



## MATERIALS AND METHODS

**Drug.** Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU were prepared as reported earlier from Chittaranjan National Cancer Institute (CNCI) [5–9]. It was characterized by <sup>1</sup>HNMR spectra in CDCl<sub>3</sub>. Purity of compound was checked by HPLC [Waters modular system, Millford, USA; (μ-bondapak C18 steel column, 30 cm×3.9 mm), isocratic mobile phase — methanol: water (v:v, 1:1) at a flow rate of 0.5 ml/min expressed in retention time (R.T.); UV detection at 254 nm; data analysis with Empower 1154 Software] at room temperature [5–9]. Anticancer drugs were purchased from Sigma-Aldrich Com. (USA).

**Mice, animal treatment and tumor cells.** Swiss albino male mice of about 6–8 weeks of age and weighting 24 ± 2 g were taken. Animals were randomly bred in the vivarium of CNCI housed in cage and maintained on standard mouse food and tap water ad libitum. All *in vivo* experiments were conducted following the guidelines of IAEC (Institutional Animal Ethical Committee) approved by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals). Physiological saline containing 2% Tween 20 (Sigma Inc., USA) was used for drug administration in respective doses through intraperitoneal (i.p.) route to each mouse in different schedules. The drug solutions were prepared daily just prior to the injection. The control groups received an equal volume of vehicle (0.2 ml) on those days. The number of death was monitored daily during the test. S-180, EC and Dalton's lymphoma (DL-60) cells initially obtained from National Centre for Cell Sciences (NCCS), Pune (India) were maintained by regular intraperitoneal passage in Swiss mice.

**In vitro screening in human and mouse tumor cell lines.** Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU were screened in human Jurkat (lymphoma) and mouse S-180, EC and Dalton Lymphoma-60 (DL-60) cell lines by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [10]. Drug stock solutions (20 mg/ml) were prepared in DMSO. These were serially 10-fold diluted with complete sterile RPMI-1640 medium prior to use to obtain different drug concentrations. Standard drugs as CCNU and 5-fluorouracil (5-FU) were also included. Cell suspension (100 μl) from the stock (2×10<sup>5</sup> cells per well) were added to 96-well cell culture plates and incubated with drug solutions (10 μl) of different concentrations for 48 hrs. All vehicle controls contained the same concentration of DMSO. After removal of 100 μl of media from each well, 10 μl of a 5 mg/ml solution of MTT in Dulbecco's PBS (GIBCO, BRL) were added and incubated for 4 hr at 37 °C. Subsequently, acid isopropanol solution (100 μl, 0.04 N HCl in isopropanol) was added to the wells to dissolve formed formazan crystals. Optical density was measured in a microplate reader (BioTek Instruments Inc., Vermont, USA) at 540 nm.

**Effect on blood mononuclear cells (MNC).** MNC was isolated from heparinized venous blood obtained from healthy mouse by Ficoll-Paque (His-

topaque 1077, Sigma Aldrich Inc., USA) density gradient centrifugation as per standard procedure [11]. MNCs (1×10<sup>5</sup> cells/well) were cultured in complete RPMI-1640 media as usual and incubated with Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU for 48 h followed by MTT assay [10].

**In vivo screening in solid mouse tumors.** Mice were inoculated subcutaneously in the hindquarter with 2×10<sup>6</sup> viable S-180 cells on day 0. Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU were administered in two schedules, either early treatment QD<sub>1-7</sub> or late treatment QD<sub>5-11</sub> on post tumor inoculation phase. In both schedules, drug solutions were administered at its optimum dose of 50 mg/kg dose for seven days continuously, keeping CCNU (50 mg/kg) as positive control. Growth of solid tumor was monitored weekly by caliper measurement and survival was noted daily. Tumor volume (in mm<sup>3</sup>) was calculated using the standard formula: (width<sup>2</sup> × length)/2 [12].

In a similar fashion, three nitrosoureas were tested in EC solid tumor model, inoculating 1×10<sup>7</sup> viable EC cells subcutaneously in the hindquarter on day 0. Then test drug (50 mg/kg) and positive control 5-FU were injected for 7 days. Percent tumor growth inhibition and mortality were calculated from the observed data.

**Apoptosis analysis.** Apoptosis was analysed using Annexin V — Propidium Iodide (PI) apoptosis detection kit (BD Pharmingen, San Diego, USA), according to manufacturer's protocol. S-180 cells in complete RPMI media (2 × 10<sup>5</sup> cells/100 μl) were seeded in six well plates. Nap-NU, Napro-NU, 5-NO<sub>2</sub>-Nap-NU and CCNU (10 μl in volume) in DMSO at different concentrations were added to wells followed by the addition of required media (total volume 1 ml). It was incubated for 24 h at 37 °C. The cells were centrifuged at room temperature at 1500 rpm for 5 min. The pellet was washed twice with cold PBS and was re-suspended in the binding buffer (1X, 100 μl) provided. The cells were stained with Annexin V-FITC (5 μl) and PI (5 μl) and incubated for 15 min in the dark at 25 °C. Finally, binding buffer (400 μl) was added to each tube and flow cytometrically analyzed (FACSCaliber, Becton Dickinson, Mountainview, CA) using Cell Quest software [13].

**Assessment of metabolic and immune toxicities.** The optimum dose of Nap-NU and 5-NO<sub>2</sub>-Nap-NU (50 mg/kg) were administered in normal and S-180 bearing mice from day 1 to day 7 (QD<sub>1-7</sub>), keeping one untreated group in each case. Livers and kidneys were collected from normal and sarcoma (untreated and treated) bearing mice for histological analysis. Tissues were fixed in 10% formal saline and paraffin sections were prepared for Hematoxylin and Eosin (H & E) staining. The histopathological changes were evaluated by bright field microscopy [12] and photographed.

Flow cytometric analysis for surface phenotypic markers of blood MNC was performed after purification of MNC from blood collected from S-180 solid tu-

mor bearing control and treated mice [both early treatment QD<sub>1-7</sub> or late treatment QD<sub>5-11</sub>] on day 27. MNCs (5×10<sup>5</sup> cells) were labeled with 20 µl of fluorescence labeled antibodies (CD4-FITC, CD8-PE and CD25-PE) for 30 min. After labeling, cells were washed in FACS buffer (PBS with 1% FBS), fixed in 1% paraformaldehyde in PBS, and cytometry was performed (FACS-Caliber, Becton Dickinson, Mountainview, CA) [14].

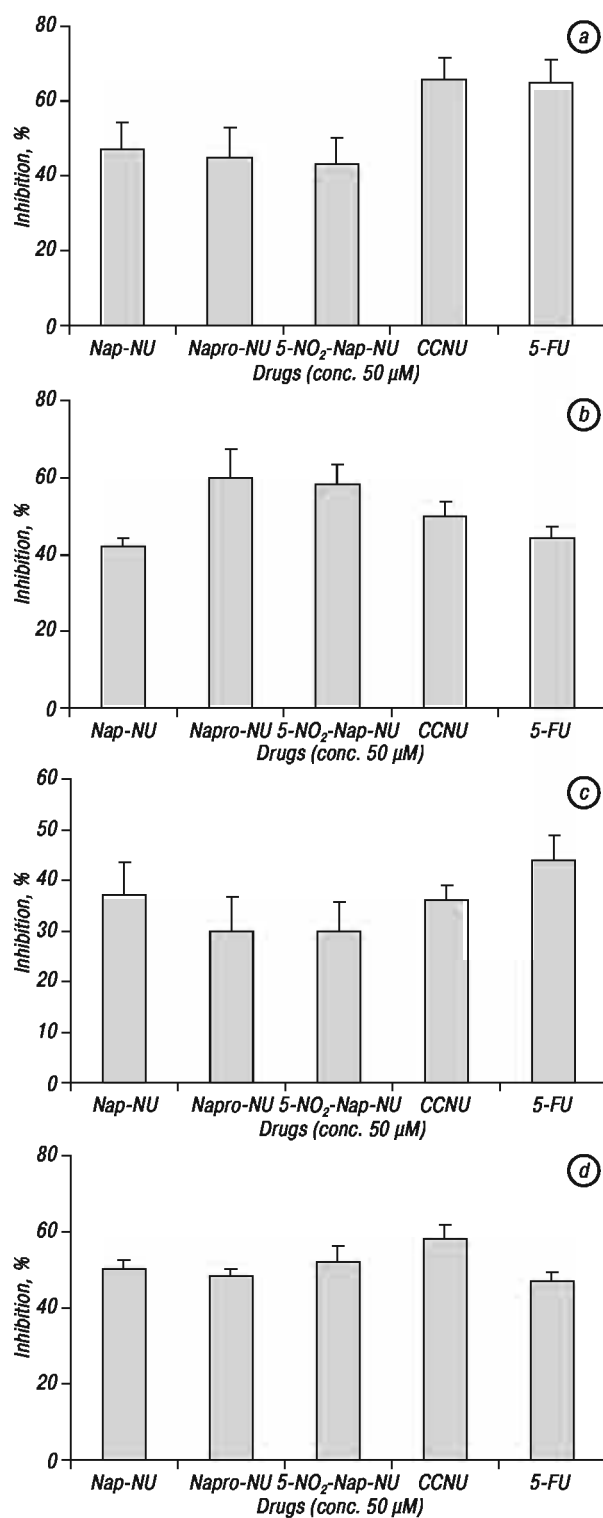
**RESULTS**

**Cytotoxicity screening in human and murine tumor cell lines.** Screening of Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU, in murine S-180, EC and DL-60 cell lines and in human Jurkat lymphoma by MTT assay revealed considerable tumor cell inhibition at different concentration (Fig. 2, a–d). In S-180 cell line the compounds showed cytotoxicity at 50 µM concentrations (Fig. 2, a). Three derivatives inhibited EC cells at 50 µM doses and that was more or less same in comparison to CCNU and 5-FU (Fig. 2, b). In DL-60 cell line percentage of inhibition by Nap-NU at 50 µM was almost equal compare to CCNU and in Jurkat lymphoma observed cytotoxicity by 5-NO<sub>2</sub>-Nap-NU was more than both CCNU and 5-FU (Fig. 2, c, d). Interestingly, the compounds have shown minimum cytotoxicity to MNCs for mouse blood at the same dose (data not shown).

**Restriction of solid sarcoma growth and increases survivability.** The growth responses in S-180 and EC solid tumors were assessed following drug treatment at the optimum dose (50 mg/kg for day 1–7). It was worthy to note that three compounds have significantly prolonged life span of S-180 tumor bearing mice with 3/6 animals having survival rates of > 100 days and also the tumor volume of treated mice was significantly reduced in comparison to control (Fig. 3, a). *In vivo* experiments in EC with this optimum dose furnished comparable results having 50% tumor growth inhibition (Table 1). It is interesting to note that tumor volume was significantly reduced and also 2 out of 6 mice were completely cured having > 80 days survivability in comparison to control group bearing highly advanced 5 days tumor (Fig. 3, b). It was also found that CCNU had exhibited highly significant anticancer activity in these tumor systems having significant tumor growth inhibition (Fig. 3, a, b). However, survivability was observed to be less than test compounds, possibly imparting some degree of toxicity. 5-FU was simultaneously assessed in EC having 50% tumor growth inhibition (see Table 1).

**Table 1.** *In vivo* anti-tumoral screening of Nap-NU, Napro-NU and 5NO<sub>2</sub>-ap-NU in EAC solid tumor

Treatment Groups	Average body weight, g				Average tumor weight on 13 <sup>th</sup> day, mg	Tumor growth inhibition, %	Mortality
	1	5	9	13			
Nap-NU	24.57	24.00	22.42	22.14	565.42	49.60	–
Napro-NU	24.85	24.14	23.00	21.85	654.88	41.63	–
5-NO <sub>2</sub> -Nap-NU	25.00	23.00	20.42	20.50	800.64	28.64	–
5-FU	25.14	24.14	22.85	22.17	531.34	52.64	0/7
Untreated	24.80	25.74	26.10	28.33	1122.00	–	0/10

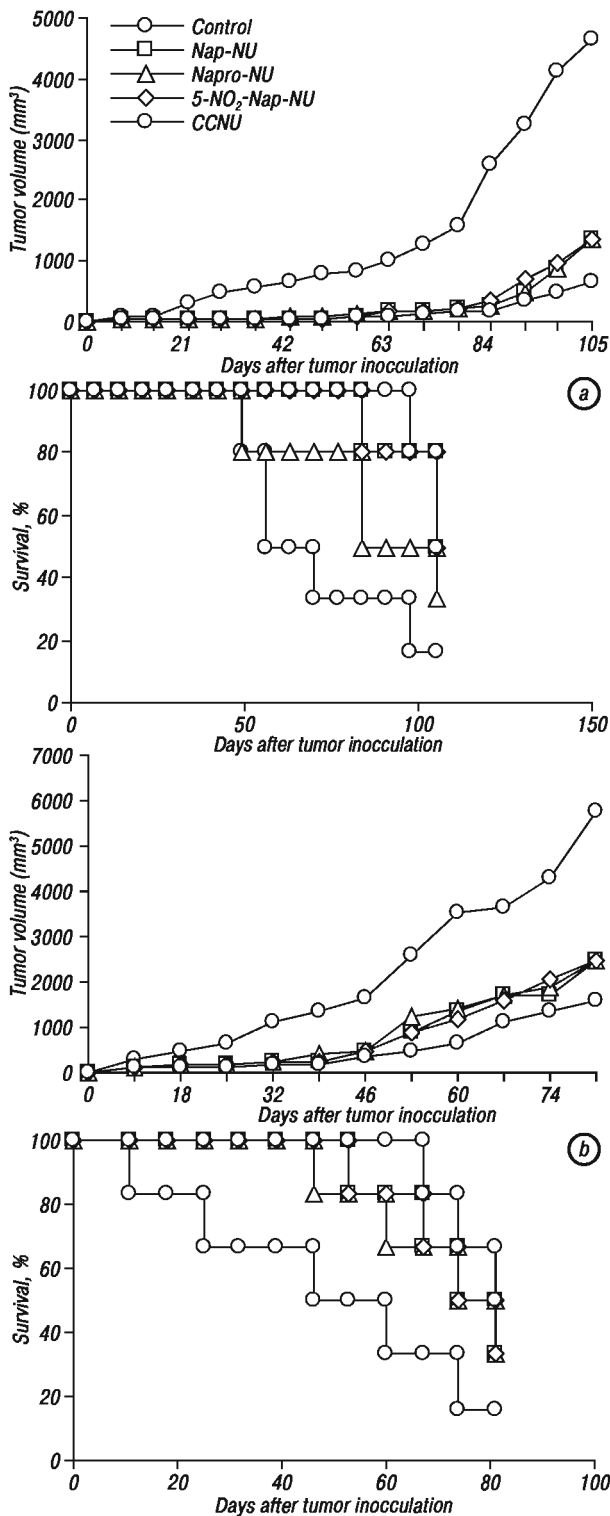


**Fig. 2.** *In vitro* cytotoxic efficacy of Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU against tumor cell lines by MTT assay; a — mouse sarcoma cells (S-180); b — mouse carcinoma cells (EC); c — mouse lymphoma cell (DL-60); d — T cell lymphoma (Jurkat) cell lines. Cells were seeded at 2×10<sup>5</sup> cells per well in 96-well cell culture plates and incubated with Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU solutions for 48 h. Each point represents M ± SD of three separate experiments (p < 0.05).

**Cytotoxicity to cancer cells by inducing apoptosis.**

S-180 cells were treated with Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU, stained with annexin V–FITC/PI. It was apparent from Fig. 4, a that the number of late apoptotic cells (AnnV<sup>+</sup>PI<sup>+</sup>) was higher in the case of sarcoma cells

treated with Nap-NU (10.61% at 50  $\mu\text{M}$ ) in comparison to CCNU (6.79%). In view of Fig 4, b, c in comparison to standard drugs CCNU, at 50  $\mu\text{M}$  the number of late apoptotic cells (AnnV<sup>+</sup>PI<sup>+</sup>) were higher in Napro-NU (30.54%) and 5-NO<sub>2</sub>-Nap-NU (12.76%).



**Fig. 3.** *In vivo* anti-tumoral screening of Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU in S-180 solid tumor in QD<sub>1-7</sub> (a) or late treatment QD<sub>5-11</sub> (b). Mice were inoculated subcutaneously with  $2 \times 10^6$  viable sarcoma cells on day 0. The derivatives were administered in treatment schedules QD<sub>1-7</sub> (a) and QD<sub>5-11</sub> (b) on post tumor inoculation phase. Tumor volume (mm<sup>3</sup>) was calculated using the standard formula: (width<sup>2</sup> × length)/2.  $p < 0.01$  obtained from three experiments

### Histopathological analysis of liver and kidney

#### Histology of liver. Treatment with Nap-NU.

Normal control (NC) group: histology of liver of normal mouse showed normal architecture having a distinct central vein within a lobule and a portal area consisting of interlobular branch of portal vein, bile duct, branch of a hepatic artery and interlobular septum (Fig. 5, a).

S-180 control (SC) group: Histology of liver of SC mouse (14<sup>th</sup> day) showed hyperactive Kupffer cells and lymphocyte infiltration in areas adjacent to central vein (Fig. 5, e-g).

Normal mice treated with Nap-NU (NT) group and S-180 mice treated with Nap-NU (ST) group: It was observed that liver sections of mice in these groups showed few inflammatory cells and hyperactive Kupffer cells on the day 9<sup>th</sup> (Fig. 5, b, h). On the day 14<sup>th</sup>, focal necrosis was observed at some region in both the groups (Fig. 5, c, i). In ST group, infiltration of lymphocytes was observed in lower numbers relative to SC mice (Fig. 5, h-j). Also the activation of Kupffer cells was enhanced compared to the day 9<sup>th</sup>. On the day 19<sup>th</sup>, a large number of regenerated hepatocytes were observed on the liver parenchyma. Infiltration of lymphocytes in both the treated groups was minimum and Kupffer cell activity was normal (Fig. 5, d, j).

Treatment with 5-NO<sub>2</sub>-Nap-NU. NC group: histology of liver of NC mouse showed normal architecture having a distinct central vein within a lobule and a portal area consisting of interlobular branch of portal vein, bile duct, branch of a hepatic artery and interlobular septum.

SC group: Histology of liver of SC mouse (14<sup>th</sup> day) showed hyperactive Kupffer cells and lymphocyte infiltration in areas adjacent to central vein.

Photomicrographs of liver of ST group of mice treated with 5-NO<sub>2</sub>-Nap-NU showed normal histology (data not shown).

Hepatotoxicity resulting from administration of a single dose of CCNU was studied by weekly determinations of liver function tests and performance of precutaneous liver biopsies [15].

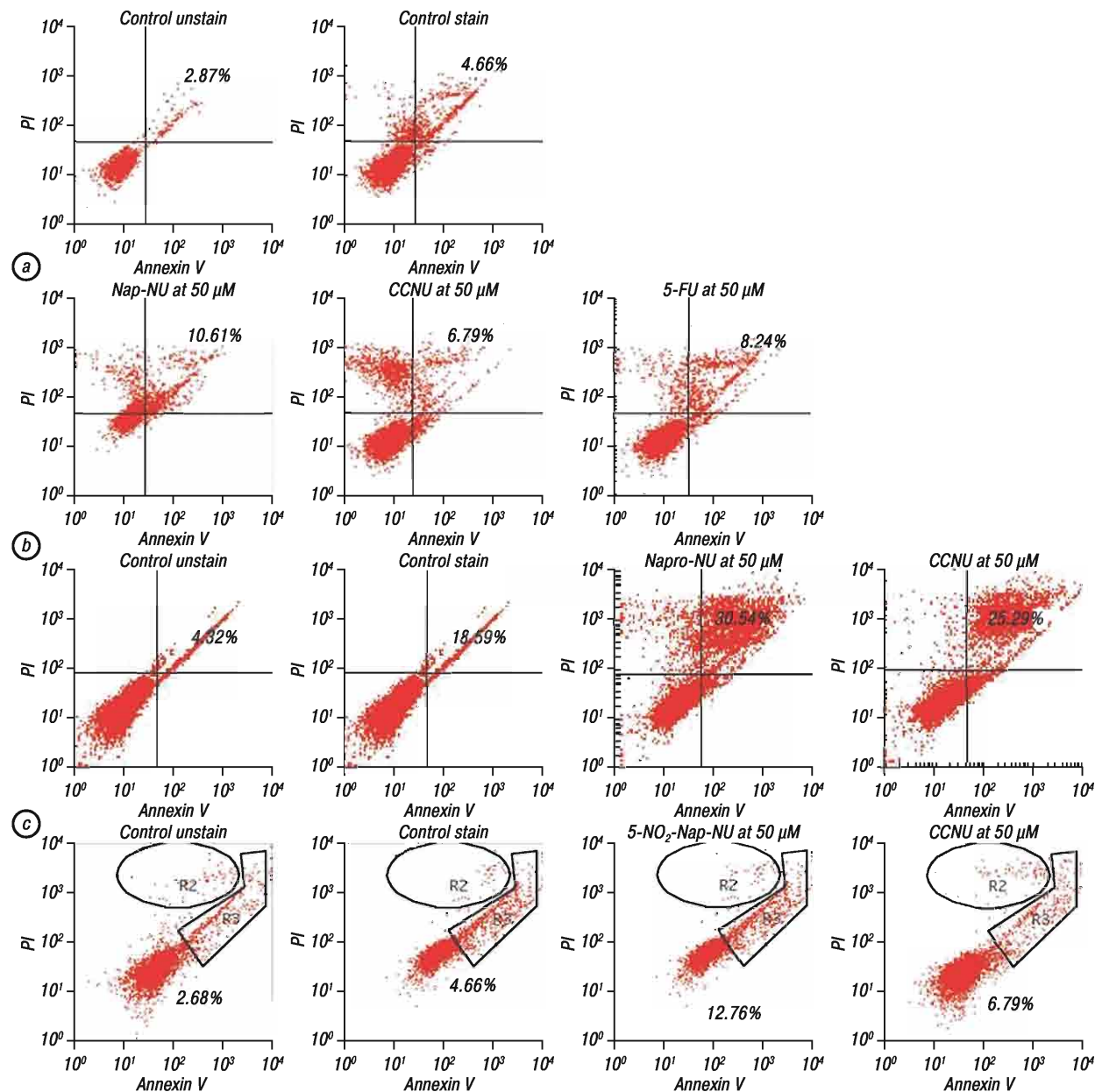
#### Histology of kidney. Treatment with Nap-NU.

In NC group renal cortex contains glomeruli, blood vessels, tubules and interstitium. In the cortex but not the medulla, the tubules are almost back to back, i.e., the tubular basement membranes are almost touching (Fig. 5, k). Photomicrographs of kidney of NC, NT and ST mice showed normal histology (Fig. 5, l-q). The cortex contained well-disposed renal corpuscles (i.e., intact Bowman's capsule and glomerulus), convoluted tubules and medullary rays. The inner medulla region contained the Henle's loop and the collecting tubules (Fig. 5, l-q).

Treatment with 5-NO<sub>2</sub>-Nap-NU. Photomicrographs of kidney of SC, ST and NT groups of mice treated with 5-NO<sub>2</sub>-Nap-NU showed normal histology (data not shown).

CCNU nephrotoxicity have been reported in cancer patients having astrocytoma of the brain [16].

**Non-cytotoxic to normal blood lymphocytes, including T cells.** It was of interest to know the effect of Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU on circulating T lymphocytes when drug was administered *in vivo*



**Fig. 4.** Analysis of apoptosis induced by Nap-NU (a), Napro-NU (b) and 5-NO<sub>2</sub>-Nap-NU (c) in sarcoma cells (2×10<sup>5</sup> cells/100 μl) by flow cytometry using annexin V-FITC and PI. Quadrant analysis of fluorescence intensity of gated cells in FL-1 (annexin V-FITC) and FL-2 (PI) channels. Three NU's at 50 μM induces apoptosis of sarcoma cells in greater extent than CCNU

in sarcoma bearing mice. It was apparent from Fig. 6 that Nap-NU had no adverse effect on CD4<sup>+</sup> and CD8<sup>+</sup> T cells. On the other hand, percentage of CD4<sup>+</sup> CD25<sup>+</sup> suppressor regulatory cells was decreased after drug treatment, though it was not statistically significant (Fig. 6, a–f). In CCNU treated mice no such stimulatory effect was observed (data not shown). Napro-NU and 5-NO<sub>2</sub>-Nap-NU also showed immunostimulatory functions (Table 2).

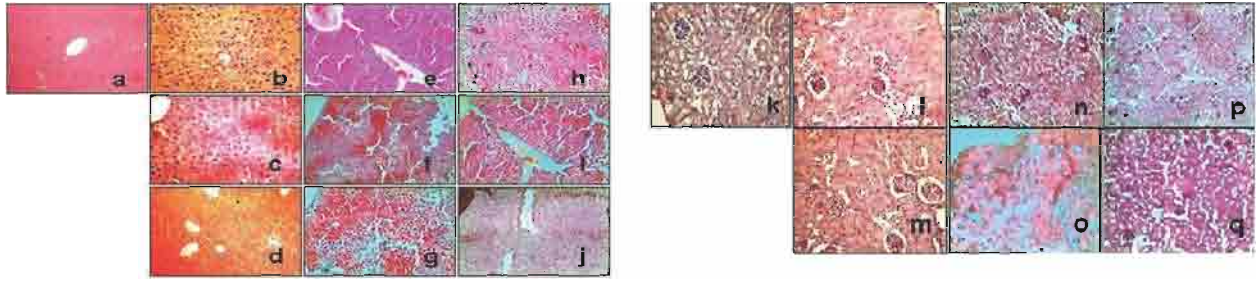
**Table 2.** Expression status of CD4<sup>+</sup>, CD8<sup>+</sup> and CD25<sup>+</sup> on MNC of Napro-NU and 5-NO<sub>2</sub>-Nap-NU

Treatment day	Cell Types	Positive cells, %				
		Unstain	Control	Napro-NU	5-NO <sub>2</sub> -Nap-NU	CCNU
1–7	CD4 <sup>+</sup>	0.70	42.46	45.01	48.51	50.04
	CD8 <sup>+</sup>	0.38	20.42	21.32	22.03	22.10
	CD4 <sup>+</sup> CD25 <sup>+</sup>	0.06	21.22	22.52	18.36	20.56
5–11	CD4 <sup>+</sup>	0.00	10.05	12.14	12.57	15.07
	CD8 <sup>+</sup>	1.84	26.61	30.11	33.12	30.10
	CD4 <sup>+</sup> CD25 <sup>+</sup>	0.00	11.20	12.37	12.81	13.94

## DISCUSSION

In our effort to develop new nitrosourea compounds, we have developed a series of compounds and assessed their antitumor activity [7–9, 17, 18]. Among them Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU were examined in *in vitro* as well as *in vivo* for their tumor lytic effects, and most promising results, in comparison to standard chemotherapeutic agents (CCNU, 5-FU), have been observed.

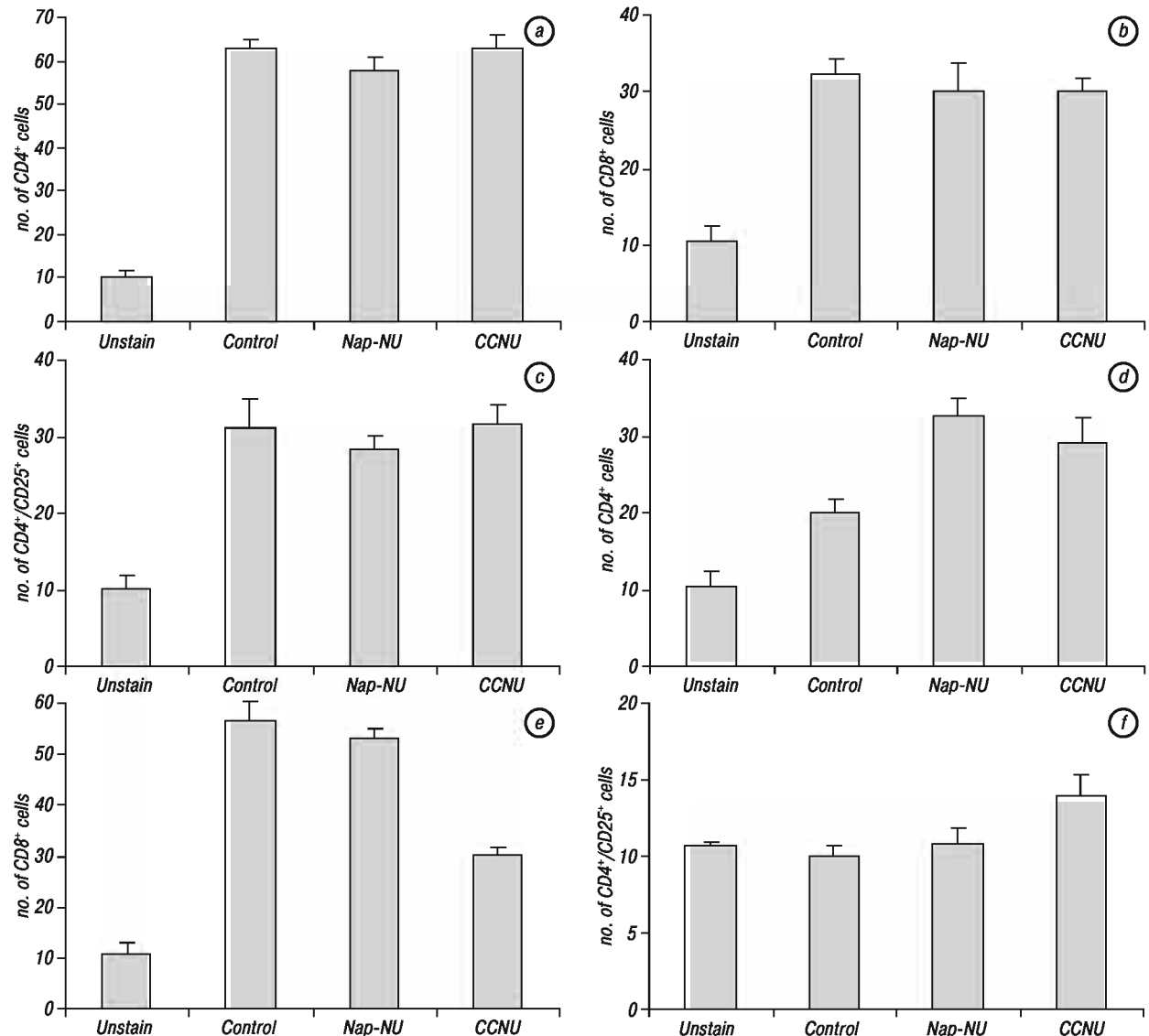
Examination of cytotoxic potential of above three NU's in different human and mouse cancer cell line revealed their considerable cytotoxicity comparable to CCNU and 5-FU. However, in SNB-78, HOP-62, HL-60 and U-937 cells, the compounds showed greater cytotoxicity than standard chemotherapeutic drugs as determined by SRB and MTT assays. *In vivo* toxicological assay conducted in normal and S-180 bearing mice revealed that these are well tol-



**Fig. 5.** Histological features of liver and kidney after Nap-NU treatment in mice. Nap-NU (50.0 mg/kg) was administered in normal and S-180 bearing mice from day 1–7<sup>th</sup>. After the completion of the treatment, animals were sacrificed on the day 9<sup>th</sup>, 14<sup>th</sup> & 19<sup>th</sup>. Liver and kidney were collected to be analyzed histopathologically. Photomicrograph of H & E stained section of liver: (a) Normal control (NC) mouse, (b–d) Normal treated (NT) mouse, (e–g) S-180 control (SC) mouse, (h–j) S-180 treated (ST) mouse at the day 9<sup>th</sup>, 14<sup>th</sup>, 19<sup>th</sup> ( $\times 200$ ). Kidney: (k) Normal control (NC) mouse, (l–m) Normal treated (NT) mouse, (n–o) S-180 control (SC) mouse, (p–q) S-180 treated (ST) mouse at the day 9<sup>th</sup>, 14<sup>th</sup> ( $\times 200$ )

rated in mice and did not adversely affect hematopoiesis at their optimum doses. CCNU produced delayed and cumulative bone marrow depression, which significantly limits its chemical usefulness [19]. In the light of those promising results, presently the experimen-

tion was extended to two solid tumor models. It was found that Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU induced significant retardation of these tumors grown in Swiss mice. The compounds not only restricted the early growth, they also effectively inhibited the tu-



**Fig. 6.** Assessment of immune toxicities of Nap-NU in S-180 solid tumor. Mice were inoculated subcutaneously with  $2 \times 10^6$  viable sarcoma cells on day 0. Nap-NU was administrated in QD<sub>1-7</sub> (a–c) or QD<sub>5-11</sub> (d–f) schedules after tumor inoculation. After the purification of MNC, blood was collected from both groups. MNCs ( $5 \times 10^5$  cells) were labeled with 20  $\mu$ l of fluorescence labeled antibodies (CD4-FITC, CD8-PE and CD25-PE) for 30 min. Nap-NU maintained CD4<sup>+</sup>, CD8<sup>+</sup> T cell and CD4<sup>+</sup>CD25<sup>+</sup> Treg cell status in Sarcoma bearing mice. Data are presented as the means  $\pm$  SD

mor growth in advanced state. Interestingly, life span of Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU treated S-180 bearing mice was more than that observed in case of CCNU. Based on our early experiment *in vivo* system it can be realized that hematotoxic and nephrotoxic effect of these tested compounds have been less than CCNU. This is possibly reason of greater life span of NU's treated mice than those received CCNU. It also induced apoptosis of sarcoma cells to a greater extent than CCNU. This observation helped to understand the efficacy of these derivatives to kill the tumor cells. Tumor burden itself alters normal physiology that was reflected in the histology of livers and kidneys of tumor host. After chemotherapy the toxic effect like unfavorable changes in hematological parameters, bone-marrow suppression, etc., observed sometimes. In continuation to earlier report of minimum toxicity of these NU compounds, here, we have examined whether they have any toxic effect that could be demonstrated by histopathological analysis. These NU compounds helped to normalize the tumor induced alteration of liver and kidney architecture and such normalizing effect is more prominent than CCNU. Moreover, in normal mice, the compounds have shown no effect on normal liver and kidney histology. *In vivo* effect of the compounds on liver enzymes and different kidney components were studied before [7–9] and no significant abnormalities were noted. Study was also extended to find its effect on important immune cells, i.e., T lymphocytes. It had no cytotoxic effect on CD4<sup>+</sup> and CD8<sup>+</sup> T cells; interestingly it regulated suppressor Treg cells. This observation suggested that Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU helped to maintain the normal immune functions of the host. Thus, this group of drugs might act perfectly and clinical outcome would be facilitated. The, antimetastatic activity of the compounds will be tested in future.

Thus, data obtained from this study holds promise of Nap-NU, Napro-NU and 5-NO<sub>2</sub>-Nap-NU as anticancer agent for treating various types of tumors. Further preclinical study is pre-requisite to move forward with this promising drug to the clinic.

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