ELSEVIER

International Journal of Pharmaceutics 222 (2001) 237–242

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Two glycine containing 2-chloroethylnitrosoureas—a comparative study on some physicochemical properties, in vivo antimelanomic effects and immunomodulatory properties

Antoaneta Zheleva [a,*], Spaska Stanilova [b], Zlatka Dobreva [b], Zhivko Zhelev [b]

[a] *Department of Chemistry and Biochemistry, Thracian University, Medical Faculty, 6000 Stara Zagora, Bulgaria*
[b] *Department of Molecular Biology and Immunology, Thracian University, Medical Faculty, 11 Armeiska Street, 6000 Stara Zagora, Bulgaria*

Received 4 July 2000; received in revised form 11 April 2001; accepted 27 April 2001

Abstract

Physicochemical properties such as alkylating and carbamoylating activity and in vivo antimelanomic effects against B16 melanoma of the spin labeled (nitroxyl free radical containing) glycine nitrosourea (SLCNUgly) and its nonlabeled analogue (ChCNUgly), synthesized in our laboratory are studied and compared to those of antitumour drug 3-cyclohexyl-1-(2-chloroethyl)-1-nitrosourea (CCNU). We have demonstrated that introducing of glycine moiety in the nitrosourea structure in practice does not affect either alkylating or carbamoylating activity. On the other hand replacement of cyclohexyl moiety in ChCNUgly structure with nitroxyl free radical leads to a decrease in carbamoylating activity and an increase in alkylating activity. Compound ChCNUgly showed in vivo a higher antimelanomic activity against B16 melanoma in comparison with CCNU and SLCNUgly. It completely inhibited B16 melanoma growth (TGI = 100%) at a dose 64.0 mg/kg. Moreover, we established that joint i.p. application in normal mice of SLCNUgly plus a new immunostimulator (C3bgp) formerly isolated in our laboratory led to a 75% restoration in immune function with respect to antibody production measured by Jerne hemolytic plaque assay. In contrast, no immunostimulation was found after joint application of C3bgp plus ChCNUgly or CCNU at the same experimental conditions. Based on these preliminary results, a possibility for developing of new combination immunochemotherapy schemes for treatment of human cancers is discussed.

Keywords: Spin labeled nitrosoureas; B16 melanoma; Immunostimulation; Nitroxyl free radical; C3bgp

1. Introduction

2-chloroethylnitrosoureas belong to the alkylating antitumour agents and some of them have

* Corresponding author. Fax: + 359-600-705.
E-mail address: stanilova@excite.com (A. Zheleva).

0378-5173/01/$ - see front matter
PII: S0378-5173(01)00714-1

found application for the treatment of human cancer, mainly lymphomas, gliomas, a few solid tumours and melanomas (Carter et al., 1988). At present, it is accepted that antitumour activity of the nitrosoureas is due to their alkylating property whereas the severe toxic effects such as myelossupression are due to the carbamoylating property (Kann, 1981). Clinical application of these drugs is limited because they show delayed and cumulative toxic effects, bone marrow suppression being prevalent and dose limiting (Green et al., 1981). Bearing in mind the following established facts: (1) L-amino acid participate in transport through cell membranes; (2) aminoxyl radicals act as a transport vehicle through cell membranes and in general possess low toxicity and are not mutagenic by themselves (Sosnovsky, 1992); (3) stable nitroxyl free radical 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl [TMPO] selective accumulates in hamster and mice melanoma tissues (Blagoeva et al., 1979), we synthesized a number of spin labeled (containing nitroxyl free radical) and non-labeled amino acid 2-chloroethylnitrosoureas. These nitrosoureas showed in vivo a high antimelanomic effect against B16 melanoma (Ilarionova et al., 1993a; Zheleva et al., 1996a,b). We also found a high in vivo antileukemic activity against L1210 lymphoid leukemia (Zheleva et al., 1995) and a good correlation between this activity and some biochemical properties for the spin labeled amino acid nitrosoureas (Ilarionova et al., 1993b). On the other hand numerous studies on the antitumour activity of various nitrosoureas and correlation of this activity with their action on the immune system of the host have been made. All these studies suggest that the outcome of the chemotherapy with nitrosoureas depends on the modulating action on the immune mechanism of the host in addition to the antitumour activity of the drugs (Clary et al., 1990). In our previous studies we reported isolation of a new glycoprotein factor from the parasitic plant *Cuscuta europea* that specifically binds to C3 complement component of the immune system (Zhelev et al., 1994). Using different immunological assays we have demonstrated that this glycoprotein (C3bgp) exhibits in vivo and in vitro strong immunomodulatory property with respect to the antibody production (Stanilova et al., 2000). Further, it was of interest to study how the replacement of the cyclohexyl moiety with the nitroxyl free radical in the amino acid nitrosourea structure would affect its in vitro alkylating and carbamoylating activity and in vivo antimelanomic effect and immunomodulating properties.

By this paper we present our comparative study on the alkylating and carbamoylating activity and in vivo antimelanomic effect against B16 melanoma of two formerly synthesized (in our laboratory) spin labeled and nonlabeled glycine containing nitrosoureas and also compared to those of the antitumour drug CCNU. We also described our preliminary results concerning immunomodulatory effect of the same nitrosoureas with respect to antibody production measured by Jerne hemolytic plaque assay and possibility C3bgp to be used as an immunostimulator after nitrosourea application in normal mice.

2. Experimental procedures

2.1. Animals

Mice, hybrid BDF1 (DBA/2xC57Bl/6) were obtained from the National Centre of Oncology, Sofia, Bulgaria and used for study antimelanomic effects of the tested nitrosoureas. BALB/c male mice (8–10 weeks old, bred in the animal facilities of Thracian University), with body weight 23–25 g were used for the study immunomodulatory properties of the nitrosoureas and C3bgp.

2.2. Drugs

Nitrosoureas, SLCNUgly and ChCNUgly (see Fig. 1) used in this study were synthesized by the procedures formerly reported (Zheleva et al., 1995, 1996b).

Antitumour nitrosourea drug CCNU was purchased from Bristol Myers Squibb. Co. Immunosuppressive drug cyclophosphamide (CY) was purchased from Sigma Chemical Co. C3bgp was isolated by affinity chromatography as previously described (Zhelev et al., 1994)

2.3. Physicochemical properties of the tested nitrosoureas

Alkylating activities of the studied nitrosoureas were determined according to Gadzheva et al. (1989). Alkylating activity was expressed through $A_{560} \times mM^{-1} \times h^{-1}$. Carbamoylating activities of the nitrosoureas were determined following the method of Gadzheva et al. (1997).

2.4. In vivo drug treatment

2.4.1. Antimelanomic effects of the tested nitrosoureas

In vivo antimelanomic effect against B16 melanoma was evaluated in accordance with routine methods (Geran et al., 1972) with slight modifications. That is: On day 0, mice (average weight, 18–22 g) were inoculated subcutaneously with 10% tumour cell suspension in saline in a volume of 0.5 ml. On day 3, various doses of compounds SLCNUgly, ChCNUgly and CCNU (see Table 2) were administered i.p. in a single injection in volume 0.01 ml per body weight, as 10% ethanol solutions in saline. The control group (22 mice) received only the same volume of 10% ethanol in saline. Six mice were used for each treated group. The antimelanomic effect was evaluated by comparing the weights of the tumours of the control animals to those of the treated mice.

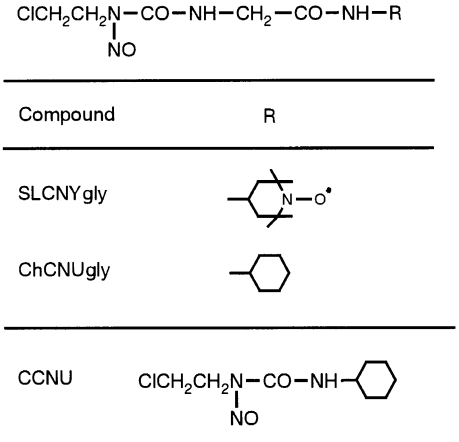

Fig. 1. Chemical s.tructures of SLCNUgly, ChCNUgly and antitumour drug CCNU

Table 1
Alkylating and carbamoylating activities of the studied nitrosoureas

Compounds (code)	Alkylating activity ($A_{560} \times mM^{-1} \times h^{-1}$)	Carbamoylating activity (%)
SLCNUgly	0.83	37.78
ChCNUgly	0.33	61.20
CCNU	0.34	62.68

The TGI parameter was calculated by the formula $[T_c - T_t/T_c] \times 100$ where T_c represents the weights of the tumours of the control mice and T_t represents the weights of the tumours of the treated mice.

2.4.2. Immunomodulatory properties of the tested compounds

For evaluation of immunomodulatory effect of the nitrosoureas an antigen stimulation has been performed using sheep red blood cells (SRBC). All studied mice in the control and tested groups (five in each group) were injected i.p. with 0.5 ml 10% suspension of SRBC in PBS. All studied compounds were i.p. injected 24 h before antigen challenge only once, alone or in combination with C3bgp in the mice of the corresponding group. All nitrosoureas have been applied at the doses they have shown the highest antimelanomic activity against B16 melanoma (see Table 2). CY was applied at a dose of 24 mg/kg. At this dose, 100% inhibition of antibody production was observed for CY (Stanilova et al., 2000). C3bgp was applied at a dose of 1.2 mg/kg. This dose exhibits the strongest immunostimulation (Stanilova et al., 2000). The immunomodulatory effect of the tested compounds on the number of antibody production cells was evaluated according to Jerne hemolytic plaque assay (Hudson and Hay, 1991).

3. Results and discussion

Results from alkylating and carbamoylating activity of the nitrosoureas are presented in Table 1. As is seen, SLCNUgly showed higher alkylating activity and lower carbamoylating activity in com-

parison with its nonlabeled analogue ChCNUgly and antitumour drug CCNU. Alkylating and carbamoylating activity of ChCNUgly were almost the same as those of CCNU. It is obvious that the introduction of glycine moiety in the nitrosourea structure in practice does not affect either alkylating or carbamoylating activity, while the replacement of cyclohexyl moiety with the nitroxyl free radical ones (see differences in the chemical structures of R in Fig. 1) leads to a decrease of carbamoylating activity and increase of alkylating activity.

Results from in vivo test against B16 melanoma of the nitrosoureas are presented in Table 2. As is seen compound ChCNUgly showed a higher antimelanomic effect against B16 melanoma than those of its spin labeled analogue SLCNUgly and CCNU. It completely inhibited B16 melanoma growth (TGI = 100%) at a dose of 64.0 mg/kg, while TGI value for SLCNUgly was 85.7% at a dose of 96.0 mg/kg and for CCNU 85.2% at a dose of 33.3 mg/kg. These results show that introducing of glycine moiety and its combination with cyclohexyl ones in the structure of ChCNUgly leads to a better antimelanomic effect when compared to those of CCNU and SLCNUgly

Results obtained by Jerne hemolytic plaque assay presented in Fig. 2 showed that all tested compounds exhibited an immunosuppressive effect after application alone. Complete immune suppression was found after application of ChCNUgly, CCNU or CY, while for the spin labeled compound SLCNUgly a 84% suppression of plaque forming cells (PFC) count was calculated. When animals were treated with 30 μg C3bgp plus CCNU or ChCNUgly the immune suppression on PFC count was retained. In contrast, after treatment with C3bgp plus CY or SLCNUgly, an 85% repaired number of hemolytic plaques compared to the control group was calculated for CY and 75% for SLCNUgly, respectively.

Results from in vivo test for evaluation of immunomodulatory effect of the tested nitrosoureas demonstrated that the replacement of the spin labeled moiety with cyclohexyl ones increased the immunosuppression with respect to antibody production measured by Jerne hemolytic plaque assay.

In vivo CY possesses alkylating property (Hoon et al., 1990), while 2-chloroethylnitrosoureas exhibit not only alkylating but carbamoylating property, as well (Montgomery et al., 1967). Bearing in mind these last mentioned properties, the well expressed immunostimulation after treatment with C3bgp plus CY and full lack of immunostimulation after C3bgp application together with CCNU or ChCNUgly, we suppose that the mechanism of nitrosourea immunosuppression at our experimental conditions is due not only to alkylating but also to their carbamoylating activ-

Table 2
[a]Antimelanomic effects of the nitrosoureas against B16 melanoma in BDF1 mice

Compounds (code)	Dose (mg/kg)	TGI (%)	Number of animals (deaths/total)
ChCNUgly	28.4	29.0	0/6
	42.7	83.9	0/6
	64.0*	100.0	0/6
	80.0	100.0	1/6
SLCNUgly	61.4	48.0	0/6
	76.8	52.0	0/6
	96.0*	85.7	0/6
	120.0	85.7	0/6
	150.0	–	6/6
CCNU	22.2	63.0	0/6
	33.3*	85.2	2/6
	50.0	85.2	2/6

[a] At these doses immunodulatory properties were studied.

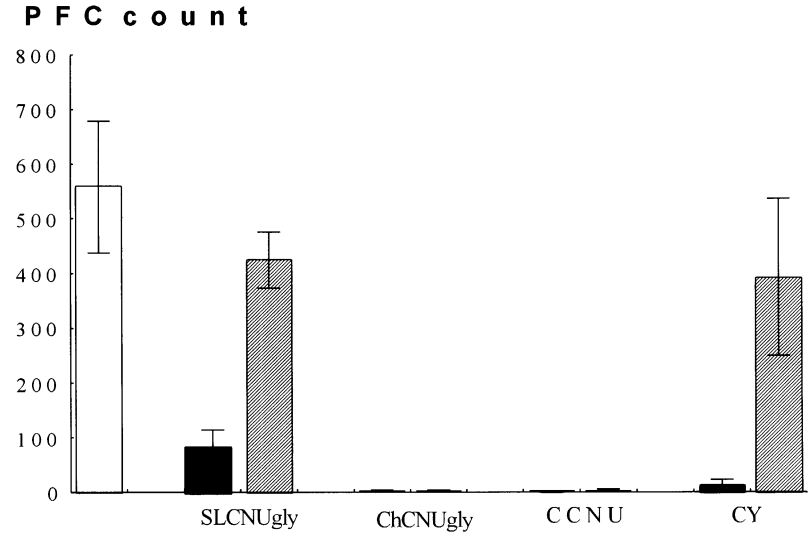

Fig. 2. Changes in plaque forming cells (PFC) response to SRBC of mice treated with antitumour drugs alone or together with C3bgp. PFC values were expressed as means ± SD per 10^6 spleen cells. ■ groups treated with different drugs at the doses as indicated in the text; ▨ groups received the same doses of drugs plus C3bgp (1.2 mg/kg); □ control group.

ity. No complete immune suppression after alone application of SLCNUgly (see Fig. 2) in comparison with complete ones for its nonlabeled analogue ChCNUgly suggests that the nitroxyl free radical moiety in SLCNUgly chemical structure in some way is involved in the lowered immunosuppressive effect. Moreover, results from joint application of C3bgp and SLCNUgly suggest also that the nitroxyl free radical moiety in the nitrosourea structure contributes to well expressed immunostimulation by C3bgp, resulting in immune restore function.

These preliminary results show that the overcoming of the nitrosourea immunosuppressive effect by joint application of spin labeled nitrosoureas and immunostimulators such as C3bgp might turn out to be a promising possibility for the development of new combination immunochemotherapy schemes for the treatment of human cancers.

References

Blagoeva, P.M., Raikov, Z.D., Chernozemsky, I., Nikolov, I., Yordanov, N.D., 1979. Distribution of 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl in normal and neoplastic animal tissues. Cancer Biochem. Biophys. 3, 169–171.

Carter, S.K., Schabel, F.M. Jr, Broder, L.E., Johnston, T.P., 1988. 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and other nitrosoureas in cancer treatment. A review. Adv. Cancer Res. 16, 273–332.

Clary, S., Nagarkatti, P.S., Nagarkatti, M., 1990. Immunomodulatory effects of nitrosoureas on the phenotype and functions of T cells in the thymus and periphery. Immunopharmacology 20, 153–164.

Gadzheva, V., Raikova, E., Raikov, Z., Ivanova, Ts, Stafanova, M., 1989. Investigation of some physicochemical properties of new nitrosourea derivatives potential antitumour agents. Compt. Rend. Acad. Bulg. Sci. 42 (7), 131–134.

Gadzheva, V., Ichimory, K., Nakazawa, H., Raikov, Z., 1997. New method for measurement of carbamoylating activity of nitrosoureas and isocyanates by EPR method. Free Radic. Res. 27, 197–206.

Geran, R.S., Greenberg, N.H., Macdonald, M.M., Schumacher, A.M., Abbott, B.J., 1972. Protocols for screening chemical agents and natural products against animal tumours and other biological systems. Cancer Chemother. Rep. 13 (2), 1–87.

Green, D., Ahlgren, J.D., Schein, P.S., 1981. Nitrosoureas in cancer treatment. In: Serrou, B., Schein, P.S., Imbach, J.I. (Eds.), INSERM Symposium 19. Elsevier, Amsterdam, p. 49.

Hoon, D.S., Foshag, L.J., Nizze, A.S., Bohman, R., Morton, D.L., 1990. Suppressor cell activity in a randomized trial of patients receiving active specific immunotherapy with melanoma cell vaccine and low dosages of cyclophosphamide. Cancer Res. 50, 5358–5364.

Hudson, L., Hay, F., 1991. Practical Immunology, 3rd edn. Blackwell, Oxford, p. 138.

Ilarionova, M., Todorov, D.K., Zheleva, A., Raikov, Z., 1993a. Third generation potential antimelanomic drugs. Oncologia 30 (Suppl.1), 73.

Ilarionova, M.V., Zheleva, A.M., Raikov, Z.D., Todorov, D.K., Dudov, A.P., 1993b. Nitrosourea antitumour agents-correlation between antitumour action and some biomedical properties of newly synthesized derivatives. J. Chemother. 5 (Suppl. 1), 786–787.

Kann, H.E., 1981. Carbamoylating activity of nitrosoureas. In: Prestayko, A.W., Grooke, S.T., Baker, L.H., Carter, S.K, Shein, P.S. (Eds.), Nitrosoureas: Current Status and New Developments. Academic Press, New York, pp. 95–104.

Montgomery, J.A., James, R., McCaleb, G.S., Johnston, T.P., 1967. The modes of decomposition of 1,3-bis(2-chloroethyl)-1-nitrosourea and related compounds. J. Med. Chem. 10, 668–674.

Sosnovsky, G., 1992. A critical evolution of the present status of toxicity of aminoxyl radicals. J. Pharmaceut. Sci. 81, 496–499.

Stanilova, S., Zhelev, Zh, Dobreva, Z.L., 2000. Preliminary studies on the immunomodulatory effect of the C3 binding glycoprotein isolated from *Cuscuta europea*. Int. J. Immunopharmacol. 22, 15–24.

Zhelev, Zh, Stanilova, S., Carpenter, B., 1994. Isolation, partial characterization and complement inhibiting activity of a new glycoprotein from *Cuscuta europea*. Biochem. Biophys. Res. Commun. 202, 186–194.

Zheleva, A., Raikov, Z., Ilarionova, M., Todorov, D., 1995. Spin labeled amino acid nitrosourea derivatives-synthesis and antitumour activity. Pharmazie 50, 25–26.

Zheleva, A., Carpenter, B., Ilarionova, M., Raikov, Z., 1996a. Synthesis and in vivo evaluation of several new nitrosoureas as potential antimelanomic drugs. Br. J. Cancer 73 (Suppl. XXVI), 32.

Zheleva, A., Raikov, Z., Ilarionova, M., Carpenter, B., Todorov, D., Armstrong, N., 1996b. Potential antimelanomic drugs. 1. Synthesis and antimelanomic effect of a spin labeled D,L-aminoacid containing a 2-chloroethylnitrosocarbamoyl group. Pharmazie 51, 602–604.