A comparative study of antiplasmin activity of some pyridine derivatives and related compounds

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Antiplasmins have therapeutic significance in arresting the uncontrolled bleeding associated with several disease states. Understanding of the structure–function relationship of antiplasmins is important in the development of coagulants as well as anticoagulants. A comparative structure–activity analysis of antiplasmin activity of some pyridine, piperidine, indole and thiocyclohexane derivatives with known antiplasmins, viz. *p*-aminomethyl benzoic acid (PAMBA) and epsilon-amino-caproic acid (EACA), has been carried out. The antiplasmin activity of pyridine, piperidine, indole and thiocyclohexanes appears to depend on over-all lipophilicity and the distance between basic centre and carboxyl function present in these compounds where the optimum separation is found to be 6.5 Å. Such spatial disposition of basic and carbonyl centres in these compounds may be responsible for binding to the sites responsible for antiplasmin activity. The lipophilicity may be responsible for making the compounds available at the site of action and also for their hydrophobic binding.

ANTIPLASMIN compounds have their therapeutic significance in arresting the uncontrolled bleeding associated with several disease states and pathophysiological conditions resulting out of excessive lysis of fibrin by plasmin[1]. The understanding of the structure–function relationship for antiplasmins is also important because sometimes the antiplasmin activity may be a side effect in conditions where acceleration in coagulation may aggravate the disorders associated with haemostatis leading to thrombotic diseases namely, myocardial infarction, stroke, pulmonary lumbolism, venus thrombosis and arterial thrombosis[2,3]. In view of this, known antiplasmin compounds, namely, epsilon-aminocaproic acid (EACA), *p*-aminomethyl benzoic acid (PAMBA) and 4-aminomethylbicyclo(2,2,2)octane-1-carboxylic acid[4,5] have been considered as the guiding structures for the selection of other compounds to study their antiplasmin activity. The most characteristic feature of the above antiplasmins has been the presence of an acidic and basic function in a particular spatial orientation for their biomolecular interactions. In PAMBA and 4-aminomethyl bycyclo(2,2,2)octane-1-carboxylic acid, the basic NH_2– and acidic COOH groups are separated by a distance of about 6.5 Å. EACA being a flexible molecule, can also exist in energetically allowed conformations in which its NH_2 and COOH groups are separated by the same distance. The hydrophobicity (log*P*) of epsilon-amino-caproic acid (EACA), PAMBA and 4-aminomethyl bicyclo(2,2,2)octane-1-carboxylic acid are 0.05, 0.64 and 1.19, respectively. In order to substantiate the importance of the above observations and to study the minimum structural and physicochemical requirements of antiplasmins, a set of molecules, viz. pyridine, piperidine, indole and thiocyclohexane carboxylic acids (Figure 1) was selected. In these compounds the acidic (COOH) and electron rich centers (N and S) are separated by a distance ranging from 2.6 to 6.5 Å and have hydrophobicity (log*P*) in the range of –2 to +2. In addition, these molecules are among the most commonly incorporated substructures in various biologically active molecules. So these compounds were evaluated for their antiplasmin activity in comparison to EACA and PAMBA to derive meaningful structure–activity relationships in terms of the above parameters. The results of this study are presented in this paper.

The chemical compounds **1–15** (Figure 1) used in these studies were either procured from commercial firms or were prepared by reported methods[6–8].

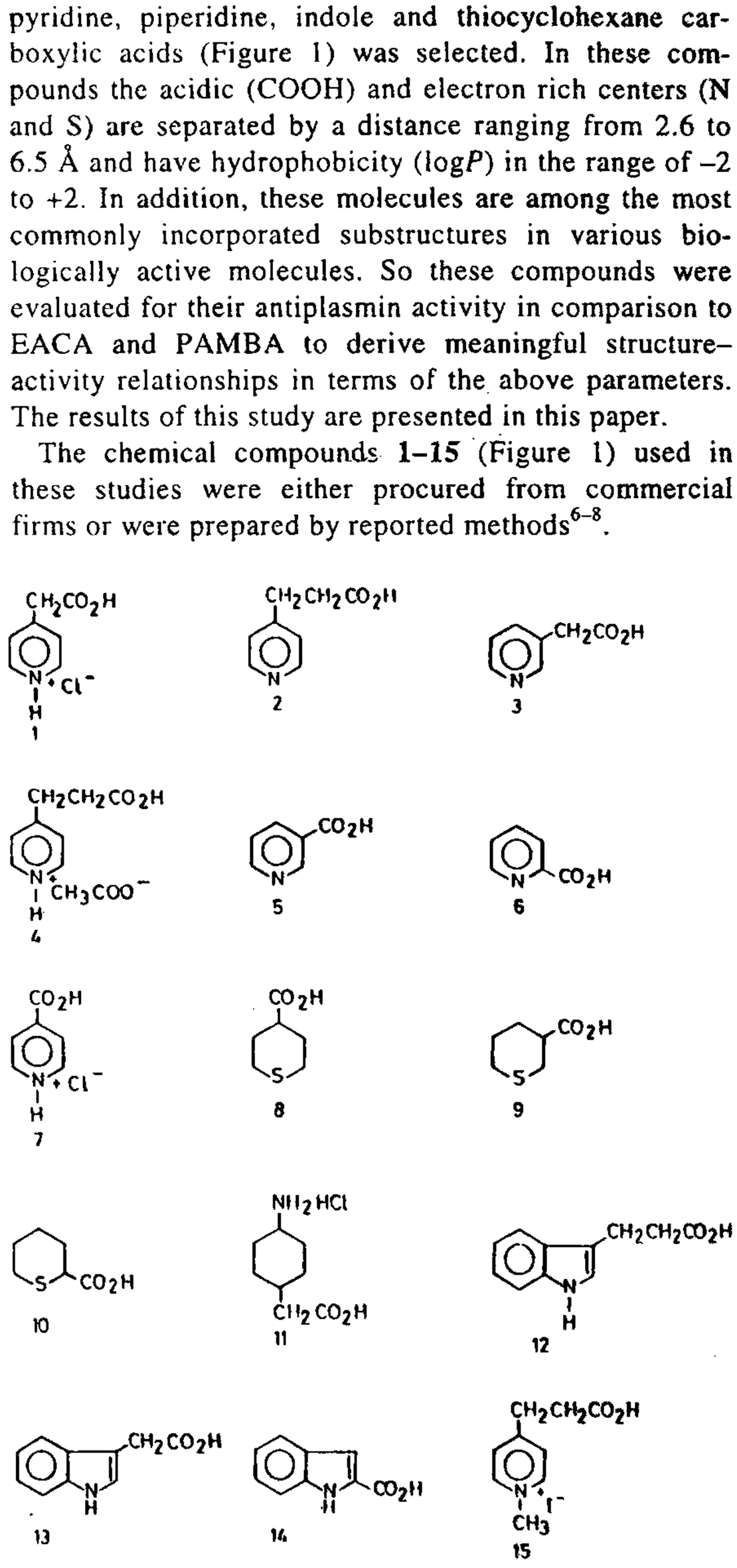


Figure 1. Various pyridine, piperidine, indole and thiocyclohexane based compounds included in the study.

Fibrinogen was fractionated according to the method of Blomback *et al.*[9] for fraction 1–0 with slight modification and thrombin was prepared according to Oser[10]. Bovine plasmin was received as gift from Sigma Chemical Co., USA. EACA was purchased from Epsamon Emser Werke, Switzerland and Riley Tar, USA, respectively. The method of Bickford *et al.*[11] was used with some modifications and is described below:

Table 1. Physicochemical properties and antiplasmin activity of pyridine and related derivatives

Compd	Log*P*	Distances (Å) N–C (COOH)	N.R.E. kcal/M	M.M.E kcal/M	Activity (%)
1.	–	5.003	0.3205	–0.5155	5
2.	0.42	6.479	0.3448	–0.4317	50
3.	–0.12	4.318	0.3198	–0.4259	10
4.	–	5.318	0.2980	0.2746	6
5.	0.51	3.760	0.2622	–0.1164	10
6.	0.60	2.399	0.2597	0.1479	–
7.	–	4.108	0.3002	0.0130	–
8.	0.88	4.027	0.2976	0.0332	–
9.	0.99	4.115	0.2900	–0.0369	2
10.	1.30	2.715	0.0289	–0.1724	–
11.	–2.52	6.546	0.2438	0.5095	–
12.	1.87	4.973	0.3933	0.6330	5
13.	1.33	4.653	0.3503	0.3008	–
14.	1.98	2.667	0.2766	1.2010	–
15.	–	5.665	0.2388	0.2134	–
EACA	0.05	–	–	–	100

The reaction mixture containing 0.25 ml of fibrinogen solution (1.0 mg protein representing 0.85–0.90 mg clottable fibrinogen in 0.3 M NaCl), 0.3 ml tris buffer (0.05 M; pH 7.2), 0.2 ml inhibitor solution in desirable concentration and 0.25 ml of enzyme solution containing 0.25 µg bovine plasmin was incubated at 37°C. At 0 h and at subsequent intervals up to 1 h, the reaction was stopped by diluting with 10 ml NaCl (0.15 M), followed by addition of 0.2 ml of bovine thrombine. The incubation was continued for another 20 min and the fibrin clot was taken out by winding around a thin-pointed glass and washed thoroughly with 0.15 M NaCl. The clot was dissolved in 0.1 M NaOH and protein was estimated according to the method of Lowry *et al.*[12]. The K_i values were determined according to the method of Dixon[13]. EACA and PAMBA were used as standard compounds.

The partition coefficient (Log*P*) of the compounds has been calculated using the constructionist approach of Hansch and Leo[14]. The Log*P* values of some ionic compounds (**1, 4, 7** and **15**) have been omitted due to the non-availability of appropriate fragment values. For geometrical parameters, the molecules were constructed from well-defined fragments on Sigmex terminal attached to Micro VAX-I computer system using CHEM-X software[15], the molecules were optimized for their geometry using MM2 routines of the software

Table 2. Antiplasmin activity of compound **2** in relation to EACA and PAMBA

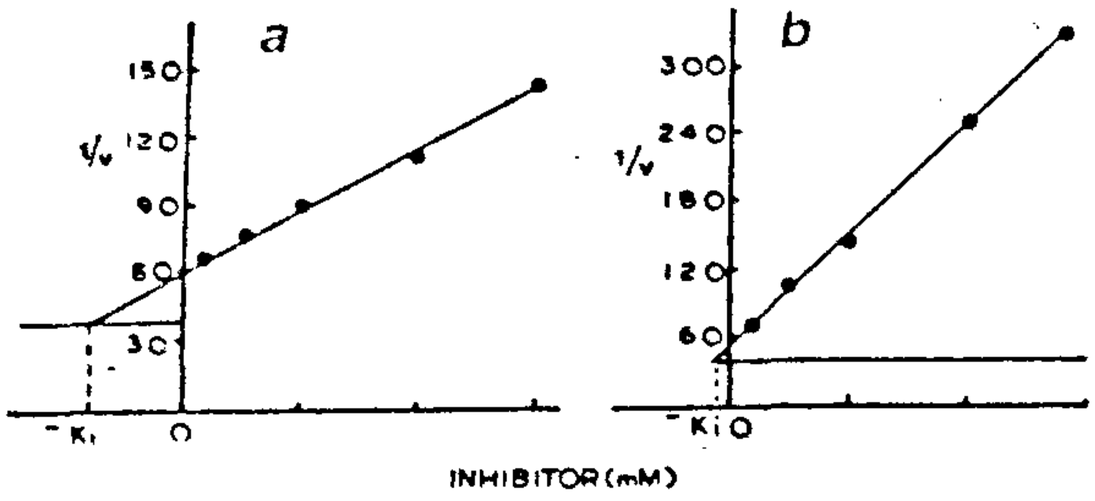
Exp. system	Residual fibrinogen after 1.0 h (µM)[a]			Plasmin activity degradation/h (µM)[b]			% Inhibition of plasmin[c]		
Control	1.28			1.57					
Inhibitor (mM)	**2**	EACA	PAMBA	**2**	EACA	PAMBA	**2**	EACA	PAMBA
1.0	1.71	1.92	1.99	1.14 (72.6)	0.93 (59.2)	0.86 (54.7)	27.4	40.8	45.3
2.5	1.75	2.03	2.20	1.10 (70.0)	0.82 (52.2)	0.65 (41.4)	30.0	47.8	58.6
5.0	1.86	2.16	3.24	0.99 (63.01)	0.69 (43.9)	0.61 (38.8)	37.0	56.1	71.2
10.0	1.99	2.28	2.60	0.86 (54.7)	0.57 (36.3)	0.25 (15.9)	45.3	63.7	84.1
15.0	2.05	2.34	2.70	0.80 (50.9)	0.51 (32.4)	0.15 (9.5)	49.1	67.6	90.5

[a]Reaction mixture containing fibrinogen and plasmin was incubated at 37°C for one hour in presence of different concentrations of inhibitor. Residual fibrinogen concentration at zero hour is 2.85 µmoles. Residual fibrinogen was determined as described in text.

[b]Difference between residual fibrinogen concentration at zero hour (2.85) and at 1 hour (0) residual. The % plasmin activity (in parenthesis) was calculated according to the formula:

$$= \frac{\text{Degradation of fibrinogen/hour in the presence of inhibitor}}{\text{Degradation of fibrinogen in the absence of inhibitor}} \times 100$$

[c]Inhibiton of plasmin activity = 100 – % plasmin activity.



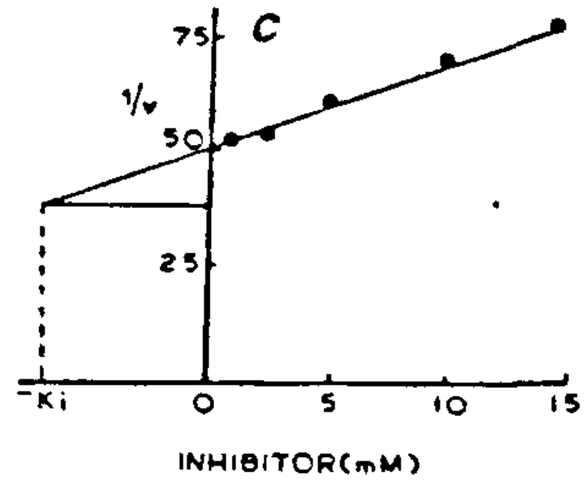


Figure 2. Determination of K_i of *a*, EACA ($K_i = 7.0$); *b*, PAMBA ($K_i = 4.2$); *c*, compound **2** ($K_i = 0.5$) by Dixon, simple graphical method. $1/v$ was determined in presence of various inhibitor concentrations keeping the substrate constant. This was plotted against inhibitor concentration.

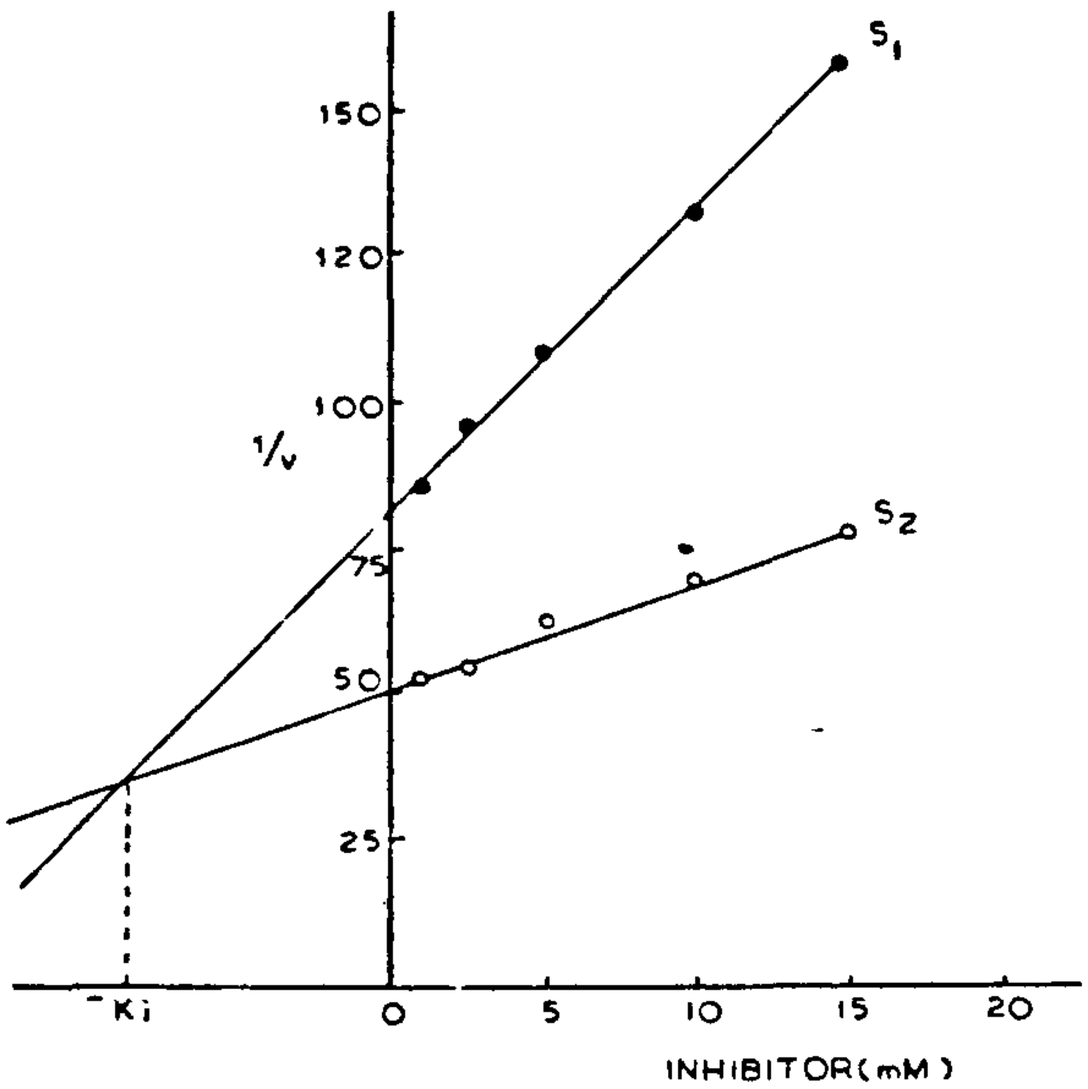


Figure 3. Evaluation of the dissociation constant by Dixon's method; $S_1 = 2$ mg/ml; $S_2 = 4$ mg/ml; i = inhibitor concentration in mM; v = velocity of the reaction, determined in presence of various concentration of inhibitors at two different substrate concentrations.

(convergence criteria 0.01 kcal). The molecular mechanical energy, net repulsion energy and the distance between N or S atom and C atom of their carboxylic group were measured for these molecules using CHEM-X software and are listed along with Log*P* and antiplasmin activity in Table 1.

It may be seen from the data (Table 1) that out of the 15 compounds tested, 7 exhibited antiplasmin activity of varying degree. However, of these, only compound **2** showed significant enzyme inhibitory activity. It was compared with the standard compounds EACA and PAMBA at 10 mM concentration (Table 2). The antiplasmin potency of these three compounds is in the order of PAMBA > EACA > 2. The comparative inhibitory potency for compound **2** and the two reference compounds EACA and PAMBA has been expressed in terms of K_i values of 7.0, 4.2 and 0.5 mM, respectively from the Dixon plot (Figure 2), suggesting the relative potencies of these compounds as 1:0.6:0.07. The relative potencies of EACA and PAMBA as reported in literature[13,16] are in agreement with the present results. The compound **2** is a competitive inhibitor like EACA as shown by Dixon plot analysis (Figure 3).

The antiplasmin activity of these compounds seems to depend mainly on the distance between the two sites, namely, basic centre and the carbon of carboxyl group and on the hydrophobicity of the molecule. The former may provide a better fit, whereas the latter may be responsible for its availability at the site of action and may also represent the forces involved in the binding. The distance between N and C atoms of carboxyl group in PAMBA has been 6.578 Å and Log*P* is of the order of 0.64. Due to structural flexibility, EACA can take a conformation within 2 kcal from its global minimum, where N–C(COOH) would be of the order of 6.5 Å. However, the geometrical advantage of EACA has been nullified by its low Log*P* (0.05) value, hence the compound is less active than PAMBA. Compound **2** of the present study has almost the same N–C (COOH) distance (6.479 Å) as PAMBA (6.478 Å) and Log*P* (0.42) a little less than PAMBA (log $P = 0.64$). The considerable loss of activity of compound **2** and its analogues may be due to the change in basicity of nitrogen. The other compounds **3** and **5** are equiactive because in case of **3**, the N–C (COOH) distance (4.648 Å) is 2 Å less than that in PAMBA but Log*P* is far lower while in compound **5**, the Log*P* is favourable but the deviation of N–C (COOH) distance is by 3 Å. Other compounds, particularly those with S at the place of N, lack the ideal basic character which is also not present in indole derivatives (**12–14**). Further, this analysis also explains the high activity of 4-aminomethylbicyclo(2,2,2)octane-1-carboxylic acid, a compound 30 times more active than PAMBA[5] in terms of its N–C (COOH) distance (6.5 Å) and Log*P* (1.19).

The significance of the reported work lies in the identification of physicochemical and spatial characteristics of molecules for antiplasmin activity. This knowledge

may be useful for designing new coagulants devoid of side effects. Also, in view of the increasing interest in anticoagulants, these studies would help to understand and overcome the undesirable antiplasmin activity of the drugs meant for other therapeutic uses.

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Homing of polyclonally activated syngeneic T cells at tumour site and their efficacy in post operative immunotherapy of malignancy in mouse

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^{3}H-Thymidine labelled and polyclonally activated syngeneic lymphocytes injected intravenously have been found to home preferentially at the fibrosarcoma site in mice from 12 h after adoptive transfer. The highest counts from infiltrating radiolabelled cells were obtained at 24 h at the centre of the tumour. Considering this value as maximum, all other values are expressed relative to this number. At 12 h lymphocyte infiltration at the periphery of the tumour mass was about 75%, followed by about 40% infiltration in the liver. After 24 h the central region of tumour mass showed the highest infiltration of radioactively labelled lymphocytes, whereas in liver it remained 50%. There was decline in homing of the activated lymphocytes after 48 h of adoptive transfer.

These tumour site seeking activated lymphocytes are likely to recognize the residual malignant cells after surgical removal of solid tumour, so these cells were adoptively transferred in conjunction with surgery. It has been observed in such experiments, the reappearance of tumour was prevented in 67% of mice and the survival of the hosts increased.

IT has been shown earlier that suppressor cell depleted syngeneic murine T lymphocytes activated polyclonally with Con A *in vivo* can kill tumour target cells[1–3]. The efficacy of such cells in curbing tumour growth after being injected at the tumour site[3,4] opened up the possibility of using such cells in tumour immunotherapy. However, it needs to be investigated whether after adoptive transfer via intravenous route, the effector cells generated either by *in vivo* Con A stimulation[5,6] or by *in vitro* sensitization with specific tumour cell lines[7–10] can infiltrate the tumour mass.

To make this immunotherapeutic measure even more effective, the proportion of effector T cells to the enormous number of rapidly dividing tumour cells needs to be taken into consideration. Therefore, in the present study, surgical removal of bulk of the solid tumour was followed by adoptive transfer of polyclonally activated effector T cells.

Inbred adult Swiss mice of both the sexes, 6–12 weeks of age were used for all the experiments. Methylcholanthrene (MCA) induced ascitic fibrosarcoma cell line is maintained in our laboratory by serial passage. Adult mice were injected subcutaneously with 10^6 ascitic fibrosarcoma cells per mouse in 0.2 ml PBS for induction of solid tumour. Mice bearing tumour of an average size of 1.4 cm^2 were used for the experiments.

Cyclophosphamide (Cy) (Sigma) at a dose of 25 mg/kg body weight was injected in a mouse intraperitoneally as this dose was found to be effective in depleting the suppressor T cells *in vivo*[6,11]. After 48 h of cyclophosphamide treatment, concanavalin A (Con A, Type IV, Sigma Co., St. Louis, USA) at a dose of 50 µg/animal via intravenous route was injected for polyclonal stimulation of T lymphocytes as described earlier[5,12]. Spleen and lymph nodes were taken out aseptically from Cy and Con A treated syngeneic normal mice 48 h after Con A injection and cell suspension was prepared in phosphate buffered saline (PBS) following standard protocol.