Anti-Cancer Drugs, 2, pp. 285–288

Anti-tumor effect of 3-amino-*N*-substituted-pyrrolidine-2,5-dione-*N*-mustard hydrochloride

MA Indap, SD Naik and RY Ambaye[CA]
Bio-Organic Unit, Cancer Research Institute, Tata Memorial Centre, Parel, Bombay-400012, India

The anti-tumor effect of 3-amino-*N*-substituted pyrrolidine-2,5-dione-*N*-mustard hydrochloride (PNM.HCl) against Ehrlich (ascites) carcinoma (EAC) was studied. A substantial increase in the survival of mice bearing EAC tumor was achieved following daily administration of PNM.HCl at subtoxic dosages. The therapeutic efficacy of PNM.HCl was maintained with changes in dosages and the schedules of administration. The effect of PNM.HCl when administered with conventional anti-cancer drugs atdifferent time schedules against EAC was also studied. The results demonstrated an augmentation of anti-tumor activity in the case of certain anti-cancer drugs against EAC tumor, thereby suggesting a potential usefulness of PNM.HCl in multi-drug therapy.

Key words: 3-Amino-*N*-substituted pyrrolidine-2,5-dione-*N*-mustard hydrochloride, anti-cancer drugs, anti-tumor effect, augmentation, Ehrlich (ascites) carcinoma.

Introduction

Synthesis of nitrogen mustard analogs of 3-amino-*N*-substituted pyrrolidine-2,5-dione yielded several promising anti-tumor compounds. Of these, (R,S)3-{*N*,*N*-[bis-(2-chloroethyl)]-amino}-1-(2-methoxyphenyl)-2,5-dione hydrochloride (PNM.HCl) has shown potent anti-cancer activity against P388 and L1210 leukemias and sarcoma 180.[1–3] Further, the cytotoxic activity of the conventional anti-cancer drugs was modulated by co-administration with PNM.HCl and its intermediate(s) against these murine tumors.[4–6] The present work assesses the anti-tumor effect of PNM.HCl and its influence on the chemotherapy of Ehrlich (ascites) carcinoma.

Materials and methods

Chemicals and drugs

Synthesis of 3-amino-*N*-substituted pyrrolidine-2,5-dione-*N*-mustard hydrochloride (PNM.HCl) has been previously reported.[1] Anti-cancer drugs such as methotrexate (MTX), 5-fluorouracil (5-FU), cyclophosphamide (CTX), 6-mercaptopurine (6-MP), cytosinearabinoside (Ara-C), mitomycin (Mit-C), vinblastine (VLB) and mechlorethane (HN2) were obtained from commercial sources and their corresponding LD_{10} doses were used.[4] The test solutions were freshly prepared either in suspension form (0.2% carboxymethylcellulose) or in normal saline.

Animals and tumor transplantation

Ehrlich (ascites) carcinoma (EAC) was maintained by propagation in Swiss (Webster) mice by weekly intraperitoneal (ip) passages of ascitic fluid from a 7-day-old EAC tumor.[7] Swiss mice, 6–8 weeks old and weighing 20–25 g, were used in these experiments. Tumor cells were harvested from the peritoneal cavity of a 7-day-old ascitic tumor under aseptic conditions. Each recipient received 2×10^7 cells by the ip route.

The animals were fed a normal colony diet, composition: cracked wheat 70%, cracked Bengal gram 20%, yeast powder 4%, fish-meal 5%, shark-liver oil 0.25% and sesame oil 0.75%, and were given water *ad libitum*.

Anti-tumor treatment protocol and evaluation

After tumor transplantation, the animals were randomized to ascertain if the weight of an individual animal varied less than 7.5% from the mean weight of all the animals. The administration of PNM.HCl or anti-cancer drug singly, and PNM.HCl with conventional anti-cancer drug, was initiated 24 h after tumor transplantation. In the first experiment, in combined therapy PNM.HCl (100 mg/kg) was simultaneously administered with

[CA] Corresponding Author

© *1991 Rapid Communications of Oxford Ltd.*

the anti-cancer drug on days 1, 5 and 9 by the ip route. In the second experiment, PNM.HCl (100 mg/kg) was administered ip to the tumor-bearing mice on days 1, 5 and 9, which was followed by anti-cancer drug on days 2, 6 and 10 (keeping a gap of 24 h between the two drugs). In the third experiment, the anti-cancer drug was given to the tumor-bearing mice on days 1, 5 and 9, and it was followed by PNM.HCl (100 mg/kg) on days 2, 6 and 10 with a gap of 24 h.

The controls received the vehicle 0.2% carboxymethylcellulose (w/v) or distilled water/N.saline by the same route. The body weights of both control and treated animals were recorded on days 1 and 5 to determine the toxicity, if any. The median survival time (MST) of animals treated with PNM.HCl alone or with PNM.HCl and anti-cancer drug administered using different schedules and dosages were compared with each other.

The efficacy of treatment was determined on the basis of increase in the survival time of the treated group (T) as compared to that of the control group (C) using the following expression:

$$\frac{\text{Median survival time (days) of treated animals } (T)}{\text{Median survival time (days) of control animals } (C)} \times 100$$

$T/C\% \geqq 125$ indicates anti-tumor activity.[7]

Statistical analysis

The statistical significance of differences between the combined treatment group and the anti-cancer drug-treated group was calculated by Student's t-test and expressed as p values.

Results

The anti-tumor effect of PNM.HCl against EAC tumor is shown in Table 1. An impressive activity has been observed with a dose of 100 mg/kg given ip for 9 consecutive days to the mice bearing EAC tumor. The survival period of mice inoculated with 2×10^7 EAC tumor cells was prolonged considerably ($T/C\% = 642$). The higher activity was also obtained with the doses of 75 mg/kg and 50 mg/kg using the same route of administration and schedule. A lower dose of PNM.HCl (25 mg/kg × 9) also exhibited fairly good anti-tumor activity against EAC tumor (Table 1).

Additional studies were performed on mice bearing EAC tumors using different routes of administration and schedules. For all ip schedules evaluated, PNM.HCl reduced the tumor burden at the end of treatment. Intravenous administration of PNM.HCl was not successful because of its lack of solubility in standard vehicles; oral administration did not have effect probably because of breakdown into inactive metabolic products.

Table 1. Anti-tumor effect of PNM.HCl against Ehrlich (ascites) carcinoma

Dose[a] (mg/kg × 9) ip	Av body wt diff[b] ($T - C$)	Median survival time (MST) (days)	$T/C\%$[c]
Control	—	14	—
100	−3.1	90 (5)[d]	642[e]
75	−2.0	90 (4)[d]	642[e]
50	+0.4	56 (2)[d]	400[e]
25	+1.3	24.5	175[e]

[a] Dose of PNM.HCl given to tumor-bearing mice on days 1–9 by ip route.
[b] Difference between average body weight change in treated (T) and control (C) animals on day 5.
[c] $\frac{\text{MST of treated animals } (T)}{\text{MST of control animals } (C)} \times 100$.
[d] Parentheses: number of survivors.
[e] $p < 0.001$.

The feasibility of combining PNM.HCl with established anti-cancer drugs and their administration using different schedules was studied. We observed that the combination of PNM.HCl with 6-MP, 5-FU and CTX definitely showed enhancement in their cytotoxic potentiality, the maximum being observed with 5-FU (Table 2).

Discussion

The present study highlights an impressive anti-tumor effectiveness of PNM.HCl.

Improvement of the therapeutic activity of conventional anti-cancer drugs could be achieved by suitable combination therapy or by association with modulating agents or with other therapeutic modalities such as irradiation, immunotherapy or hyperthermia.[6] The ability of PNM.HCl to kill EAC tumor cells *in vivo* appears to be a major rationale for combining PNM.HCl with conventional anti-cancer drugs on the premise that PNM.HCl would make the tumor more responsive *in vivo* to chemotherapeutic drugs.

Our results show: (i) consistent anti-tumor activity maintained by systemic administration of PNM.HCl even at low doses; (ii) enhancement in

Table 2. Influence of the combination of PNM.HCl and anti-cancer drugs on the chemotherapy of Ehrlich (ascites) carcinoma

Drug	Dose (mg/kg × 3) ip	Treatment[a]			Treatment[b]		
		Av body wt change (g)[c]	Median survival time (MST)	*T/C*%	Av body wt change (g)	Median survival time (MST)	*T/C*%[d]
Control	—	—	14.5	—	—	15	—
PNM.HCl	50	+0.2	31.5	217	+0.4	26.5	177
6-MP	100	−1.1	15	103	−0.5	15.5	103
6-MP + PNM.HCl	100 + 50	−2.1	39	269[e]	−1.2	29	193[e]
5-FU	20	+0.3	19	137	+0.9	25	167
5-FU + PNM.HCl	20 + 50	−0.1	90	620[e]	−0.7	90	600[e]
CTX	50	+0.1	27.5	189	+0.2	30.5	203
CTX + PNM.HCl	50 + 50	−0.8	48.5	334[e]	−0.9	51	340[e]

[a] PNM.HCl on days 1, 5 and 9 followed by anti-cancer drugs on days 2, 6 and 10.
[b] Anti-cancer drug on days 1, 5 and 9 followed by PNM.HCl on days 2, 6 and 10.
[c] Difference between average body weight change in treated (*T*) and control (*C*) animals on day 5.
[d] *T/C*% ≧ 125 indicates anti-tumor activity.
[e] Statistical significance, $p < 0.001$.

the activity of certain anti-cancer drugs, particularly 5-FU, by PNM.HCl; (iii) superadditive action as a result of independent antitumor action of two agents without interaction between them; and (iv) no increased toxicity observed in any of the combinations, suggesting a reasonable candidate for multi-drug therapy.

The findings also provide evidence for the improved treatment of EAC tumor using anti-cancer drugs with the administration of investigational agents like PNM.HCl. This is evident in the case of 6-MP, 5-FU and CTX with a highly pronounced effect in case of 5-FU. Several possible explanations for this enhanced phenomenon in case of 5-FU could be put forward. 5-FU exerts its cytotoxicity through 5-fluoro-2′-deoxyuridine-5-monophosphate (5-FdUMP) which inhibits DNA synthesis by blocking thymidylate synthetase (TS) and/or through its incorporation into RNA via 5-fluorouridine.[6,8] The anti-tumor effect is mediated through base pair transformation and changes in RNA structure processing and modification.[8] The increase in the intracellular level of thymidine triphosphate (dTTP) inhibits thymidine kinase[9] and ribonucleoside diphosphate reductase.[10] Both these enzymes act to increase the level of 5-FdUMP, thus allowing more of 5-FU to be incorporated in RNA. The proportion of nucleotides from the 'salvage' metabolic pathways could be increased using different agents that block steps in the endogenous pathways.[11] The production of an active metabolite could be raised by increasing the availability of the necessary substrate or cofactor.

The cyclic imide moiety in PNM.HCl may selectively manipulate intratumoral biochemical mechanisms to amplify the tumor cytotoxicity of 5-FU by inhibiting the enzyme levels concerned in metabolic pathways in pyrimidine biosynthesis.[6] The enhancement of 5-FU with administration of PNM.HCl might be due to the increased uptake of 5-FU into tumor cells.[12] PNM.HCl might have modulated the permeability of the cell membranes of the EAC tumor or stimulated the antitumor immunity, suggesting that other mechanisms should also be considered.[13]

In conclusion it may be said that PNM.HCl produced significant anti-tumor effect in mice bearing EAC tumor indicating that there is a potential usefulness for PNM.HCl in multi-drug combination therapy.

Acknowledgments

The authors wish to thank Mr M. G. Nirmalkar for technical assistance during the work and Ms Premlata M. Kotenkar for preparing the typescript.

References

1. Naik SD, Ambaye RY, Gokhale SV. Anticancer activity of new 3-amino-pyrrolidinedione nitrogen mustard derivatives on murine Sarcoma 180. *Anticancer Res* 1987; 7: 87–90.
2. Naik SD, Ambaye RY, Gokhale SV. Antitumour effect of α-amino-N-substituted pyrrolidino-2,5-dione-N-mustards

on the growth of P388 lymphocyte leukaemias. *J Cancer Res Clin Oncol* 1987; **113**: 151–154.
3. Naik SD, Khadse BG, Ambaye RY. Synthesis and anticancer activity of pyrrolidin-2,5-dione-N-mustards. *Med Sci Res (Biochem)* 1987; **15**: 27–28.
4. Ambaye RY, Indap MA, Naik SD. Enhanced activity of anticancer drugs in murine tumours by co-administration with 3-amino-pyrrolidine-2,5-dione-N-mustard derivative. *J Cancer Res Clin Oncol* 1989; **115**: 379–382.
5. Ambaye RY, Indap MA, Naik SD. Modulating effect of α-[N,N-[bis(2-hydroxyethyl)]-amino-N-(o-methoxyphenyl-pyrrolidin-2,5-dione on the chemotherapy of murine tumour. *Cancer Letters*, 1990; **51**: 119–125.
6. Ambaye RY, Indap MA. Influence of N-(o-methoxyphenyl-maleimide) on 5-fluorouracil cytotoxicity in Ehrlich (ascites) carcinoma. *Cancer Letters* (in press).
7. Geran RI, Greenberg NH, MacDonald MM, *et al.* Protocols for screening chemical agents and natural products against animal tumours and other biological systems. *Cancer Chemother Rep*, 1972; **Part 3(2)**: 1–103.
8. Ardalan B, Glazer R. An update on the biochemistry of 5-fluorouracil. *Cancer Treat Rev*, 1981; **8**: 157–167.
9. Finan PJ, Koklitis PA, Chisholm EM, *et al.* Comparative levels of tissue enzymes concerned in the early metabolism of 5-fluorouracil in normal and malignant human colorectal tissue. *Br J Cancer*, 1984; **50**: 711–715.
10. Jato J, Windhenser JJ. 5-Fluorouracil and derivatives in cancer chemotherapy III: *In vivo* enhancement of antitumour activity of 5-fluorouracil (5-FU) and 5-fluoro-2′-deoxyuridine (FUDR). *J Pharm Sci* 1973; **62**: 1975–1978.
11. Spiegelman S, Sawyer R, Nayak R, *et al.* Improving the antitumour activity of 5-fluorouracil by increasing its incorporation into RNA via metabolic modulation. *Proc Natl Acad Sci (USA)* 1980; **77**: 4966–4970.
12. Zupi G, Marangolo M, Arancia G, *et al.* Modulation of the cytotoxic effect of 5-fluorouracil by N-methylformamide on human colon carcinoma cell line. *Cancer Res* 1988; **48**: 6193–6200.
13. Ning S, Hahn GM. Cytotoxicity of lonidamine alone and in combination with other drugs against murine RIF-1 and human HT 1080 cells *in vitro*. *Cancer Res* 1990; **50**: 7867–7870.

(Received 11 March 1991; accepted 13 March 1991)