Cancer Chemother Pharmacol (2002) 50: 291–298
DOI 10.1007/s00280-002-0491-x

ORIGINAL ARTICLE

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Therapeutic advantage from combining 5-fluorouracil with the hypoxia-selective cytotoxin NLCQ-1 in vivo; comparison with tirapazamine

Received: 27 February 2002 / Accepted: 7 June 2002 / Published online: 24 July 2002

Abstract *Purpose*: The antitumor effect and bone marrow toxicity of 5-fluorouracil (5FU) in combination with the hypoxia-selective cytotoxins NLCQ-1 or tirapazamine (TPZ) were investigated in vivo. *Methods*: Using appropriate intraperitoneal administration schedules for optimal synergistic interactions, the antitumor effect and the bone marrow toxicity of combinations of NLCQ-1 or TPZ and 5FU were determined in EMT6/BALB/c and SCCVII/C3H models in terms of dose modification factors (DMF) using the in vivo-in vitro clonogenic assay as endpoint. Bone marrow toxicity studies were performed in parallel using a modified CFU-GM assay. The antitumor efficacies of each combination treatment under optimal administration conditions were evaluated in the SCCVII/C3H model using also the tumor regrowth assay as endpoint. *Results*: A schedule-dependent and tumor-specific synergistic interaction was observed for NLCQ-1 plus 5FU and DMFs of 2.0–2.3 and 1.0 were obtained for the antitumor effect and bone marrow toxicity, respectively, in both tumor models. The antitumor effect of 5FU was slightly potentiated (DMF 1.2) by TPZ in the EMT6/BALB/c model but not in the SCCVII/C3H model when the in vivo-in vitro assay was used as the endpoint. Significant additional tumor regrowth delays (about 11 and 6 days for NLCQ-1 and TPZ, respectively) were observed, compared to the effect of 5FU alone, when an equitoxic dose of NLCQ-1 (10 mg/kg) or TPZ (23 mg/kg) was administered 1 h before 5FU (50 mg/kg) twice a day at 4-h intervals on days 0 and 9. *Conclusions*: These results corroborate the therapeutic advantage of combining hypoxia-selective cytotoxins such as NLCQ-1 and TPZ with chemotherapy.

Keywords NLCQ-1 · 5-Fluorouracil · Tirapazamine · Potentiation · Hypoxic cytotoxins

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Introduction

An increasing number of reports suggests that hypoxia-induced proteome and genome changes in tumors can lead to a more aggressive phenotype, malignant progression [7, 13, 18] and resistance towards radiation or chemotherapy [42, 50, 56]. Additional reasons for the resistance of hypoxic tumor tissues to chemotherapeutic drugs include their distance from the viable blood vessels and their slower rate of proliferation [14, 20, 44]. Hypoxia-mediated tumor chemoresistance may be overcome with the use of hypoxia-activated bioreductive drugs [25, 26, 27, 28, 48, 49]. It has been previously shown that hypoxia-selective cytotoxins such as alkylaminoanthraquinone di-N-oxide (AQ4N), 3-amino-1,2,4-benzotriazine-1,4-dioxide (tirapazamine, SR-4233, TPZ), and 4-[3-(2-nitro-1-imidazolyl)propylamino]-7-chloroquinoline hydrochloride (NLCQ-1) can significantly enhance the antitumor effect but not the systemic toxicity of commonly used chemotherapeutic agents, both in vivo and in vitro [8, 9, 11, 12, 19, 21, 30, 31, 35, 39, 47]. The results of recent clinical trials of TPZ combined with cisplatin (cisDDP) confirm that a therapeutic gain in terms of overall response rates and survival may be achieved in the clinic as well as in animal models [2, 23, 24, 52, 54]. On the other hand, NLCQ-1 is considered for phase I clinical trials due to the promising results obtained by the NCI in studies against human prostate PC-3 xenografts in athymic nude mice, in combination with paclitaxel or cyclophosphamide (unpublished results; [38]).

NLCQ-1 is a weakly DNA-intercalating hypoxia-selective cytotoxin developed in our laboratory [29] which

demonstrates an increasing hypoxic potency and selectivity with time [32], like the so-called "bis-bioreductive agents", compounds that bear two reducible centers [15]. NLCQ-1 synergistically enhances the effect of radiation against hypoxic cells in vitro and murine tumors in vivo [33] and optimizes the effect of radioimmunotherapy in human xenografts [3]. Importantly, NLCQ-1 substantially enhances, in a schedule-dependent manner, the antitumor effect of alkylating agents against murine tumors, without a concomitant enhancement in bone marrow or hypoxia-dependent retinal toxicity [30, 34, 37].

Potentiation of chemotherapy by NLCQ-1 and TPZ seems to be both chemotherapeutic agent- and administration schedule-dependent [8, 9, 31, 35] even though there is some evidence that the timing of TPZ administration may be less important than previously thought [22]. Thus, NLCQ-1 and TPZ have to be given before an alkylating agent (e.g. melphalan, cyclophosphamide or cisDDP) or after the antimitotic Taxol, for example, so that an optimal synergism can occur, and the timing is different for each of the agents mentioned [35]. Another factor that may play a significant role in the potentiation of chemotherapy by NLCQ-1 and TPZ, a factor that has not been adequately addressed, is tumor specificity. The present study was undertaken to further investigate potential synergistic interactions between NLCQ-1 or TPZ and 5-fluorouracil (5FU). 5FU was chosen because: (a) it has been the mainstay of treatment in combined-modality therapy, in particular against colorectal cancer, for nearly four decades [45], (b) its toxicity remains an obstacle to the achievement of overall clinical benefit in many patients [53], and (c) both NLCQ-1 and TPZ yield a similar and significant potentiation of 5FU (55.5% and 53.0%, respectively) in the EMT6/BALB/c model [35].

Materials and methods

Drugs

NLCQ-1 (provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute) and TPZ (provided by Sanofi-Winthrop, Malvern, Pa.) were dissolved in saline at 1 and 0.75 mg/ml, respectively, and 5FU was used as the formulated solution (50 mg/ml) provided by the company (Pharmacia, Kalamazoo, Mich.). All drugs were injected intraperitoneally (i.p.) on the basis of animal body weight and the total injected volume was ≤ 0.7 ml.

Mice and tumors

EMT6 mammary tumor cells (a gift from Dr. B. Teicher, Dana-Farber Cancer Institute, Boston, Mass.) [43] were inoculated (s.c.) into both legs (2×0^5 cells in 0.2 ml per leg) of 18–20 g female BALB/c mice (Jackson Laboratories, Bar Harbor, Me.). SCCVII tumor-cells (squamous carcinoma; a gift from Dr. D. Siemann, University of Florida, Gainesville, Fl.) [17] were inoculated (s.c.) into both legs (in vivo-in vitro assay; 5×10^4 cells in 0.05 ml per leg) or into one leg (tumor regrowth assay) of 18–20 g female C3H mice (Jackson Laboratories). Two to three (in vivo-in vitro assay) or eight mice (tumor regrowth assay) were used per group. Treatment was initiated when the tumor geometrical mean diameter reached about 0.8 cm (in vivo-in vitro assay) or about 0.5 cm (tumor regrowth assay). All animals were housed under germ-free conditions and the studies were conducted in accordance with the guidelines set by the Evanston Northwestern Healthcare Institutional Animal Care.

In vivo-in vitro clonogenic assay (antitumor effect)

When the EMT6/BALB/c model was used, we only determined the dose modification factor (DMF) values for the antitumor effect and bone marrow toxicity, since the optimal administration schedule for potentiation of 5FU by NLCQ-1 and TPZ is known from our previous work [35]. In the SCCVII/C3H model, the optimal timing was first determined at 150 mg/kg 5FU. Subsequently, dose response survival curves were generated at the optimal administration time for various 5FU doses (up to 175 mg/kg) to determine DMF values for the antitumor effect and bone marrow toxicity. NLCQ-1 and TPZ were given at equitoxic doses of 10 mg/kg (0.027 mmol/kg) and 23 mg/kg (0.168 mmol/kg), respectively, which represent 28% [9, 30] of their single LD_{50} value.

The in vivo-in vitro assay was used as the endpoint as described previously [30]. Briefly, tumors were excised 18 h after the last treatment, minced, dissociated with an enzyme cocktail (0.5 mg/ml pronase, 0.2 mg/ml collagenase, 0.2 mg/ml DNAase in RPMI-1640 with 10% FBS) added in the proportion of 1 ml per 60 mg of tumor, mixed together and incubated at 37°C with magnetic stirring for 40 min. The single-cell suspension was washed twice by centrifugation and the cell density determined with a Coulter counter. The cells were then plated at various concentrations in quadruplicate to form colonies. After 2 weeks of incubation at 37°C in a humidified atmosphere containing 5% CO_2, tumor cell colonies were stained with crystal violet and counted. The plating efficiencies of untreated EMT6 and SCCVII tumor cells were 36.5% (32.5–40.5%) and 29.4% (28.5–30.3%), respectively.

Survival of the treated groups is expressed as a fraction in relation to that of the untreated control group. Additive effects were calculated as the product of the surviving fractions (SFs) following treatment with each drug alone [9]. When DMF values were determined, the survival curves obtained from the NLCQ-1 plus 5FU and TPZ plus 5FU treatments were normalized for the hypoxic toxicity of NLCQ-1 and TPZ alone. The degree of potentiation (P), the percentage of tumor cells that were killed due to clear synergism [55], was calculated by subtracting the SF obtained in the combination treatment (SFc) from the calculated additive effect (SFa) and multiplying by 100: $P = (SFa–SFc)\times100$.

A therapeutic index (ThI) was also calculated for NLCQ-1 and TPZ as the ratio between the DMF obtained for the antitumor effect (DMF_T) divided with the DMF obtained for bone marrow toxicity (DMF_{BM}): $ThI = DMF_T/DMF_{BM}$.

Bone marrow toxicity

Bone marrow toxicity studies were performed in parallel with the antitumor efficacy studies, when the in vivo-in vitro assay was used, by flushing marrow from the femurs of treated and untreated mice and using a modified CFU-GM [46]. A complete methylcellulose-based medium with growth factors for murine clonogenic hematopoietic progenitor cells (MethoCultGF M3434; StemCell Technologies, Vancouver, Canada) was used for the formation of colonies, which were detected and counted using an inverted microscope.

Tumor regrowth assay

To assess the response of SCCVII tumors to treatment, when the tumor regrowth assay was used as the endpoint, their size was measured every day or every other day using a vernier caliper. Tumor volumes were calculated using the formula $V = \pi(x\times y\times z)/6$, where x, y and z are orthogonal diameters minus folded skin

thickness (1 mm). At the time of treatment, the tumor volumes ranged from 47 to 82 mm^3. The doubling time of untreated control tumors (Td) was calculated as the mean time required for a tumor to double from 200 to 400 mm^3. Tumors were evaluated at a volume of 600 mm^3 and the specific growth delay (SGD) for each treatment group was calculated [4] from the formula: SGD = (T–C)/Td, where T is the time taken for the treated tumor to reach a volume of 600 mm^3 and C is the time taken for the control tumor to reach a volume of 600 mm^3. Also, the log kill corresponding to this growth delay was calculated from the formula: log kill = 0.301×SGD [41]. Groups were compared using Student's *t*-test.

Results

In vivo-in vitro assay

The chemical structures of NLCQ-1 and TPZ are shown in Fig. 1B and C, respectively. The antitumor effects and bone marrow toxicities when NLCQ-1 (10 mg/kg) or an equitoxic dose of TPZ (23 mg/kg) was administered i.p. 30 min after or 3 h before various doses of 5FU to EMT6 tumor-bearing BALB/c mice are also shown in Fig. 1. These schedules were suggested by the results of our previous studies [35] to be the best time intervals for optimal potentiation [55] in this particular tumor model. It is clear that neither bioreductive drug modified the bone marrow toxicity induced by 5FU, thus yielding a DMF value of 1 (Fig. 1B, C), whereas NLCQ-1 significantly potentiated the antitumor effect (Fig. 1A).

When schedule-dependent potentiation was investigated in the SCCVII/C3H model, the results were quite different from those obtained in the EMT6/BALB/c model [35]. Thus, NLCQ-1 optimally potentiated 5FU when administered 1 h before rather than after 5FU, whereas no indication of potentiation was observed with TPZ for any of the time intervals studied (Fig. 2A, B). Almost identical results were obtained from two different experiments with three mice per group (six tumors). Again, equitoxic doses of NLCQ-1 and TPZ (10 mg/kg, 0.027 mmol/kg, and 23 mg/kg, 0.168 mmol/kg, respectively) representing 28% of their single LD_{50} values were administered [9, 30]. In the plots, for clarity only the effect of the combination treatment is depicted as well as the calculated additive effect. The mean SFs following treatment with NLCQ-1, TPZ and 5FU alone were 0.924 ± 0.023, 0.715 ± 0.023 and 0.478 ± 0.018, respectively. The degrees of potentiation were 21.5 ± 1.9 and 2.9 ± 2.2 for NLCQ-1 and TPZ, respectively (Table 1). It is apparent that less potentiation was seen for NLCQ-1 in the less-hypoxic SCCVII tumors than in the EMT6 tumors [1]. On the other hand, no potentiation of 5FU was seen with TPZ in this tumor.

Dose-response curves were generated for NLCQ-1 with or without 5FU, but not for TPZ with or without 5FU, in terms of the antitumor effect and bone marrow toxicity in the SCCVII/C3H model (Fig. 3). The antitumor effect and the bone marrow toxicity were modified by NLCQ-1 by a factor of 2.3 and 1, respectively. Therefore, a therapeutic index (ThI) of 2.3 was obtained, identical with that for the more hypoxic EMT6 tumors (Table 1). The SF following treatment with 5FU at 150 mg/kg in this experiment was greater than the corresponding SF in the timing experiment (0.685 ± 0.077 vs 0.478 ± 0.018).

Tumor regrowth assay

Figure 4 shows the mean tumor responses of groups of SCCVII tumor-bearing mice untreated or treated with

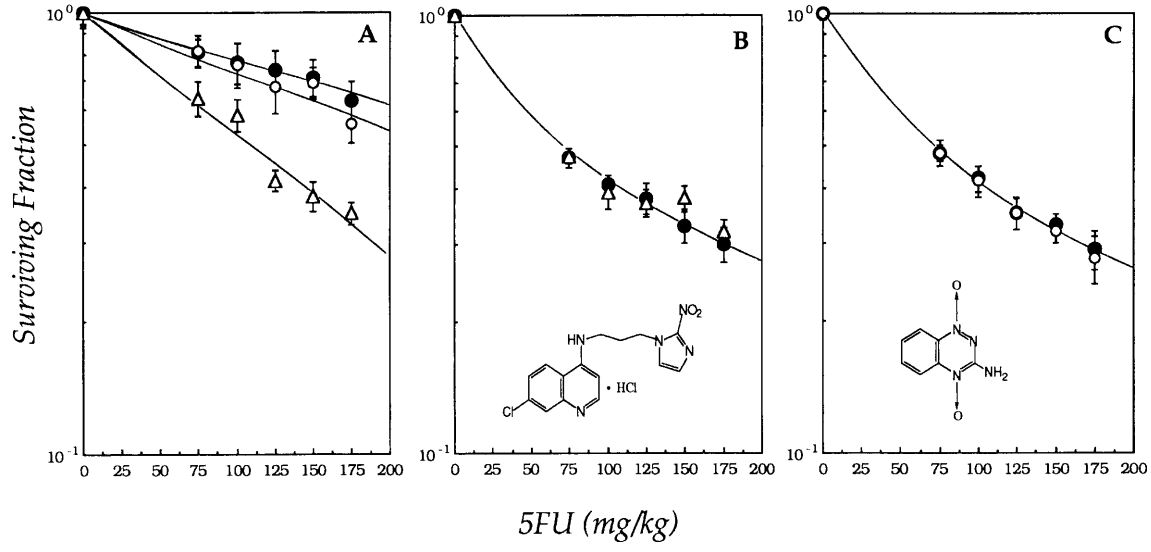

Fig. 1A–C. Antitumor effect (**A**) and bone marrow toxicity (**B**, **C**) of 10 mg/kg NLCQ-1 (**A**, **B**) or 23 mg/kg TPZ (**A**, **C**) given i.p. 30 min after and 3 h before 5FU, respectively, in BALB/c mice bearing EMT6 tumors (*closed circles* 5FU alone, *triangles* NLCQ-1 plus 5FU, *open circles* TPZ + 5FU). Assays were carried out 18 h after the last drug administration. Bone marrow toxicity was assayed by CFU-GM. Three mice per point were used (six tumors). Single-cell suspensions of tumor obtained after each tumor dissociation or of bone marrow cells retrieved from the three mice were combined and plated in quadruplicate to form colonies (*bars* SD of quadruplicate measurements)

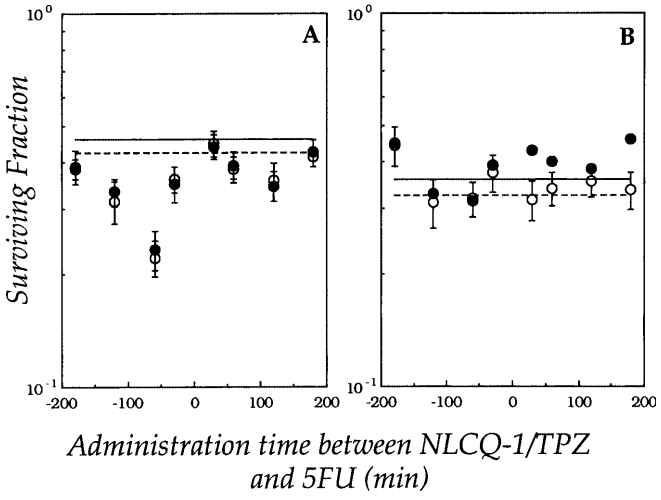

Fig. 2A, B. Schedule-dependent modification of the antitumor effect of 150 mg/kg 5FU by (**A**) NLCQ-1 (10 mg/kg; 0.027 mmol/kg) and (**B**) TPZ (23 mg/kg; 0.129 mmol/kg) in C3H mice bearing SCCVII tumors. Only the effects of combined drugs are shown from two independent experiments (*open circles*, *closed circles*); *dashed and solid lines* represent the additive effects (calculated as the product of the surviving fractions obtained with the two independent agents) corresponding to the *open and solid symbols*, respectively. Six tumors per point were used. Single-cell suspensions obtained after each tumor dissociation were combined and plated in quadruplicate to form colonies (*bars* SD of quadruplicate measurements)

NLCQ-1 or TPZ with or without 5FU. NLCQ-1 (10 mg/kg) or TPZ (23 mg/kg) was administered i.p. 1 h before 5FU (50 mg/kg) twice a day at 4-h intervals on days 0 and 9. Tumors in mice treated with NLCQ-1, TPZ or 5FU alone grew essentially at the same rate and at a slightly slower rate than the control group (saline-treated). Tumors treated with TPZ plus 5FU showed a decrease in size to 72% of their original mean volume on day 3, whereas tumors treated with NLCQ-1 plus 5FU showed a decrease in size to 68% of their original mean volume on day 5. Similarly, tumors showed a decrease in size after the second treatment to 85% of their volume on day 9 following treatment with TPZ plus 5FU and to 48% following treatment with NLCQ-1 plus 5FU on days 11 and 12, respectively.

The doubling time of untreated SCCVII tumors was 2 days. Tumor growth delays relative to the control group were compared at a mean volume of 600 mm^3 for all treated groups and the results are shown in Table 2. The differences in the mean time delay for the tumors to reach 600 mm^3 between each treated group and the control group were greater than would be expected by chance ($P < 0.001$, and in one case $P < 0.02$). Similarly, the P value was < 0.001 for the comparison between the groups treated with NLCQ-1 plus 5FU and TPZ plus 5FU. On the other hand, there was no significant difference between the groups treated with 5FU, TPZ or NLCQ-1 alone. From the data in Table 2, it is apparent that synergistic interaction occurred between NLCQ-1 and 5FU (7.3 days extra delay beyond additivity), whereas in the case of TPZ plus 5FU combination treatment, the extra delay was only 1 day.

Normal tissue toxicity

At the given dose and schedule, there was no weight loss or lethality observed in the C3H mice treated with NLCQ-1 alone or NLCQ-1 plus 5FU. Mice treated with TPZ alone or TPZ plus 5FU had 94% and 100%, respectively, of the corresponding mean body weight of the control group on day 9. Two animals died on days 13 and 14, respectively, in the group treated with TPZ alone, presumably due to the large size of the tumors. However, these two mice were sick already on day 11, even though mice with larger tumors in the control group did not develop signs of illness. Mice treated with 5FU alone (50 mg/kg ×2) had 85.2% of the corresponding mean body weight of the control group on day 9. After the second treatment with 5FU alone (another 50 mg/kg ×2), five mice became ill and eventually 50% of the animals in this group had died by day 13, presumably due to drug-related toxicity. Unexpectedly, mice treated with NLCQ-1 or TPZ plus 5FU

Table 1. Potentiation data of 5-FU by NLCQ-1/TPZ in murine tumors. The data were obtained using the optimal administration schedules from two experiments in quadruplicate. Values are means ± SD (*ND* not determined)

Parameter	NLCQ-1		TPZ	
	EMT6/BALB/c	SCCVII/C3H	EMT6/BALB/c	SCCVII/C3H
SFa[a]	0.755 ± 0.022[b]	0.442 ± 0.028	0.600 ± 0.043[b]	0.342 ± 0.023
SFc[c]	0.200 ± 0.032[b]	0.227 ± 0.008	0.070 ± 0.006[b]	0.313 ± 0.001
Potentiation[d]	55.5 ± 1.5[b]	21.5 ± 1.9	53.0 ± 1.1[b]	2.9 ± 2.2
DMF_T[e]	2.3	2.3	1.2	ND
DMF_{BM}[e]	1	1	1	ND
ThI[f]	2.3	2.3	1.2	ND

[a]Surviving fraction calculated for the additive effect
[b]Data taken from reference 35
[c]Actual surviving fraction obtained in the combination treatment
[d]Percentage of cells killed due to pure potentiation by the bioreductive compound (see Materials and methods)
[e]DMF_T and DMF_{BM} are the dose modification factors (ratio between two doses of the chemotherapeutic agent in the presence and absence of NLCQ-1/TPZ for the same effect) for the antitumor effect (T) and the bone marrow toxicity (BM), respectively
[f]Therapeutic index (DMF_T/DMF_{BM})

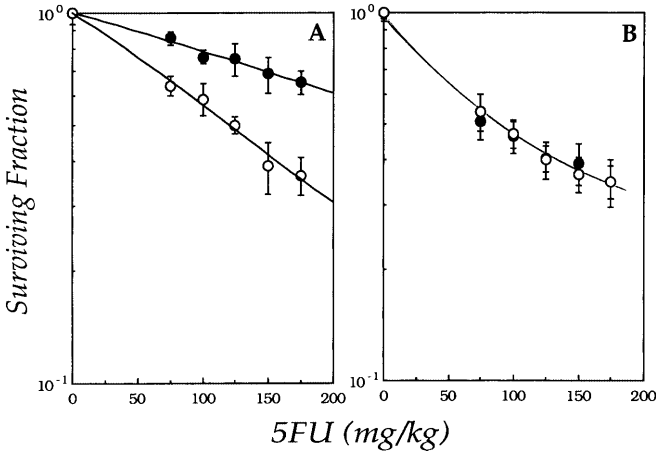

Fig. 3A, B. Antitumor effect (**A**) and bone marrow toxicity (**B**) of 10 mg/kg NLCQ-1 given i.p. 1 h before 5FU in C3H mice bearing SCCVII tumors (*closed circles* 5FU alone, *open circles* NLCQ-1 plus 5FU). Assays were carried out 18 h after the last drug administration. Bone marrow toxicity was assayed by CFU-GM. Three mice per point were used (six tumors). Single cell suspensions from tumor obtained after each tumor dissociation or of bone marrow cells retrieved from the three mice were combined and plated in quadruplicate to form colonies (*bars* SD of quadruplicate measurements)

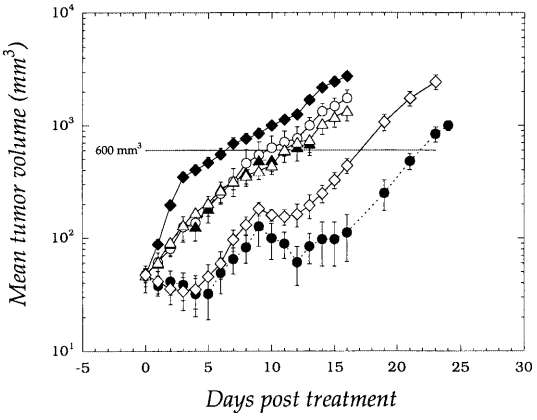

Fig. 4. Response of SCCVII tumors to NLCQ-1 or TPZ treatment combined with 5FU. Relative mean tumor volume is plotted as a function of time after treatment. NLCQ-1 (10 mg/kg) or TPZ (23 mg/kg) was given 1 h before 5FU (50 mg/kg) twice a day 4 h apart on days 0 and 9. Eight mice per point were used (*bars* SD). Tumor growth delay was evaluated at tumor volume of 600 mm^3 (*closed diamonds* saline, *open circles* NLCQ-1 alone, *open triangles* TPZ alone, *closed triangles* 5FU alone, *open diamonds* TPZ plus 5FU, *closed circles* NLCQ-1 plus 5FU)

did not develop any sign of sickness, and 5FU-related cachexia was not seen.

Discussion

The results of the present study showed that TPZ failed to significantly potentiate the antitumor effect of 5FU in the EMT6/Balb/c model (clonogenic assay), in contrast to our previous results in the same model [35]. Thus, a DMF_T value of only 1.2 was obtained (Table 1). It is not certain if this failure reflects lack of hypoxia, since a DMF_T of 2.3 was obtained with an equitoxic dose of NLCQ-1 (Table 1), or if the administration schedule should be reconsidered in the case of TPZ. In our previous study [35], TPZ potentiated 5FU when administered 3 h before or 1 h after 5FU. In the present study only the 3 h before schedule was used. Similarly, in the EMT6/BALB/c model, TPZ potentiated Taxol when administered before or after Taxol [35]. However, in DMF-determining experiments in the same model, TPZ potentiated Taxol only when it followed Taxol (unpublished results).

The results in the SCCVII/C3H model, when the clonogenic assay was used as the endpoint, again showed that TPZ failed to potentiate 5FU regardless of the administration schedule, yielding a degree of potentiation value only 2.9 ± 2.2 (Table 1). Even though oxygen was not measured in this study, we assumed that the tumors in the clonogenic experiments were large enough to ensure significant hypoxia. The degree of potentiation obtained following treatment with NLCQ-1 in SCCVII tumors was less than half the corresponding degree of potentiation obtained in EMT6 tumors (Table 1), consistent with the report that SCCVII tumors are more oxygenated than EMT6 tumors [1]. However, it should be emphasized that the ThI values obtained following treatment with NLCQ-1 in both tumor models were identical (Table 1), which implies that therapeutic benefits can be achieved with NLCQ-1 plus 5FU in tumors with different degrees of hypoxia. On the other hand, potentiation of 5FU by NLCQ-1 seemed to be not only administration schedule-dependent but also tumor model-specific.

In the tumor regrowth studies NLCQ-1 and TPZ were given at the non-toxic total doses of 4×10 mg/kg and 4×23 mg/kg, respectively [6, 33]. Pilot toxicity studies in non-tumor-bearing mice were not conducted for 5FU, but the total 5FU dose given was much less than the reported LD_{50} value (437 mg/kg; 95% confidence limits) for 5FU (given as a single i.p. dose) in C3H mice [9]. However, in our experiments, 5FU alone given at 2×50 mg/kg on day 0 and day 9 to a final dose of 200 mg/kg was quite toxic in SCCVII tumor-bearing C3H mice, resulting in 50% deaths by day 13. Despite this lethality, no biologically significant tumor growth delay was observed following treatment with 5FU alone. Thus, the corresponding log kill was < 1 (Table 2). On the other hand, at the given schedule, treatment with 5FU in combination with either bioreductive compound NLCQ-1 or TPZ did not result in any lethality or 5FU-associated cachexia, suggesting that each bioreductive drug played a protective role against systemic toxicity. With regard to TPZ, this is contrary to previous reports, in which the combination of TPZ and 5FU always increased lethality, even at a final dose of 4×40 mg/kg 5FU [9, 22]. However, in the first reference, TPZ plus 5FU was given as a single dose whereas in the latter, both the schedule (administration for four consecutive days) and

Table 2. Time for tumors to grow to 600 mm^3, tumor growth delay and specific growth delay (SGD) relative to saline-treated control, and corresponding log kill

Treatment group	Time to grow to 600 mm^3 (days ± SD)	Tumor growth delay (days)	SGD	Log kill
Control (saline)	6.4 ± 0.7	–	–	–
5FU	11.1 ± 2.4*	4.7	2.35	0.71
NLCQ-1	9.7 ± 2.7*	3.3	1.65	0.50
TPZ	11.1 ± 2.0*	4.7	2.35	0.71
TPZ + 5FU	16.8 ± 1.4**	10.4	5.20	1.56
NLCQ-1 + 5FU	21.7 ± 1.8***	15.3	7.65	2.30

*$P < 0.001$ vs control
**$P < 0.001$ vs 5FU alone
***$P < 0.001$ vs TPZ + 5FU

the model (human xenografts in nude mice) were different than the ones we used.

The fact that TPZ causes blood flow alterations [10] may explain the observed protection, especially when 5FU is given at relatively small doses. Protection in cisplatin-induced renal toxicity has also been reported when TPZ is combined with cisplatin, but the mechanism of such protection is unclear [47]. However, in another experiment in which the same regimen was kept for all drugs except that 5FU was given at 75 mg/kg instead of 50 mg/kg, lethality was observed not only in the group treated with 5FU alone (33% by day 14) but also in the groups receiving combination treatment. Thus, 16% of the mice treated with NLCQ-1 plus 5FU or TPZ plus 5FU had died by days 15 and 7, respectively (the results of this experiment are not shown).

Concerning tumor growth delay, a marked effect was noted only in the group treated with NLCQ-1 plus 5FU. Thus, the tumor growth delay compared to the saline-treated control group was 15.3 days, of which 7.3 days were due to potentiation. This tumor growth delay corresponds to 2.3 log kill and thus it can be considered biologically significant. Furthermore, this biologically significant effect was obtained by combining marginally active doses of NLCQ-1 and 5FU with log kill values of 0.50 and 0.71, respectively. In contrast, TPZ enhances the activity of chemotherapeutic agents such as bleomycin when the latter is given at an already active dose [22]. It should be mentioned that the NLCQ-1 plus 5FU combination treatment appeared more efficacious in terms of log kill in the tumor regrowth assay experiments than in the in vivo-in vitro assay experiments. This discrepancy could be attributed to the slightly higher 5FU dose used, but mainly to the different administration schedule followed in the tumor regrowth assay experiments, which could lead to improved pharmacokinetics. It is known that 5FU has a short plasma half-life and that increased response rates are seen with infusional protocols [51]. Also, it is known that thymidylate synthase inhibition by 5FU is limited to the S-phase of the cell cycle [51] and thus the probability of tumor cells being in this phase could be increased in a fractionated administration protocol.

Significant tumor growth delay was also observed in the group treated with TPZ plus 5FU compared to those treated with TPZ or 5FU alone. However, the delay was very close to that expected from the mere additive effect of the two drugs alone, consistent with the results obtained in the clonogenic assay. Thus, the extra delay beyond additivity was only 1 day. However, the TPZ plus 5FU combination treatment led to a log kill of 1.56, which is biologically significant, once again indicating the advantage of combining inactive modalities to produce active treatments.

With regard to mechanism(s), preliminary in vitro results showed that enhancement of apoptosis (i.e. increased caspase 3 activation and nucleosome formation), unrepairable DNA damage and persistent inhibition of DNA, RNA and protein synthesis are some of the mechanisms involved in the potentiation of 5FU by NLCQ-1 [36]. TPZ alone is known to produce DNA single and double strand breaks, and chromosome aberrations [5], and to also dramatically inhibit DNA replication [40] in vitro. Therefore, TPZ could theoretically potentiate the antitumor effect of 5FU by similar mechanisms. However, tumor penetration by TPZ might be compromised by its high metabolic rate [16]. Alternatively, blood flow alterations caused by TPZ [10] might have a negative impact on 5FU pharmacokinetics and efficacy, especially when TPZ is given before 5FU.

Our present findings also suggest that potentiation of chemotherapy by NLCQ-1 or TPZ might be both tumor- and administration schedule-dependent. This makes sense if we take into account the variability in tumor-related hypoxia and enzymatic profile which affect bioreductive drug metabolism, as well as possible alterations that one combined agent might cause in the pharmacokinetics of the other. Thus, NLCQ-1 potentiates Taxol in PC-3 xenograft-bearing athymic mice and in FSaIIC-bearing C3H mice when given 90 min before or 90 min after Taxol, respectively (unpublished results). Similarly, it has been suggested that sequencing of TPZ with other agents requires careful consideration in the clinic [10].

In summary, the present investigation demonstrated that an appropriately scheduled NLCQ-1 plus 5FU combined treatment regimen could effectively increase the antitumor efficacy of this chemotherapeutic agent without concurrent enhancement in systemic toxicity, at least in murine tumor models. Moreover, a therapeutic advantage was obtained even with combinations of NLCQ-1 and 5FU at marginally active doses. Therefore,

experiments with even lower and thus less-toxic 5FU multiple doses combined with this bioreductive agent should be considered for future investigation.

References

1. Aboagye EO, Maxwell RJ, Kelson AB, Tracy M, Lewis AD, Graham MA, Horsman MR, Griffiths JR, Workman P (1997) Preclinical evaluation of fluorinated 2-nitroimidazole N-(2-hydroxy-3,3,3-trifluoropropyl)-2-(2-nitro-1-imidazolyl) acetamide (SR-4554) as a probe for measurement of tumor hypoxia. Cancer Res 57:3314
2. Bedikian AY, Legha SS, Eton O, Buzaid AC, Papadopoulos N, Coates S, Simmons T, Neefe J, von Roemeling R (1997) Phase II trial of tirapazamine combined with cisplatin in chemotherapy of advanced malignant melanoma. Ann Oncol 8:363
3. Blumenthal RD, Taylor A, Osorio L, Ochakovskaya R, Raleigh J, Papadopoulou M, Bloomer WD, Goldenberg DM (2001) Optimizing the use of combined radioimmunotherapy and hypoxic cytotoxin therapy as a function of tumor hypoxia. Int J Cancer 94:564
4. Bremner JCM, Stratford IJ, Bowler J, Adams GE (1990) Bioreductive drugs and the selective induction of tumor hypoxia. Br J Cancer 61:717
5. Brown MJ (1993) SR 4233 (tirapazamine): a new anticancer drug exploiting hypoxia in solid tumours. Br J Cancer 67:1163
6. Brown MJ, Lemmon MJ (1990) Potentiation by the hypoxic cytotoxin SR4233 of cell killing produced by fractionated irradiation of mouse tumors. Cancer Res 50:7745
7. Cuvier C, Jang A, Hill RP (1997) Exposure to hypoxia, glucose starvation and acidosis: effect on invasive capacity of murine tumor cells and correlation with cathepsin (L + B) secretion. Clin Exp Metastasis 15:19
8. Dorie MJ, Brown JM (1993) Tumor-specific, schedule-dependent interaction between tirapazamine (SR4233) and cisplatin. Cancer Res 53:4633
9. Dorie MJ, Brown JM (1997) Modification of the antitumor activity of chemotherapeutic drugs by the hypoxic cytotoxic agent tirapazamine. Cancer Chemother Pharmacol 39:361
10. Durand RE, Olive PL (1997) Physiologic and cytotoxic effects of tirapazamine in tumor-bearing mice. Radiat Oncol Investig 5:213
11. Friery OP, Gallagher R, Murray MM, Hughes CM, Galligan ES, McIntyre IA, Patterson LH, Hirst DG, McKeown SR (2000) Enhancement of the anti-tumor effect of cyclophosphamide by the bioreductive drugs AQ4N and tirapazamine. Br J Cancer 82:1469
12. Gallagher R, Hughes CM, Murray MM, Friery OP, Patterson LH, Hirst DG, McKeown SR (2001) The chemopotentiation of cisplatin by the novel bioreductive drug AQ4N. Br J Cancer 85:625
13. Graham CH, Forsdike J, Fitzgerald CJ, MacDonald-Goodfellow S (1999) Hypoxia-mediated stimulation of carcinoma cell invasiveness via upregulation of urokinase receptor expression. Int J Cancer 80:617
14. Grau C, Overgaard J (1988) Effect of cancer chemotherapy on the hypoxic fraction of a solid tumor measured using a local tumor control assay. Radiother Oncol 13:301
15. Hay MP, Wilson WR, Moselen JW, Palmer BD, Denny WA (1994) Hypoxia-selective antitumor agents. 8. Bis(nitroimidazolyl)alkanecarboxamides: a new class of hypoxia-selective cytotoxins and hypoxic cell radiosensitisers. J Med Chem 37:381
16. Hicks KO, Fleming Y, Siim BG, Koch CJ, Wilson WR (1998) Extravascular diffusion of tirapazamine: effect of metabolic consumption assessed using the multicellular layer model. Int J Radiat Oncol Biol Phys 42:641
17. Hirst DG, Brown JM, Hazlehurst JL (1983) Effect of partition coefficient on the ability of nitroimidazoles to enhance the cytotoxicity of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea. Cancer Res 43:1961
18. Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P (1996) Association between tumor hypoxia and malignant progression in advanced cancer of uterine cervix. Cancer Res 56:4509
19. Holden SA, Teicher BA, Ara G, Herman TS, Coleman CN (1992) Enhancement of alkylating agent activity by SR-4233 in FSaIIC murine fibrosarcoma. J Natl Cancer Inst 84:187
20. Kennedy KA (1987) Hypoxic cells as specific drug targets for chemotherapy. Anticancer Drug Des 2:181
21. Kovacs MS, Hocking DJ, Evans JW, Siim BG, Wouters BG, Brown JM (1999) Cisplatin anti-tumour potentiation by tirapazamine results from a hypoxia-dependent cellular sensitization to cisplatin. Br J Cancer 80:1245
22. Lartigau E, Guichard M (1996) The effect of tirapazamine (SR-4233) alone or combined with chemotherapeutic agents on xenografted human tumors. Br J Cancer 73:1480
23. Marshall JL, Figueira M, Dahut W, Rizvi N, von Roemeling R (1998) Tirapazamine plus cisplatin in the treatment of patients with breast cancer and other platinum resistant tumors. A phase II clinical trial. Cancer Ther 1:237
24. Miller VA, Ng KK, Grant SC, Kindler H, Pizzo B, Heelan RT, von Roemeling R, Kris MG (1997) Phase II study of the combination of the novel bioreductive agent, tirapazamine, with cisplatin in patients with advanced non-small cell lung cancer. Ann Oncol 8:1269
25. Papadopoulou MV, Miller A, Seskey T, Epperly MW, Bloomer WD (1993) Potentiation of antineoplastic drugs in vitro and in vivo by intercalating bioreductive agents. Radiat Oncol Investig 1:206
26. Papadopoulou MV, Ji M, Bloomer WD (1996) THNLA-1: a DNA-targeted bioreductive agent as chemosensitizer in vitro and in vivo. In Vivo 10:49
27. Papadopoulou MV, Ji M, Bloomer WD (1996) THNLA-1 as radio/chemosensitizer of EMT6 tumors in mice. Br J Cancer 74 [Suppl XXVII]: S267
28. Papadopoulou MV, Ji M, Rao MK, Bloomer WD (1997) 9-[3-(2-Nitro-1-imidazolyl)propylamino]-cyclopenteno[b]quinoline hydrochloride (NLCPQ-1): a novel DNA-affinic bioreductive agent as chemosensitizer. I. Oncol Res 9:249
29. Papadopoulou MV, Ji M, Rao MK, Bloomer WD (1997) 4-[3-(2-Nitro-1-imidazolyl)-propylamino]-7-chloroquinoline hydrochloride (NLCQ-1), a novel bioreductive compound; cytotoxicity and reduction studies. Second International Conference on Redox Processes and Cancer: Molecular Biology and Therapeutics. Banff, Alberta, Canada, 7–10 April. Proceedings
30. Papadopoulou MV, Ji M, Bloomer WD (1998) NLCQ-1, a novel hypoxic cytotoxin: potentiation of melphalan, cisDDP and cyclophosphamide in vivo. Int J Radiat Oncol Biol Phys 42:775
31. Papadopoulou MV, Ji X, Xue C, Bloomer WD (2000) In vitro schedule-dependent potentiation of taxol, 5FU and cisDDP by the hypoxic cytotoxin NLCQ-1. Comparison with tirapazamine. 11th International Conference on Chemical Modifiers of Cancer Treatment. Tumor Physiology and Cancer Treatment. Banff, Alberta, Canada, 5–7 October. Proceedings
32. Papadopoulou MV, Ji M, Rao MK, Bloomer WD (2000) 4-[3-(2-Nitro-1-imidazolyl)-propylamino]-7-chloroquinoline hydrochloride (NLCQ-1), a novel bioreductive compound as a hypoxia-selective cytotoxin. Oncol Res 12:185
33. Papadopoulou MV, Ji M, Rao MK, Bloomer WD (2000) 4-[3-(2-Nitro-1-imidazolyl)-propylamino]-7-chloroquinoline hydrochloride (NLCQ-1). A novel bioreductive agent as radiosensitizer in vitro and in vivo. Comparison with tirapazamine. Oncol Res 12:325
34. Papadopoulou MV, Ji M, Bloomer WD (2000) Potentiation of chemotherapeutic drugs by the bioreductive compound NLCQ-1 against SCCVII tumors in mice. Proc Am Assoc Cancer Res 41:766
35. Papadopoulou MV, Ji M, Bloomer WD (2001) Schedule-dependent potentiation of chemotherapeutic drugs by the

bioreductive compounds NLCQ-1 and tirapazamine against EMT6 tumors in mice. Cancer Chemother Pharmacol 48:160
36. Papadopoulou MV, Ji X, Bloomer WD (2001) Mechanisms involved in the potentiation of taxol or 5-FU by the hypoxic cytotoxin NLCQ-1. Clinical Cancer Res [Suppl] 7:3679s
37. Papadopoulou MV, Ji X, Bloomer WD (2002) Hypoxia-dependent retinal toxicity of NLCQ-1 and tirapazamine in BALB/c mice. Proc Am Assoc Cancer Res 43:1092
38. Papadopoulou MV, Ji M, Bloomer WD, Hollingshead MG (2002) Enhancement of the antitumor effect of cyclophosphamide with the hypoxia-selective cytotoxin NLCQ-1 against murine tumors and human xenografts. J Exp Ther Oncol 2:(in press)
39. Patterson LH, McKeown SR (2000) AQ4N: a new approach to hypoxia-activated cancer chemotherapy (review). Br J Cancer 83:1589
40. Peters KB, Wang H, Brown MJ, Iliakis G (2001) Inhibition of DNA replication by tirapazamine. Cancer Res 61:5425
41. Plowman J, Dykes DJ, Hollingshead M, Simpson-Herren L, Alley MC (1997) Human tumor xenograft models in NCI drug development. In: Teicher B (ed) Anticancer drug development guide: preclinical screening, clinical trials, and approval. Humana Press, Totowa, pp 101–125
42. Rice GC, Hoy C, Schimke RT (1986) Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells. Proc Natl Acad Sci U S A 83:5978
43. Rockwell SC, Kallman RF, Fajardo LF (1972) Characteristics of a serially transplanted mouse mammary tumor and its tissue-culture-adapted derivative. J Natl Cancer Inst 49:735
44. Sartorelli AC (1988) Therapeutic attack of hypoxic cells of solid tumors: presidential address. Cancer Res 48:775
45. Schmoll HJ, Buchele T, Grothey A, Dempke W (1999) Where do we stand with 5-fluorouracil? (review). Semin Oncol 26:589
46. Siemann DW, Allalunis-Turner MJ (1988) Potentiation of combination chemotherapy by nitroheterocyclics. Int J Radiat Oncol Biol Phys 16:973
47. Siemann DW, Hinchman CA (1998) Potentiation of cisplatin activity by the bioreductive agent tirapazamine. Radiother Oncol 47:215
48. Siemann DW, Mulcahy RT (1986) Sensitization of cancer chemotherapeutic agents by nitroheterocyclics. Biochem Pharmacol 35:111
49. Siemann DW, Sutherland RM (1992) Potentiation of alkylating chemotherapy by dual function nitrofurans in multicell spheroids and solid tumors. Radiother Oncol 24:239
50. Teicher BA, Holden SA, al-Achi A, Herman TS (1990) Classification of antineoplastic treatments by their differential toxicity toward putative oxygenated and hypoxic tumor subpopulations in vivo in the FSaII murine fibrosarcoma. Cancer Res 50:3339
51. Thomas DM, Zalcberg JR (1998) 5-Fluorouracil: a pharmacological paradigm in the use of cytotoxics (review). Clin Exp Pharmacol Physiol 25:887
52. Treat J, Johnson E, Langer C, Belani C, Haynes B, Greenberg R, Rodriquez R, Drobins P, Miller W Jr, Meehan L, McKeon A, Devin J, von Roemeling R, Viallet J (1998) Tirapazamine with cisplatin in patients with advanced non-small-cell lung cancer: a phase II study. J Clin Oncol 16:3524
53. Vincent M, Labianca R, Harper P (1999) Which 5-fluorouracil regimen? – the great debate (review). Anticancer Drugs 10:337
54. von Pawel J, von Roemeling R, Gatzemeier U, Boyer M, Elisson LO, Clark P, Talbot D, Rey A, Butler TW, Hirsh V, Olver I, Bergman B, Ayoub J, Richardson G, Dunlop D, Arcenas A, Vescio R, Viallet J, Treat J (2000) Tirapazamine plus cisplatin versus cisplatin in advanced non-small-cell lung cancer: a report of the international CATAPULT I study group. J Clin Oncol 18:1351
55. Zaider M (1990) Concepts for describing the interaction of two agents. Radiat Res 123:257
56. Zhivotovsky B, Joseph B, Orrenius S (1999) Tumor radiosensitivity and apoptosis. Exp Cell Res 248:10