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N-Sulfonylpyrimidine derivatives and hyperthermia treatment of anaplastic mammary carcinoma *in vivo*

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ABSTRACT

The aim of this study was to investigate *in vivo* antitumor activity of newly synthesized *N*-sulfonylpyrimidine derivatives applied as a single agent and in combination with hyperthermia (43 °C/60 min). Antitumor activity was examined for: 1-(*p*-toluenesulfonyl)cytosine (4H), 1-(*p*-toluenesulfonyl)cytosine hydrochloride (4HxHCl), complex (Zn(II) [1-(*p*-toluenesulfonyl)cytosine]$_2$) (4K) and 5-bromo-1-(methanesulfonyl)uracil (8H). In the study we used transplantable anaplastic mammary carcinoma (AMCa). All experiments were performed on 10-14-week-old female CBA mice weighing 22-25 g at the time of treatment. The obtained data show that *N*-sulfonylpyrimidine derivatives (4H, 4HxHCl, 4K, 8H) possess antitumor activity. In combination with hyperthermia, the highest enhancement was obtained when hyperthermia was applied with 4HxHCl (300 mg/kg). The antitumor activity of *N*-sulfonylpyrimidine derivatives was similar or better than the antitumor effect of 5-fluorouracil (positive control). These findings provide a good reason for further research of these compounds, both in experimental and preclinical studies.

Key words: hyperthermia, mammary carcinoma, mice, *N*-sulfonylpyrimidine derivatives

Introduction

A number of recent innovative research approaches, targeting malignant abnormalities of tumor cells are under development. However, new derivatives of presently known "small molecule" cytotoxic agents, possessing improved properties, such as broader activity and lower toxicity, will continue to be an essential part of cancer therapy in the

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near future. Therefore, a strong need for new "small molecule" cytotoxic agents, with improved properties still exists.

Modified nucleosides and nucleic acid bases have been the subject of many studies due to their potential antitumor activity (BADAWEY, 1996; HATTORI et al., 1996; HATSE et al., 1999). The pyrimidine derivatives that are currently used as first-line chemotherapeutic agents for treatment of a wide range of solid tumors are 5-fluorouracil (5-FU) and capecitabine (ISAAC et al., 2002; ASSIKIS et al., 2003). HARADA et al. (1995), and SAKURAI et al. (1996) have shown that 5-fluorouracil, in combination with hyperthermia, has much better activity in comparison to 5-fluorouracil treatment only. A good synergistic effect was also obtained when hyperthermia was applied with cisplatin (STOJKOVIĆ and RADAČIĆ, 2002), dacarbasine and cyclophosphamide (STOJKOVIĆ and RADAČIĆ, 2002), paclitaxel (CIVIDALLI et al., 1999) and nicotinamide (EIKESDAL et al., 2001).

Recently, KAŠNAR et al. (1997), ŽINIĆ et al. (1999; 2003a; 2003b), KAŠNAR-ŠAMPREC et al. (2005) and GLAVAŠ-OBROVEC et al. (2005a) designed and synthesized *N*-sulfonylpyrimidine derivatives as a new type of sulfonylcyclourea. The *N*-sulfonylpyrimidine derivatives showed potent growth inhibitory activity against human tumor cell lines *in vitro* (GLAVAŠ-OBROVAC et al., 2001, 2005a and 2005b). Some of them showed the ability to induce apoptosis in treated tumor cells (GLAVAŠ-OBROVAC et al., 2001) and have strong antiproliferative activity and good selective effect in regard to normal cells (SUPEK et al., 2008). These types of nucleic base derivatives were found to inhibit DNA, RNA and protein synthesis (ŽINIĆ et al., 2003b; GLAVAŠ-OBROVAC et al., 2005a), and *in vivo* studies showed that *N*-1-sulfonylpyrimidine derivatives have strong antitumor activity against mouse mammary carcinoma (PAVLAK et al., 2005a; GLAVAŠ-OBROVAC, 2005a).

These novel *N*-1-sulfonylpyrimidine derivatives represent a valuable contribution to the development of new anticancer chemotherapeutic agents capable of improving (anti)tumor therapy. With respect to these observations and the positive influence of hyperthermia on the recently used cytostatic drugs, we investigated the antineoplastic activity of these derivatives in combination with local hyperthermia.

Materials and methods

Animals. Mice were obtained from the Ruđer Bošković Institute's breeding colony. During the experiment, three to four animals were kept in a cage. Food and water were supplied *ad libitum*. All experiments were performed on 10-14 week-old female CBA mice weighing 22 - 25 g at the time of treatment. Eight to ten animals were used in each group per experiment. Animals were treated with examined drugs alone and in combination with local hyperthermia. Each experiment was repeated twice.

All procedures performed in this study were according to the European document entitled "Directive for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes" (86/609/EEC) as well as the Croatian Act on Animal Protection in experimental work (NN19/99).

Tumors. In this study we used anaplastic mouse mammary carcinoma (AMCa) arising spontaneously in a female CBA mouse and maintained by serial transplantation in syngenic recipients (CBA mice) (PAVLAK et al., 2005a). The tumor was transplanted by injection of 10^6 tumor cells (0.02 mL) into the footpad of the right hind leg. Tumor volume was measured by caliper and calculated according to the formula $A \times B \times C \times \pi/6$ where A, B, C represent three orthogonal tumor diameters. The end point of tumor response was tumor growth time (TGT) *i.e.* the time needed for an individual tumor to increase its volume five times over the volume at the beginning of the tumor volume measurement. The effect of therapy was evaluated by comparing tumor growth time in the treated groups of animals versus tumor growth time in the control (untreated) group of animals.

New agents: N-Sulfonylpyrimidine derivatives. The synthesis of *N*-sulfonylpyrimidine derivatives, by attaching the sulfonyl fragment onto N-1 of pyrimidine bases, was described for the first time in its patent (ŽINIĆ et al., 2003a). The compounds were synthesized in the Laboratory of Supramolecular and Nucleoside Chemistry, Rudjer Bošković Institute (Zagreb, Croatia).

Antitumor activity was investigated for cytosine derivatives: 1-(*p*-toluenesulfonyl)cytosine (4H), 1-(*p*-toluenesulfonyl)cytosine hydrochloride (4HxHCl) and complex (Zn(II) [1-(*p*-toluenesulfonyl)cytosine]$_2$) (4K) and uracil derivative 5-bromo-1-(methanesulfonyl)uracil (H8). The structures of investigated *N*-sulfonylpyrimidine derivatives are shown in Figure 1.

Hyperthermia. Local hyperthermia (HT) was given by immersing the tumor-bearing leg into a water bath (Heto water bath, Birkerad, Denmark), which gives temperature of 43 ^{o}C. A total heating time of 60 min was applied in our experiments. Hyperthermia treatment started when the tumor reached a volume of about 200 mm^3 which was usually between 10 and 16 days after tumor cell implantation into the footpad. All tested compounds were administrated 15 min prior to application of hyperthermia.

Treatment. In this work all tested compounds were used alone and with local hyperthermia. The treatment groups were as follows: Group I: control/untreated animals received vehicle; Group II: animals received 1200 mg/kg of derivative 4H alone; Group III: animals received 300 mg/kg of derivative 4HxHCl alone; Group IV: animals received 300 mg/kg of derivative 4K alone; Group V: animals received 50 mg/kg of derivative H8 alone; Group VI: animals received 100 mg/kg of 5FU alone; Group VII: control/HT animals received HT alone; Group VIII: animals received 1200 mg/kg of derivative 4H with HT; Group IX: animals received 300 mg/kg of derivative 4HxHCl with HT; Group

X: animals received 300 mg/kg of derivative 4K with HT; Group XI: animals received 50 mg/kg of derivative H8 with HT; Group XII: animals received 100 mg/kg of 5-FU with HT.

The doses of new compounds used were chosen according to previous studies (PAVLAK et al., 2005a, 2005b). 5-FU was used as a positive control. All test compounds were dissolved in distilled water immediately prior to intraperitoneal injection. All dissolved compounds were given intraperitoneally according to the body weight of mice, *i.e.* 0.5 mL of solution was given per 25 g of body weight.

Statistical analysis. All results were presented as arithmetic mean ± standard deviation (X ± SD) of TGT. The effect of therapy was evaluated by comparing TGT in the treated groups of animals versus TGT in the control (untreated) group of animals. A comparison was also made with the positive control group (5-FU) and comparing TGT in the group of animals treated with new agents alone, versus TGT in the group of animals treated with hyperthermia plus new agents. The T/C ratio was calculated for all treated groups and presented as a percentage [(TGT-treated group/TGT-control) × 100].

The results were tested by the ANOVA test for independent samples. The level for statistical significance was set at the level of<0.05.

Results

The results presented in Tables 1 and 2 and in Fig. 2 show antitumor activity of all investigated *N*-sulfonylpyrimidine derivatives applied alone (Table 1) and in combination with a local hyperthermia (Table 2).

Table 1. Antitumor activity of 4H (1-(p-toluenesulfonyl)cytosine), 4HxHCl (1-(p-toluenesulfonyl)cytosine hydrochloride and complex 4K (Zn(II) [1-(p-toluenesulfonyl)cytosine]$_2$) and 8H (5-bromo-1-(methanesulfonyl)uracil) on the tumour growth time (TGT) of anaplastic mammary carcinoma

Treatment (mg/kg)	TGT X ± SD (days)	Therapy: Control P	T/C* (%)
Control/untreated	9.6 ± 1.9	1.0000000	100
4H (1200 mg/kg)	19.0 ± 3.4	0.000017**	198
4HxHCl (300 mg/kg)	17.2 ± 3.1	0.000131**	179
4K (300 mg/kg)	22.4 ± 4.2	0.000002**	233
8H (50 mg/kg)	18.3 ± 2,3	0.000003**	191
5-FU (100 mg/kg)	17.3 ± 4.1	0.000393**	180

**statistically significant; *T represents arithmetic mean TGT of treated animals and C represents arithmetic mean TGT of control animals

From the data shown in Table 1 and Fig. 2 all tested *N*-sulfonylpyrimidine derivatives significantly reduced the speed of mouse tumor growth. TGT was prolonged in all examined groups by two or more times compared with the control group ($P<0.01$). From the data shown in Table 2 and Fig. 2 it can be seen that the best effect was achieved when 4HxHCl was given with local hyperthermia ($P<0.05$), and this is statistically still significant.

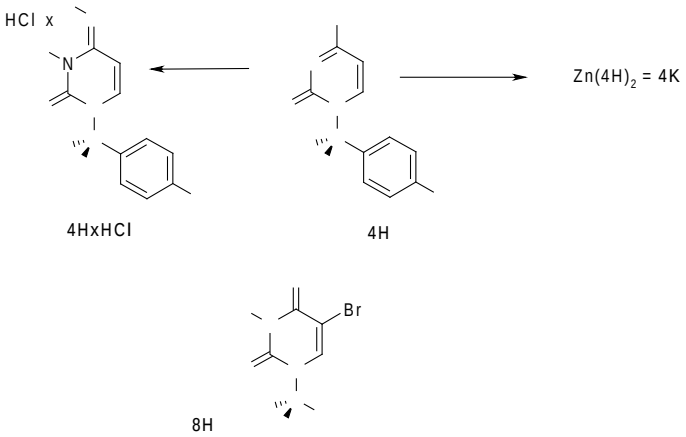

Fig. 1. Structure of *N*-sulfonylpyrimidine derivative.

Table 2. Antitumor activity of 4H (1-(p-toluenesulfonyl)cytosine), 4HxHCl (1-(p-toluenesulfonyl)cytosine hydrochloride and complex 4K (Zn(II) [1-(p-toluenesulfonyl)cytosine]$_2$) and 8H (5-bromo-1-(methanesulfonyl)uracil) with local hyperthermia (HT) on the tumour growth time (TGT) of anaplastic mammary carcinoma

Treatment(mg/kg)	TGT X ± SD (days)	Combined therapy: HTP	T/C(%)
Control/HT (43 °C)	21.3 ± 1.4	1.0000000	100
4H (1200 mg/kg) + HT	20.9 ± 1.3	0.6672070	98
4HxHCl (300 mg/kg) + HT	24.2 ± 1.7	0.006462**	114
4K (300 mg/kg) + HT	22.3 ± 3.9	0.4049870	105
8H (50 mg/kg) + HT	25,5 ± 6,4	0.0753120	120
5-FU (100 mg/kg) + HT	21.9 ± 5.6	0.5672070	103

** statistically significant; * T represents arithmetic mean TGT of treated animals and C represents arithmetic mean TGT of control animals

TGT in the group of animals treated with 1-(*p*-toluenesulfonyl)cytosine (4H) was about 2 times prolonged in comparison to the control/untreated group (P<0.01) (Table 1, Fig. 2). On the other hand, in 4H plus HT (TGT 20.9 ± 1.3 days) no statistical differences were found in tumor growth time when compared to the control/HT group (TGT 21.3 ± 1.4 days) (Table 2, Fig. 2). Derivative 4H showed similar antitumor effect to 5-FU (Table 1 and 2, Fig. 2).

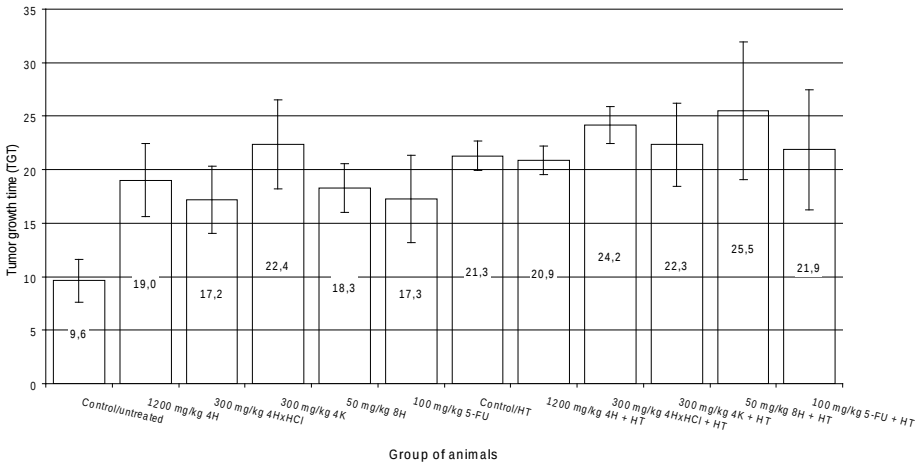

Fig. 2. Antitumor activity of *N*-sulfonylpyrimidine derivatives (H4, H4xHCl, K4 and H8) and local hyperthermia (43 °C) on the growth of anaplastic mammary carcinoma

In the group of animals treated by 1-(*p*-toluenesulfonyl)cytosine hydrochloride (4HxHCl), TGT was also about 1.8 times prolonged in comparison to the control/untreated group (P<0.01) and 4HxHCl had similar antitumor activity as the positive control 5-FU (Table 1, Fig. 2). Statistically significant results (P<0.05) were obtained in the group of animals treated with this derivative and HT (TGT 24.2 ± 1.7 days) in comparison to the group of animals treated with HT only (TGT 21.3 ± 1.4 days) (Table 2, Fig. 2). The results of combined treatment of 4HxHCl and HT were also statistically significant in comparison to the antitumor activity produced by 5-FU plus HT (TGT 21.9 ± 5.6 days) (P<0.05) (Table 2, Fig. 2).

The tumor growth time of complex (Zn(II) [1-(*p*-toluenesulfonyl)cytosine]$_2$) (4K) (TGT 22.4 ± 4.2 days) was statistically prolonged compared to TGT in the control/ untreated group (P<0.01). Derivative 4K has also showed some better antitumor activity against mouse mammary carcinoma than 5-FU (TGT 17.3 ± 4.1 days), but the result was not statistically significant (Table 1 and Fig. 2). Tumor growth time in the protocol with 4K and HT (TGT 22.3 ± 3.9 days) was similar to tumor growth time in the control/HT

group (TGT 21.3±1.4 days) as well as in the group of animals treated with 5-FU and HT (TGT 21.9 ± 5.6 days) (Table 2 and Fig. 2).

TGT in the group of animals treated with 5-bromo-1-(methanesulfonyl)uracil (H8) was 18.3 ± 2.3 days, which is 2 times better than in the control/untreated group (TGT 9.6 ± 1.9 days) ($P<0.01$). Comparing with positive control (5-FU), antitumor effect of this derivative was similar to antitumor activity of 5-FU ($P>0.05$) (Table 1 and Fig. 2). In combination with HT, tumor growth time of H8 (TGT 25.5 ± 6.4 days) was prolonged in comparing with the group of animals treated with HT only (TGT 21.3 ± 1.4 days) as well as in the group of animals treated with 5-FU plus HT (TGT 21.9 ± 5.6 days) (Table 2, Fig. 2), but there was no statistical significance.

Discussion

Since *N*-1-sufonylpyrimidine derivatives showed good cytotoxic activity against various human tumour cells *in vitro* (GLAVAŠ-OBROVAC et al., 2001, 2005a and 2005b) as well as promising results in preliminary studies *in vivo* (PAVLAK et al., 2005a), we compared them with antitumor activity of fluorinated pyrimidine 5-FU which is currently used in the therapy of breast cancer (ISAAC et al., 2002; ASSIKIS et al., 2003; ZHANG et al., 2008) and with local hyperthermia (HT) because it is known that HT in combination with some drugs may improve the antineoplastic effect and reduce the effective dose of drugs (CIVIDALLI et al., 1999; EIKESDAL et al., 2001; STOJKOVIĆ and RADAČIĆ, 2002; McCORMICK, 2007; KANAYA et al., 2008).

In this study mouse mammary carcinoma was used because it may serve as a good screening model for clinically potential and predictive drug(s) and the applied doses for test compounds were chosen on the basis of previous data obtained in LD_{50} experiments (McELHINNEY et al., 1989; BIBBY et al., 1993). The antitumor effects of *N*-1-sulfonilpyrimidine derivatives were evaluated by comparing the antitumor effect of 5-FU and combined use of *N*-1-sulfonilpyrimidine derivatives and HT, applying them 15 minutes before HT.

Our presented data show that *N*-1-sulfonilpyrimidine derivatives: 1-(*p*-toluenesulfonyl)cytosine (4H), 1-(*p*-toluenesulfonyl)cytosine hydrochloride (4HxHCl), complex (Zn(II) [1-(*p*-toluenesulfonyl)cytosine]$_2$) (4K) and 5-bromo-1-(methanesulfonyl)uracil (8H) reduced the growth of AMCa ($P<0.01$). Tumour growth time in the group of animals treated with *N*-1-sulfonilpyrimidine derivatives was prolonged 1.8 (4HxHCl), 1.9 (8H) 2.0 (4H) and 2.3 (4K) times in comparison to control group ($P<0.01$) (Table 1).

When comparing antitumor activity of *N*-sulfonylpyrimidine derivatives (4H, 4HXHCl, 4K and 8H) with antitumor activity of 5-FU (positive control), it can be seen

that the antitumor activity of these derivatives was similar to the antitumor activity of 5-FU (Table 1, Fig. 2).

Tumour growth time in the group of animals treated with HT was longer than in the control/untreated group ($P<0.01$). Antitumor activity of HT is in agreement with the data of other authors achieved with different tumour models (RADAČIĆ, 1995; STOJKOVIĆ and RADAČIĆ, 2002). Tumour growth time in the group of animals treated with *N*-1-sulfonilpyrimidine derivatives plus HT was prolonged from 2.1 to 2.7 times in comparison to the control/untreated group.

The best antitumor effect of the tested derivatives applied with hyperthermia was obtained in the group of animals treated with 4HxHCl plus HT. A statistically significant difference was found in the group of animals treated with 4HxHCl alone and with 4HxHCl plus HT ($P<0.05$) (Table 2, Fig. 2). The additive effect between 4HxHCl plus HT could be explained by the adequate time interval between administration of derivatives 4HxHCl plus HT, which was not found between other derivatives (4H, 4K and 8H) applied with HT. The importance of the influence of the time interval between drug administration and application of HT was demonstrated in different antineoplastic drugs applied by some other authors (CIVIDALLI et al., 2000; RAU et al., 2000; PASSETO et al., 2008).

The antitumor effect of 5-FU in combination with HT was also much better in comparison to 5-FU treatment only, which corresponds to the results of some other authors (HARADA et al., 1995). When comparing the antitumor activity of 5-FU and HT with the antitumor activity of derivatives applied with HT, statistically significant differences in TGT were found in the group of animals treated with 4HxHCl plus HT in comparison with the TGT of 5-FU plus HT.

On the basis of the present results, it may be concluded, that new *N*-1-sulfonylpyrimidine derivatives are promising antitumor agents with antineoplastic activity. Hyperthermia in combination with 4HxHCl derivative may enhance the antitumor activity, which may be explained by the adequate time interval between administration of derivatives 4HxHCl plus HT, which was not found between the other derivatives (4H, 4K and 8H) applied with HT.

It may be concluded that our results obtained with N-sulfonylpyrimidine derivatives are similar to those obtained with 5-FU. However, while 5-FU, a clinically used cytostatic drug, has serious side effects, in our study no side effects were observed.

Therefore, further research on new *N*-sulfonylpyrimidine derivatives and hyperthermia, as well as new compounds with other modalities is a valuable contribution to the development of new anticancer chemotherapeutic agents.

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SAŽETAK

Cilj rada bio je istražiti protutumorski učinak novosintetiziranih N-sulfonil-derivata pirimidinskih nukleobaza i hipertermije (43 °C/60 min) na rast anaplastičnoga mamarnoga karcinoma. Protutumorski učinak ispitan je u sljedećih N-sulfonil-pirimidinskih derivata: 1-(p-toluensulfonil)citozina (4H), 1-(p-toluensulfonil)citozin hidroklorida (4HxHCl), kompleksa (Zn(II) [1-(p-toluensulfonil)citozin]2) (4K) i 5-brom-1-(metansulfonil)uracila (8H). Dobiveni rezultati upućuju na protutumorsko djelovanje svih pretraženih N-sulfonil-pirimidinskih spojeva (4H, 4HxHCl, 4K, 8H). U odnosu na pozitivnu kontrolu (5-fluorouracil) pretraživani derivati pokazali su podjednako ili čak nešto bolje djelovanje od 5-fluorouracila. Rezultati ispitivanja združenoga djelovanja derivata i hipertermije upućuju na značajniji protutumorski učinak 4HxHCl (300 mg/kg) i hipertermije u odnosu na pojedinačni učinak.

Ključne riječi: hipertermija, mamarni karcinom miša, *N*-sulfonil-derivati pirimidinskih nukleobaza