOH solution.

The amperometric titrations were performed on a manually operated set up, a polyflex galvanometer (sensitivity 8.1×10^{-9} amp./div.) and an AJCO varnier potentiometer, a DME was used as an indicator electrode and a calomal electrode served as reference electrode. The capillary characteristics of a DME had a $m^{2/3}$, $t^{1/6}$ = 2.13 $mg^{2/3}$ $Sec^{-1/2}$ at 50 cm effective height of mercury column.

The pH of all the test solutions was measured on an Elico digital pH meter model LI-108.

Experimental sets, each having different but known amount of the drugs under study were prepared in appropriate quantity of supporting electrolyte (potassium chloride) at pH 7.0 ± 0.02 were prepared and titrated separately against the standard solution of the titled Fe (III) ions whose pH has been adjusted to that of the titrate (7.0±0.02).

Synthesis procedure of the solid complex – Ferric chloride and Dacarbazine (drug) solutions were separately prepared in ethyl (40 : 60 v/v) alcohol and were mixed in 1:1 molar ratio. The mixture was then refluxed in a round bottom flask for one-two hours. The residue complex was filtered and washed thoroughly to remove any unreacted materials. The complex was drieed at low temperature (40°C) and stored over P_4O_{10}.

The elemental, C, H, N and O analysis of the complex was done on a Heraeus Varlo Erba elemental analyser model–1108, at CDRI, Lucknow, whereas gravimetric method was used for the estimation of iron in the complex (7).

The IR spectrum of the solid complex was recorded using KBr pallets on a perkin-Elmer IR spectrophotometer, model-379.

Biological study of Fe(III) – Dacarbazine complex

Microbial study – Paper disc method (12) was followed for the microbial screening of Fe(III) – dacarbazine complex against various bacteria viz. *Pseudomonas mangiferae, Staphylococus aureus, Salmonella typhi* and *Vibreo colarae* and fungi i.e. *Trichothesium* and *Chrysosporium* s.p. sterilized filter paper discs (6 mm) were dipped into the complex solutions of 0.01M

concentration. Prior to this, the bacteria and fungi were separately homogenised with nutrient agar and potatodextrose media (at 27–30°C) plated onto the sterilised petri dishes. Dipped filter paper discs were placed on seeded medium. After 24 hour of incubation antimicrobial activities were recorded by measuring the inhibition zone against complex under study. Similar experiment was repeated with the control drug (dacarbazine).

The number of replicates in each case of three, percentage inhibition was calculated using the following formula –

$$\% \text{ inhibition} = \frac{A - B}{A} \times 100$$

Where 'A' represents the diameter of the inhibition zone for control (dacarbazine) and `B` represents the diameter of the inhibition zone for sample (Fe(III)– dacarbazine complex).

Pharmacological studies – In vitro and *in vivo* study of anticancer activity of prepared drug metal complex have been done by following procedure (13–15).

In vitro – Mouse Sarcoma Cell line-180, obtained from National Center for Cell Science, Pune, India, as a monolyer culture in Roux bottles (Corning Plastics, USA).

Cell culture – The cells obtained were cultured in 5 ml 24 well culture plate (Corning Plastics, USA). The cells were seeded in 2×10^5 cells per well and 1.0 ml of Dulbecco`s modified Eagles medium (DMEM) containg 10% (v/v) Foetal Calf serum, penicillin 100 µg/ml and streptomycin 100 µg/ml was added to each well. The cells were kept in incubator at 37°C for 4h in 5% CO_2 atmosphere and 95% humidity. The cell count was made on Neubaurs Chamber (Fine Optik, Germany).

Three dilutions viz. 1 µM, 10 µM and 100 µM/ml of pure drug and its Fe complex was made and then the cells were treated as follows:

Column	Free drug	Column	Metal complex
A	1 µM (1 ml)	D	1 µM (1 ml)
B	10 µM (1 ml)	E	10 µM (1 ml)
C	100 µM (1 ml)	F	100 µM (1 ml)

After addition of the respective solutions, the culture plate was incubated at 37°C for 4 hours. Finally the cell counts were made as under. These are compared with the cell cultured in DMEM without treatment.

Cell viability counts – Cell viability counts were made by Trypan blue dye exclusion test. Two drops of Trypan blue were added to each cell culture well and kept for 15 minutes. Now, a drop of culture was added to hemocytometer (Neubaurs Chamber) and the number of stained, non–stained and total number of cells were counted. Then, the % inhibition was calculated using the equation.

$$\frac{\text{No. of viable cells} - \text{No. of viable cells after treatment}}{\text{No. of viable cells without treatment}} \times 100$$

The experiment of each concentration of the drug and the complex was repeated thrice and statistical conclusions were drawn.

In vivo – The comparative efficiency of pure and complex forms of dacarbazine drug evaluated from the difference in response after treatment with the two forms of drug.

Animal model : Balb/C mice.
weight 30–40 gm.

Tumor model : Sarcoma cell line –180

Drug : Dacarbazine and its iron complex

Cell growing in nutrient medium (DMEM) were obtained from NCCS, Pune. They were brought into single cell suspension by trypsinization (0.2% trypsin). The cell suspension was centrifuged to obtain concentrated suspension (1–2 × 10^5 cell/ml). Approximately 10^5 cells of tumor were injected on the dorsal surface of the mouse and allowed to grow. Palpable size was reached by 6–8 days.

The time required to double the tumor volume (volume doubling time (VDT) from 100 to 200 mm^3 was taken as a criterion to assess the antitumor efficacy of pure and complexed drug in S–180 tumor bearing mice. The treatment was started after tumor size reached 100±10 mm^3. Indicated dose (equivalent to 0.2 mg) of free drug and drug complex were injected intravenously and tumor growth was monitored. Tumor size was calculated by the formula ½ LW2. Where L–long diameter and W-short diameter of the tumor, the above *in vivo* experiment was repeated on two other sets of mice groups.

RESULTS AND DISCUSSION

Polarographic behaviour of dacarbazine with Fe(III) – In 1.0M KCl at pH 7.0 ± 0.02 the Fe(III) and its complex with ligand under study were found to be reversibly reduced involving three electrons which was evidenced from the plots of log i/(id–i). The reduction was found to be diffusion controlled, which was evidenced by the plot i_d Vs. $\sqrt{h}$ corr.

On gradual increase of the dacarbazine concentrations, the half wave potential of Fe(III) metal ion shifted to more negative value and the diffusion current also decreased thereby showing complex formation between Fe(III) with dacarbazine (Fig. 1).

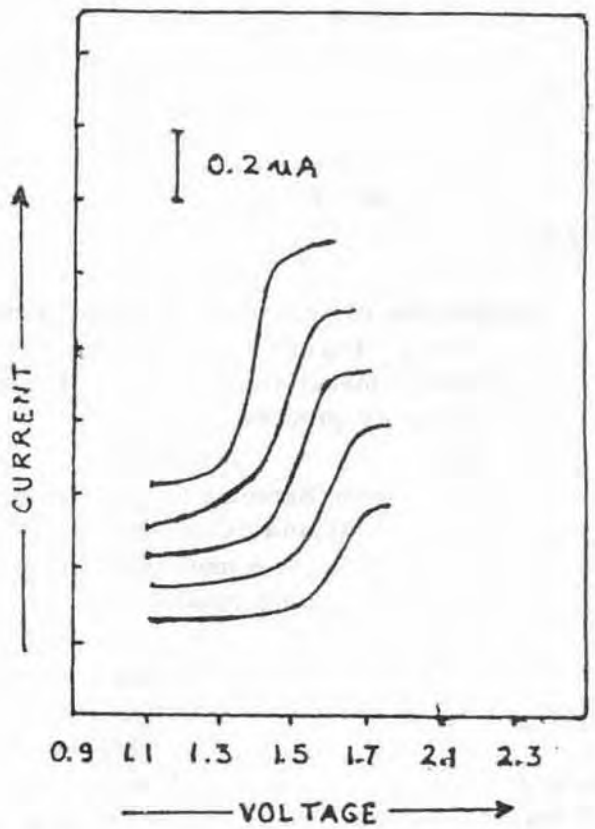


Fig. 1 : Polarograms of Fe(III) (1 mM) in 1M KCl supporting electrolyte at pH 7.0±0.02 and
A– without dacarbazine
B–1 mM dacarbazine
C, D and E–2, 4, and 5 mM dacarbazine

To study the composition and formation constant of the complex, plots of $\Delta E_{1/2}$ (shift in the $E_{1/2}$) i.e. $\Delta E_{1/2} = (E_{1/2})_C - (E_{1/2})_S$ against log C_X (logarithm of the concentration of the ligand) were drawn. The plots were linear lines showing the formation of single complex species in solution. Lingane treatment (8) of the observed polarographic data reveals 1:1 metal : dacarbazine complex formation with log $\beta = 5.1$.

Amperometric determination of Dacarbazine with Fe(III) – Fe(III) gives a well defined polarographic wave in 1.0M KCl at pH 7.0±0.02. The diffusion current was found proportional to the concentration of Fe(III). The dacarbazine drug does not produce and wave under the said experimental conditions. The platue potential for the polarographic wave of Fe(III) (–1.4v) Vs Hg pool was applied for carrying out amerometric titration. On performing the amperometric titration of drug solution with standard solution of Fe(III), the current volume plots resulted in ⌐ shapped curves (Fig. 2). The end point as located by graphical method revealed metal to drug ratio of 1:1, which is in agreement with the author`s observations on the metal:ligand equilibria using polarographic method.

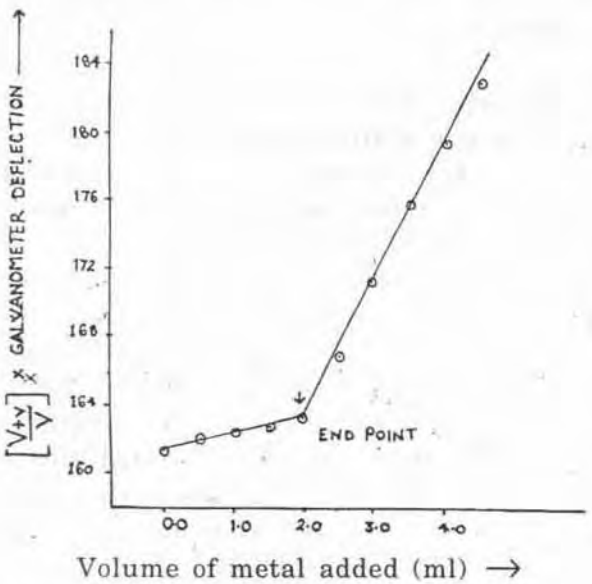


Fig. 2 : Amperometric titration of (2 mM/10 ml) Dacarbazine (1 mM/10 ml) Fe(III) solution

Characterization of Fe(III) – Dacarbazine complex

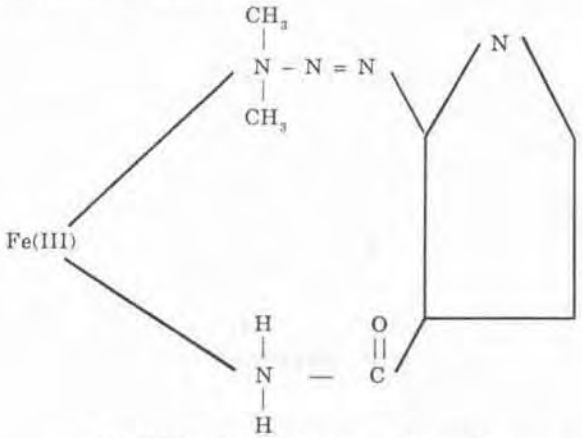
Elemental analysis – The results of elemental analysis (Table I) of the drug and it`s complex with Fe(III) revealed 1:1, metal:drug ratio in this complex, which supports author`s findings using polarographic and amperometric method.

TABLE I : Analytical data of Dacarbazine and its complex with Fe(III) Analysis/Calculated/ (Found).

	Element	*Dacarbazine*	*Fe(III)–Dacarbazine complex*
1.	Fe	–	23.52 (23.60)
2.	C	39.61 (39.70)	30.28 (30.32)
3.	H	5.40 (5.38)	4.22 (4.02)
4.	N	46.20 (46.15)	35.24 (35.36)
5.	O	8.79 (8.78)	6.74 (6.80)

IR Spectra – The structurally important frequencies of IR bands for dacarbazine and its complex with Fe(III) metal ion have been tabulated in Table II. A comparison of the IR data for the drug and its Fe(III) complex reveals that the bands at 880 (9) cm^{-1} and 1601 (10) cm^{-1} in the spectrum of the drug are shifted to 860 cm^{-1} and 1575 cm^{-1}

respectively in the spectrum of the complex, indicating the involvement of the two nitrogens, one each of primary amide and triazo (attached to dimethyl group) groups of the drug in complex formation (11). On the basis of above data attentative structure of the Fe(III)–dacarbazine complex may be given as under.



Fe(III)–Dacarbazine Complex

TABLE II : Principal IR frequencies (cm^{-1}) and their assignment for Dacarbazine and its complex,

	Ligand cm^{-1}	*Assignment*	*Fe(III) Dacarbazine*
1.	620, 685 S	Imidazole vibrations	620, 685 S
2.	880 S	$CONH_2$ streching vibrations	860 S
3.	1180, 1225, 1340 (W)	$-N_3$ streching vibrations	1180, 1225, 1340 W
4.	1430 (S)	–N = N streching vibration	1430 S
5.	1601 (br)	C – N aliphatic vibration	1575 br

S–sharp, W–weak, br–broad

Microbial study – Results of antimicrobial activities of the Fe(III)–decarbazine complex are shown in Table III. A perusal of the data in table clearly shows that iron dacarbazine complex is found to be more toxic as compared to the control drug against above mentioned bacteria and fungi.

Pharmacological studies

In vitro – The results of *in vitro* experiments of pure drug and its complex are shown in Table IV. A perusal of the results show that iron dacarbazine complex was found to be more effective than pure drug. The complex under study showed an increased inhibition against the S–180 tumor cells at all the test concentrations i.e. 1, 10 and 100 µm/ml. The increased inhibition activity of the complex was 52.1 ± 1.0%, 69.8 ± 1.0% and 92.5 ± 0.9% as against 36.4 ± 1.0%, 54.7 ± 0.6% and 78.6 ± 0.8% shown by the drug, respectively. The statistical treatment of the observed inhibition data i.e. standard deviation and coefficient of variance which never exceeded 0.9 and 1.8% respectively, speaks the reliability of the observed inhibition data.

In vivo – The results of the average of mice tumor against dacarbazine drug and its iron complex under study are shown in Fig. 3. The results indicated that the tumor volume was 0.05 cm^2 on the tumor cell injected mice without administering drug or complex after 20 days, percentage which was reduced to 0.035 cm^2 on tumor injected mice who were also administered the dacarbazine drug. However, in case of Fe(III)–dacarbazine administered mice (tumor cell injected) shows significant decrease in the tumor volume of 0.018 cm^2 was observed. Thus indicating the *in vivo* tumor inhibition power of the complex over the drug under study over the experimental time periods i.e. 20 days.

TABLE III : Antimicrobial study of Dacarbazine Fe(III) complex.

Organism	*Inhibition-zone* (mm)*		*Control Fe(III) metal (A) 1.0 mM/10 ml*	*Percentage change over control metal (A-B/A) × 100*	*Control drug (Y) 1.0 mM/ 10 ml*	*Percentage change over control drug (Y-B/Y) × 100*
	Concentration of camplex (per 10 ml)					
	5 mM	*1.0 mM(B)*				
1. Bacterial						
a. Pseudomonas mangiterae	5.8	12.8	57.0	77.54	12.2	0.00
b. Staphylococus aureus	6.4	14.0	41.2	66.02	15.0	6.66
c. Salmonella typhi	8.4	15.3	53.1	71.18	22.0	30.45
d. Vibreo colarae	–	14.0	53.0	73.58	11.0	–27.27
2. Fungal						
a. Trichothesium	8.0	15.0	40.2	62.68	–	–
b. Chryosposium sp.	7.0	17.1	39.0	56.15	–	–

*Including diameter of filter paper disc, 6mm.

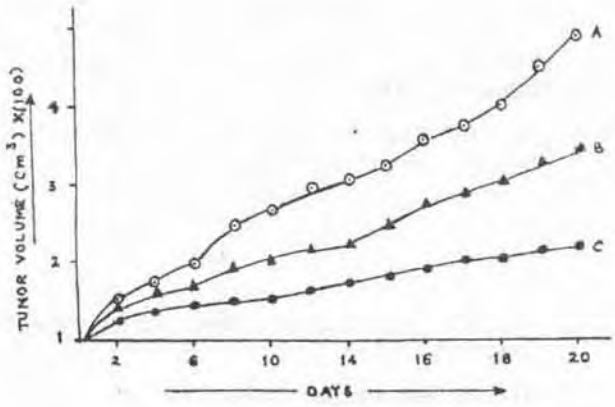


Fig. 3 : Effect of dacarbazine and Fe(III)–Dacarbazine complex on tumor volume
A–Without drug
B–With dacarbazine
C–With Fe(III)–Dacarbazine

Similar results were observed with the other two mice groups. However, the statistical treatment of the observed inhibition data i.e. standard deviation and coefficient of variance for the three mice groups, which never exceeded 1.0 and 2.3% respectively clearly reveals the reliability of the observed data.

TABLE IV: *In vitro* cytotoxicity of dacarbazine and Fe(III)–Dacarbazine complex against S-180 tumor cells.

Compound	*Concentration μM/ml*	*% inhibition after 4h*
Dacarbazine	1.0	36.4±1.0 (a) (b)
	10.0	54.7±2.6
	100.0	78.6±1.8
Fe(III) - Dacarbazine	1.0	52.1±1.0
complex	10.0	69.8±1.6
	100.0	92.5±1.9

(a) Composite results of three experiments.
(b) Mean ± standard error at mean.

From the above *in vitro* and *in vivo* results, it could be concluded that dacarbazine complex with Fe is seen to be more effective to control the multiplication of cells as compared to the dacarbazine drug, thus Fe(III)–dacarbazine complex may be recommended to the therapeutic experts as

a more potent anticancer drug in lieu of the drug taken for the present study.

ACKNOWLEDGEMENTS

The authors are thankful to Prof. S.P. Banerjee, Head, Chemistry Department, Dr. Harisingh Gour University, Sagar, for providing necessary laboratory facilities in the department. Thanks are also due to M.P. Council of Science & Technology, Bhopal (M.P.), for the award of Research Fellowship to one of the author (JS).

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