RESEARCH PAPER

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Synthesis and Anticancer Activity of Some L-Cystine Derivatives

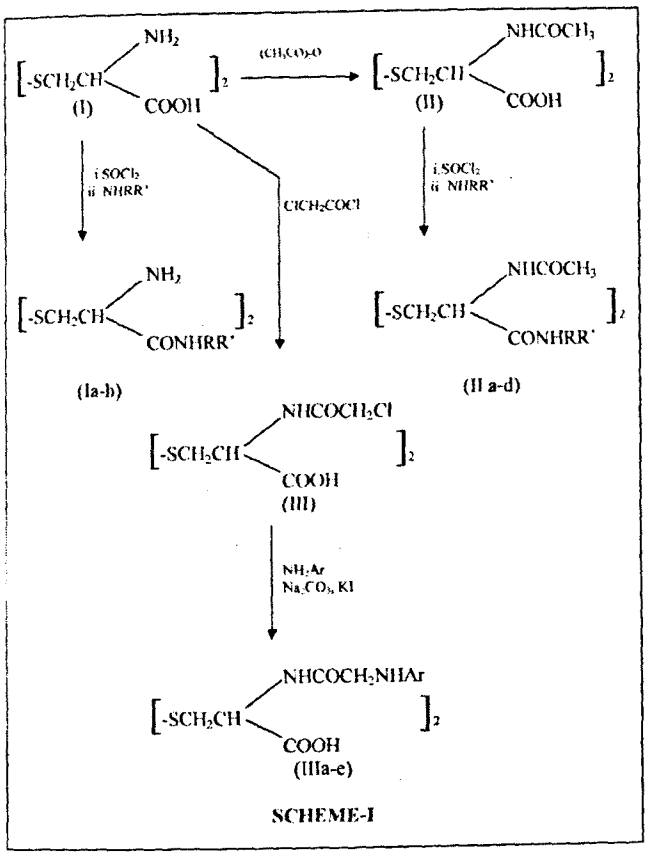
S. V. SHARMA*, S. SANKAR, G. V. S. RAMA SARMA, GIRIJA KUTTAN[1] AND B. SURESH
Department of Pharmaceutical Chemistry, J. S. S. College of Pharmacy, 'Rocklands', P.B. No. 20, Ootacamund-643 001.
[1]Cancer Research Center, Amla Cancer Hospital, Amla Nagar, Thrissur-680 553.

Eight 3,3'-dithio bis-(2-amino-N-alkyl/aryl propanamides) (Ia-h), four 3,3'-dithio bis-[2-(N-acetamido)-N-aryl propanamids] (IIa-d) and five 3,3'-dithio bis-[2-(aryl amino) acetamido propanoic acids] (IIIa-e) were synthesised from appropriate L-cystine or N,N'-bis acetyl cystine in satisfactory yields. All these compounds were purified and characterised by analytical and spectral data. Antitumour activity of selected drugs was studied by employing *in vitro* cytotoxicity assays and *in vitro* tumour growth inhibition studies. *In vitro* short term cytotoxicity studies indicated that six of the test compounds (Ia,b,d; IIb; IIIa,b) produced significant cytotoxicity in Ehrlich ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) tumour cells at a concentration upto 400 μg/ml. Tissue culture studies using L_{929} tumour cells resulted in more than 50% growth inhibition at 1.25 μg/mg concentration for the four test compounds. Administration of selected drugs (25 μg i.p. for 5 alternate days) resulted in an increase in life span of ascites tumour-bearing mice upto 102% (EAC tumour) and 68% (DLA tumour) treated with IIIb as compared with control. All the test compounds were found to possess significant antitumour activity against experimental tumours.

Most amino acids have been used as a source to discover new compounds with antimicrobial and anticancer properties, however, the disulfide amino acid, cystine and its reduced aminothiol form, cysteine remain the least explored[1]. N-Acetylcystine is, at present, considered one of the most promising second-generation cancer chemopreventive agents, which can protect platinum-induced nephrotoxicity in humans[1]. L-Cysteine can serve as an oxygen radical scavenger in extracellular fluid[2]. A large body of evidence indicates that certain aspects of T-cell response require the action of active oxygen, while other aspects require action of antioxidants such as cysteine or glutathione. Role of cyst(e)ine in T-cell mediated immune responses and in AIDS is under extensive investigation[3]. Cyst(e)ine and its several derivatives are known to possess antimutagenic, anticarcinogenic and radioprotective properties[4,5] and antitumour activity of certain derivatives has been reported in experimental tumours[6,9]. Therefore, it was felt worthwhile to synthesise some new amide derivatives of L-cystine and N,N'-bisacetyl cystine with a view to evaluate them for their cytotoxic and antitumour properties. In the present investigation, some simple analogues of L-cystine with substitution only on acid group (Type-I), on both acid and amino groups (Type-II) and only on amino group (Type-III) were designed and their syntheses have been effected as shown in Scheme 1.

Intermediates, 3,3'-dithio bis-[2-amino/N-acetamido propanoyl chlorides] were synthesised by treating corresponding L-cystine (I) and N,N'-bis acetyl cystine (II) with pure thionyl chloride and condensed with different aliphatic and primary aromatic amines in appropriate conditions. Similarly 3,3'-dithio bis-(2-chloro acetamido propanoic acid) (III) was synthesised by refluxing L-cystine with chloroacetyl chloride in dry dimethyl formamide (DMF), which was

*For correspondence:
E-mail: sunilsv@hotmail.com



SCHEME-I

further condensed with various primary aromatic amines in appropriate conditions. Products obtained were purified and characterised by analytical/spectral data and elemental analysis, which confirmed the assigned structures. Selected compounds were screened for their *in vitro* cytotoxicity and *in vivo* antitumour properties by preparing drug solutions in DMF, diluted appropriately with phosphate buffer saline (PBS).

EXPERIMENTAL

Melting points were determined in open capillaries and are uncorrected. IR spectra were recorded on Perkin-Elmer model 1600 spectrometer. Elemental analysis was carried out using Heraus Carlo Erba 1108 elemental analyser. L-Cystine, trypan blue dye were procured from E-Merck (India) Ltd., Chennai and Minimum Essential Medium (MEM) from Sigma-Aldrich Ltd., Bangalore. L_{929} cell cultures were obtained from National Facility for Animal Tissue and Cell Culture (NFATCC), Pune while other cell lines (DLA, EAC) used in the present investigation were obtained from the Amla Cancer Research Institute, Trissur. 3,3'-Dithio bis-(2-amino/acetamido propanoyl chlorides) were prepared from corresponding L-cystine (I) or N,N'-bis acetyl cystine (II) by adapting standard procedures. These inteamediates were unstable and were condensed with appropriate amine immediately.

Synthesis of 3,3'-dithio bis-(2-amino-N-alkyl propanamides):

3,3'-Dithio bis-(2-amino propanoyl chloride) (0.01 mol) was treated with a molar excess of primary or secondary aliphatic amine. Resultant mixture was cooled, concentrated under vacuum and solid product thus obtained was filtered, washed with hot water and recrystallized from acetone-ethanol (1:4) mixture. For instance, reaction of the acid chloride intermediate with methylamine yielded a colourless solid. It was characterised as 3,3'-dithio bis (2-amino-N-methyl propanamide). Its IR (KBr) spectrum showed characteristic absorption in cm^{-1} at 3478 (NH_2), 1687 (CO), 1295 (C-N, secondary) and 1452 (CH_2, alkyl). Similarly, three compounds were synthesised and characterised (Ia-c).

Synthesis of 3,3'-dithio bis-[2-(amino/N-acetamido)-N-aryl propanamides]:

Appropriate amount of 3,3'-dithio bis [2-(amino/N-acetamido) propanoyl chloride] (0.003 mol) in dry ether (30 ml) was refluxed with corresponding primary aromatic amine for 1 h on a water bath. Reaction mixture in each case was concentrated and poured onto crushed ice with constant stirring. The solid thus obtained was washed with cold water and purified from hot water and ethanol. For example, reaction of 3,3'-dithio bis-(2-amino propanoyl chloride) with aniline in dry ether yielded a pale pink crystalline product, which was recrystallized from alcohol. It was characterised as 3,3'-dithio bis-(2-amino-N-phenyl propanamide) (Id). Its IR (KBr) spectrum showed characteristic absorption in cm^{-1} at 3460 (NH_2); 1673 (CO); 1311 (CN, secondary); 3025 (CH, aromatic) and 1472 (CH_2, alkyl). Similarly, nine compounds were synthesised and characterised (Id-h, IIa-d).

Synthesis of 3,3'-dithio bis-[2-(aryl amino) acetamido propanoic acids]:

L-Cystine (I; 0.02 mol) was refluxed with molar excess of chloroacetyl chloride in dry DMF for 30 min. Reaction mixture was concentrated and kept overnight in cold to get a colourless solid which was recrystallised from acetone-ethanol (2:1) mixture. It was characterised as 3,3'-dithio bis (2-chloro acetamido propanoic acid) (III; Yield: 89%; m.p. 160°). It was condensed with appropriate primary aromatic amine (1:2 mol ratio) by refluxing for over 15 h in dry methanol in the presence of anhydrous sodium carbonate (0.001 mol) and a minute amount of sodium iodide. All the reactions were

TABLE 1: PHYSICAL AND ANALYTICAL DATA OF L-CYSTINE DERIVATIVES.

Compound	Substitution	Molecular Formula	Melting Point (°)	Yield (%)	R_f value[a]	Nitrogen Analysis % Obs. (Cal.)
Ia	Methylamino	$C_8H_{18}N_4O_2S_2$	236	86	0.72	21.15(21.05)
Ib	Ethylamino	$C_{10}H_{22}N_4O_2S_2$	208	83	0.65	19.08(19.04)
Ic	Diethylamino	$C_{14}H_{30}N_4O_2S_2$	182	79	0.62	15.92(16.00)
Id	Anilino	$C_{18}H_{22}N_4O_2S_2$	235	69	0.80	14.38(14.35)
Ie	o-Methylanilino	$C_{20}H_{26}N_4O_2S_2$	225	60	0.84	13.26(13.33)
If	o-Chloroanilino	$C_{18}H_{20}N_4O_2S_2Cl_2$	248	74	0.60	12.25(12.20)
Ig	p-Bromoanilino	$C_{18}H_{20}N_4O_2S_2Br_2$	238	56	0.45	10.21(10.22)
Ih	Phenyl ethylamino	$C_{22}H_{30}N_4O_2S_2$	167	42	0.25	12.68(12.55)
IIa	Anilino	$C_{22}H_{24}N_4O_4S_2$	232	81	0.85	11.71(11.86)
IIb	o-Cloroanilino	$C_{22}H_{22}N_4O_4S_2Cl_2$	228	92	0.55	10.45(10.35)
IIc	p-Bromoanilino	$C_{22}H_{22}N_4O_4S_2Br_2$	202	72	0.68	08.81(08.86)
IId	o-Methylanilino	$C_{24}H_{28}N_4O_4S_2$	196	67	0.78	11.28(11.20)
IIIa	Anilino	$C_{22}H_{26}N_4O_6S_2$	230	56	0.45	11.22(11.06)
IIIb	o-Chloroanilino	$C_{22}H_{24}N_4O_6S_2Cl_2$	212	64	0.62	09.71(09.75)
IIIc	o-Methylanilino	$C_{24}H_{30}N_4O_6S_2$	206	62	0.38	10.60(10.48)
IIId	p-Bromoanilino	$C_{22}H_{24}N_4O_6S_2Br_2$	190	78	0.40	0.75(08.63)
IIIe	p-Nitroanilino	$C_{22}H_{24}N_6O_{10}S_2$	198	48	0.52	14.14(14.09)

Compounds (Ia-c) were recrystallised from acetone:ethanol (1:4) mixture (TLC mobile phase:ethanol, acidified with acetic acid), compounds (IIIa-e) were recrystallised from acetone:ethanol (2:1) mixture (TLC mobile phase:chloroform), while rest of the compounds were purified from hot water or ethanol (TLC mobile phase:acetone/chloroform), a denotes retardation factor in corresponding TLC system.

monitored by TLC using chloroform. Reaction mixture was acidified with dilute hydrochloric acid, concentrated and kept overnight. The solid thus obtained was recrystallised from acetone-ethanol (2:1) mixture. For instance, reaction of 3,3'-dithio bis-(2-chloro acetamido propanoic acid) (III) and aniline yielded a white crystalline product. It was characterised as 3,3'-dithio bis [2-(phenyl amino) acetamido propanoic acid] (IIIa). Its IR (KBr) spectrum showed characteristic absorption in cm^{-1} at 3483 (OH); 3390 (NH), 1648 (CO); 1300 (CN, secondary); 3050 (CH, aromatic) and 1478 (CH_2, alkyl). Similarly, five compounds were synthesised and characterised (IIIa-e). Physical and analytical data of all the synthesised compounds are given in Table 1.

Anticancer studies; *In vitro* short term cytotoxicity assay[10]:

Eleven compounds (Ia-f; IIa,d; IIIa-c) were tested for their cytotoxicity against Ehrlich ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) tumour cells using trypan blue dye exclusion method. At 37°, tumour cells ($1x10^6$) were incubated for 3 h with various concentrations of test compounds (100-500 µg, in triplicate) in phosphate buffered saline (PBS). Concentration required for 50% cytotoxicity ($cyto_{50}$) was determined graphically. Percentage of dead cells after 3 h incubation with or without DMF (solvent control) remained the same (<2%).

***In vitro* antitumour screening[11]:**

In view of the above observations, cytotoxicity of four test compounds (Ia; IIIa-c) was determined against mouse

lung fibroblastoid cell culture (L_{929}) grown in MEM supplemented with 10% goat serum in the presence of antibiotics (penicillin 100 units/ml and streptomycin 100 µg/ml). At 37°, tumour cells ($5x10^4$) were incubated with different concentrations of test compound (1-5 µg/ml, in triplicate) for 7 d and total viable cells were determined by trypan blue dye exclusion method. Concentration required for 50% inhibition of cell growth (IC_{50}) was determined graphically by plotting drug concentration against percentage of control (live cells).

In vivo antitumour studies:

From the results of *in vitro* studies, six compounds (Ia,d; IIb; IIIa-c) were selected for *in vivo* studies. Acute toxicity studies were carried out in Swiss albino mice by standard method[12] by i.p. administration of various doses of test compounds (5-20 mg/kg body weight). *In vivo* antitumour studies were carried using standard ascites tumour models in Swiss mice[13]. Tumour cells ($1x10^6$) were transplanted i. p. in the mice of either sex (6 mice/group) weighing between 18-24 g. After 24 h, 25 µg drug was administered i. p. on five alternate days. Mortality of the animals dying due to tumour was observed and percentage increase in life span (ILS) compared with control was calculated.

RESULTS AND DISCUSSIONS

Of all the 17 compounds synthesised, eleven were subjected for short-term cytotoxicity studies (Table 2). Compounds Id and IIa were found to exhibit superior cytotoxicity potential with more than 50% cytotoxicity at a concentration of 175 µg/ml. These results revealed that six of the test compounds (Ia,b,d; IIa; IIIa,b) possessed significant cytotoxicity and have shown 100% cell death at a concentration of 600 µg/ml against both tumour cell lines tested. In general, all the six compounds were more toxic to DLA cells than EAC in short term incubation.

All the four test compounds significantly inhibited growth of L_{929} cells in tissue culture and the IC_{50} were found to be less than 1.25 µg/ml (fig. 1). Compound IIIb was the most active with only 18% cell growth at 1 µg/ml concentration. *In vitro* studies indicated dose dependent cytotoxicity for the compounds tested (Table 2 and fig. 1).

Acute toxicity studies indicated that all the six test compounds were highly toxic (Table 3). However, no CNS toxicity symptoms could be observed at the doses used for the study. Due to this high toxicity, very low dose (25 µg) was selected for treatment. In *in vivo* antitumour studies, all the test compounds showed significant inhibitory effect on the

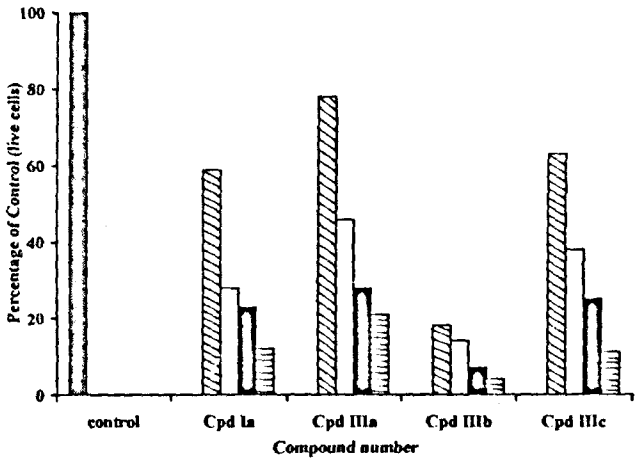


Fig. 1: Effect of test compounds on growth of L_{929} cell culture.

Growth of L_{929} cells in culture was determined in the presence of solvent control (▨), 5.00 µg/ml (▨), 2.50 µg/ml (□), 1.25 µg/ml (■) and 1.00 µg/ml (□) of the test compounds Ia, IIIa, IIIb and IIIc that were tested.

TABLE 2: *IN VITRO* ANTITUMOUR STUDIES.

Compound	*In vitro* cytotoxicity studies		
	$Cyto_{50}$ (µg/ml)[a]		IC_{50} (µg/ml)[b]
	EAC	DLA	L929
Ia	370	235	1.09
Ib	320	315	-
Ic	>400	325	-
Id	165	140	-
Ie	>400	430	-
If	>400	495	-
IIa	175	130	-
IIb	>400	265	-
IIIa	370	235	1.22
IIIb	320	315	<1.00
IIIc	>400	325	1.13

a and b denote the concentration required for 50% cytotoxicity against Ehrlich ascites carcinoma (EAC) and Daltons lymphoma ascites (DLA) tumour cells in short term incubation and 50% growth inhibition of L929 in cell culture respectively. Values are average of three readings.

TABLE 3: *IN VIVO* ANTITUMOUR STUDIES.

Compound Number	Acute Toxicity Studies LD_{50} (mg/kg)	*In vivo* Ascites Tumour Reduction Studies[a]			
		Ehrlich ascites carcinoma (EAC)		Daltons lymphoma Ascites (DLA)	
		MST (±S.D.)	%ILS	MST (±S.D.)	%ILS
Control	-	10.2±1.2	-	13.0±2.2	-
Ia	8.3	14.6±1.9	46*	20.0±2.3	54*
Id	6.0	15.6±1.5	56*	15.8±1.6	22
IIa	5.8	16.2±1.9	62*	19.0±2.0	46*
IIIa	12.3	20.0±2.0	100*	21.2±3.7	63*
IIIb	12.0	20.2±1.3	102*	21.8±1.6	68*
IIIc	7.4	18.6±1.3	86*	20.8±2.1	60*

a denotes that tumour cells (10^6) were transplanted i. p. and drugs (25 µg) were administrered i. p. for 5 alternate days. Values are average of six animals/set. MST and %ILS denote Mean Survival Time (in d) and Percent Increase in Life Span respectively; * Values are significant at $P<0.01$.

growth of ascites tumour in experimental animals (Table 3). Compound IIIb was most potent with the highest ILS of 102% (EAC) and 68% (DLA) when compared with control. Compound IIIa was next in the order of potency. In general, all the test compounds showed better *in vivo* antitumour activity against EAC tumour than DLA.

It is interesting to note that compounds having N-aryl substitution on the acid group of L-cystine (Ia,d; IId) showed marked *in vitro* cytotoxicity which probably contributes towards their high *in vivo* toxicity. But, N-(2-substituted anilino) acetamides having free acid functional group on L-cystine (IIIa-c) were comparatively less toxic both in *in vitro* as well as *in vivo* studies. A compound with electron withdrawing group at 2 position on phenyl ring (IIIb) showed better tumour reduction than one with electron releasing group (IIIc). In general, all the test compounds possess significant antitumour activity against the experimental tumours.

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