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ORIGINAL ARTICLE

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Schedule-dependent potentiation of chemotherapeutic drugs by the bioreductive compounds NLCQ-1 and tirapazamine against EMT6 tumors in mice

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Abstract *Purpose*: Comparisons of schedule-dependent interactions between the hypoxic cytotoxins NLCQ-1/tirapazamine (TPZ) and various chemotherapeutic drugs in BALB/c mice bearing EMT6 tumors. *Methods*: The antitumor effects of the single or combined drugs were assessed with various administration time intervals using the in vivo-in vitro clonogenic assay as the endpoint. The chemotherapeutic drugs tested were cisplatin (cisDDP), melphalan (L-PAM), cyclophosphamide (CPM), 5-fluorouracil (5-FU), doxorubicin (Doxo), etoposide (VP-16) and Taxol at doses of 8, 5, 100, 150, 12, 35 and 20 mg/kg, respectively. NLCQ-1 was given at 10 mg/kg (28% of its single LD_{50} value) and TPZ was given at 30 mg/kg (38% of its single LD_{50} value). All drugs were given by i.p. injection in saline or as commercially available pharmaceutical solutions. *Results*: Schedule-dependent synergistic interactions with different patterns for each bioreductive drug were observed with almost all of the chemotherapeutic agents examined. Potentiation accounting for more than 25% of the total tumor cell killing was observed with NLCQ-1/TPZ and cisDDP, L-PAM, CPM, 5-FU and Taxol at the optimal administration intervals. Potentiation accounting for 70% of the total tumor cell killing was found with NLCQ-1 and CPM. *Conclusions*: These results suggest a potential clinical use of NLCQ-1/TPZ as adjuvants to certain chemotherapeutic agents.

Keywords NLCQ-1 · Tirapazamine · Chemotherapeutic agents · Potentiation

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Introduction

Tumor-associated hypoxia can affect cure rates in both radiotherapy and chemotherapy [7, 8, 34]. There are various reasons for the resistance of hypoxic tumor tissues to chemotherapeutic drugs, including their distance from the viable blood vessels, their slower rate of proliferation, as well as the hypoxic environment itself [5, 11, 33]. For example, most chemotherapeutic agents target rapidly proliferating cells and therefore fail to efficiently kill viable, slowly proliferating or nonproliferating hypoxic cells, especially since such cells are not in proximity to the blood vessels. Ample preclinical data have shown that hypoxia-selective cytotoxins, which are activated to toxic metabolites in areas of reduced oxygenation, not only directly kill hypoxic cells but also sensitize them to the antitumor effect of conventional chemotherapy [1, 2, 9, 14, 17, 18, 19, 20, 21, 24, 26, 28, 36]. It has also been suggested that the most potent and selective hypoxic cytotoxins might be superior chemopotentiators [37, 38]. Thus, it was soon realized that a therapeutic gain could be achieved, at least in animal models, by combining hypoxia-selective cytotoxins with chemotherapeutic agents due to a synergistic interaction [1, 2, 9, 20, 26, 28, 36]. Recent clinical trials of the hypoxic cytotoxin tirapazamine (3-amino-1,2,4-benzotriazine-1,4-dioxide, SR-4233, TPZ) combined with cisplatin (cisDDP) have shown that a therapeutic gain can also be achieved in the clinic from such combinations in terms of overall response rates and survival [16, 40, 41].

4-[3-(2-Nitro-1-imidazolyl)-propylamino]-7-chloroquinoline hydrochloride (NLCQ-1, Fig. 1), is a novel, weakly DNA-intercalating hypoxic cytotoxin developed in our laboratory [22, 23, 30], which demonstrates an increasing hypoxic potency and selectivity with time, normally only seen with the so-called ''bis-bioreductive agents'', compounds bearing two reducible centers [6]. Thus, the hypoxic selectivity of NLCQ-1 in V79 cells at 50% survival is increased from 5- to 388-fold by increasing the exposure time from 1 to 4.5 h as the result

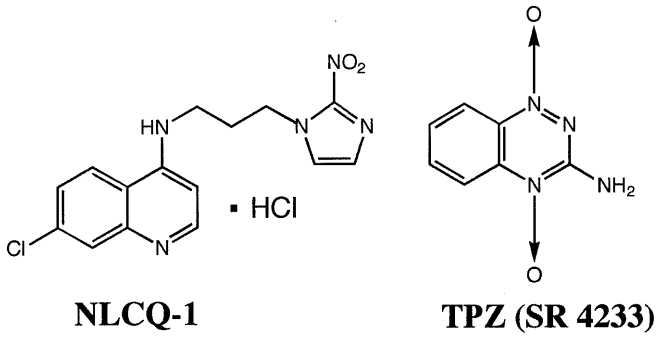

Fig. 1 Chemical structure of NLCQ-1 and TPZ

of a concomitant increase and decrease in its hypoxic and aerobic potency, respectively, over time [22, 30]. NLCQ-1 also synergistically enhances the effects of radiation against hypoxic cells in vitro and murine tumors in vivo (unpublished results; [23]). Most importantly, however, 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 (unpublished results; [26, 28]).

Because of these promising interactions, a further investigation was undertaken by the NCI to develop NLCQ-1 as an adjuvant to radio/chemotherapy in the clinic, under the RAID (Rapid Access to Intervention Development) Program (http://dtp.nci.nih.gov/docs/raid/raid_pp.html). Studies at the NCI showed that NLCQ-1 potentiates the antitumor effect of Taxol, cisDDP and cyclophosphamide (CPM) in human xenografts without a concomitant increase in systemic toxicity, exhibits good stability in human plasma and shows favorable pharmacokinetics in mice (unpublished results; [39]).

Thus, the present study was undertaken in order to complete our investigation with regard to possible synergistic interactions between NLCQ-1 and various chemotherapeutic drugs including Taxol, doxorubicin (Doxo), etoposide (VP-16) and 5-fluorouracil (5-FU), besides the alkylating agents melphalan (L-PAM), cisDDP and CPM in BALB/c mice bearing EMT6 tumors. Such investigations give insights into the mechanism(s) involved in the potentiation and help in the proper designing of a beneficial drug delivery in the clinic. The leading bioreductive drug TPZ was also used in a parallel comparison study. Similar studies have been conducted previously with TPZ and chemotherapeutic drugs in less-hypoxic RIF-1 fibrosarcoma tumors in C3H/Km mice [1, 2] and a schedule-dependent potentiation was observed.

Materials and methods

Drugs

NLCQ-1 (synthesized in our laboratory [23, 30], purified by recrystallization from methanol and checked for purity by TLC and HPLC), TPZ (generously provided by Sanofi-Winthrop, Malvern, Pa.) and CPM (Aldrich, Milwaukee, Wis.) were dissolved in saline at 1, 0.75 and 10 mg/ml, respectively. L-PAM (Sigma, St. Louis, Mo.) was first dissolved in 1% HCl in ethanol and subsequently diluted 100-fold with PBS at 0.5 mg/ml. cisDDP (Sigma) was dissolved in PBS at 0.5 mg/ml. Taxol (Bristol-Myers Squibb Co., Princeton, N.J.) at 6 mg/ml, Doxo (Bedford Laboratories, Bedford, Ohio) at 2 mg/ml, VP-16 (Bristol-Myers Squibb Co.) at 20 mg/ml, and 5-FU (Pharmacia, Kalamazoo, Mich.) at 50 mg/ml were used as formulated solutions from the companies. All drugs were injected intraperitoneally (i.p.) on the basis of animal body weight. A hyaluronidase solution (15 U/mouse in 0.05 ml saline; Wyeth Laboratories, Philadelphia, Pa.) was administered i.p. to the mice immediately after Taxol administration as a local adjuvant to reduce Taxol ulcerative toxicity [3]. Also, mannitol (100 µl, 50 mg/ml; Abbott Laboratories, North Chicago, Ill.) was injected i.p. into each mouse before and after cisDDP administration to eliminate potentially lethal and unacceptable kidney damage [15].

Mice and tumors

EMT6 tumor cells (murine mammary carcinoma; a gift from Dr. B. Teicher, Dana-Farber Cancer Institute, Boston, Mass.) [32], which were maintained as exponentially growing monolayer cultures in RPMI-1640 medium supplemented with 10% FBS, were inoculated (s.c.) into both legs (2×10^5 cells in 0.2 ml per leg) of female BALB/c mice (Harlan Sprague-Dawley, Indianapolis, Ind.) weighing 16–17 g and housed under germ-free conditions. All studies were conducted according to the guidelines set by the Evanston Northwestern Healthcare Institutional Animal Care.

Chemosensitization

Treatment was initiated when tumors (four per group) were 8–10 mm (about 2 weeks after inoculation). All drugs were given by i.p. injection and the total injected volume was $\leq$ 0.5 ml. NLCQ-1 was always given at 10 mg/kg (0.027 mmol/kg) and TPZ at 30 mg/kg (0.168 mmol/kg) which represent 28% [26] and 38% [2] of their single LD_{50} values, respectively. Each bioreductive drug was given at various times before or after a single chemotherapeutic agent. The in vivo-in vitro assay was used as the endpoint as described previously [20]. Tumors were excised 18 h after the final 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 at a rate of 1 ml per 60 mg of tumor 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. Then the cells were 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 efficiency of untreated EMT6 tumor-cells was 32.5–40.5% and was taken into account in the calculation of survival. Survival of the treated groups was calculated as the fraction in relation to the untreated control. The additive effect was calculated as the product of the surviving fractions following treatment with each combined drug alone [2]. The percentage of tumor cells that were killed due to pure potentiation is the degree of potentiation (P) which was determined by subtracting the surviving fraction obtained with the combination treatment (SFc) from the calculated additive effect (SFa) and multiplying by 100: $P = (SFa\text{-}SFc) \times 100$.

Results

The chemical structures of NLCQ-1 and TPZ are shown in Fig. 1. In all presented studies the NLCQ-1 dose used was sixfold less (in millimoles per kilogram) than and

slightly less than equitoxic to the TPZ dose. The surviving fractions obtained following treatment with each drug alone have been omitted in most of the plots for clarity purposes. In general, the surviving fraction obtained with NLCQ-1 alone was not less than 0.890 (1.00–0.890) whereas the mean surviving fraction with TPZ was 0.770 (0.920–0.620).

A schedule-dependent synergistic interaction was observed between NLCQ-1/TPZ and the crosslinking agent cisDDP, given at 8 mg/kg, against EMT6 tumors. The optimum potentiation was obtained when NLCQ-1/TPZ were administered 45 or 120 min before cisDDP, respectively, and P values of 35.1% and 26.5% were calculated, respectively (Fig. 2; Table 1). The synergistic effect was larger with NLCQ-1 even though NLCQ-1 was given at a dose slightly less than equitoxic to the TPZ dose. The pattern of cisDDP potentiation observed with TPZ in EMT6 tumors was similar to that obtained in RIF-1 tumors [1]. However, in that case, the additive effect resulted in 99% killing, perhaps because a 1.6–2-fold higher TPZ dose was used, and therefore only the remaining 1% of the RIF-1 tumor cells were available to be killed due to potentiation by TPZ. Synergistic interactions were not investigated for intervals longer than ± 5 h between TPZ and cisDDP.

Figure 3 shows the results obtained from the study between NLCQ-1/TPZ and the alkylating agent L-PAM given at 5 mg/kg. A schedule-dependent interaction was observed with each bioreductive drug and L-PAM. Thus, optimum potentiation occurred when NLCQ-1/TPZ were given before L-PAM. Killing due to potentiation at the optimal administration time was 68.5% and 30.0% for NLCQ-1 and TPZ, respectively (Table 1).

Synergistic interactions were observed between NLCQ-1/TPZ and the alkylating agent CPM given at 100 mg/kg against EMT6 tumors (Fig. 4). In an analogous fashion to cisDDP and L-PAM, optimal synergism was obtained when NLCQ-1/TPZ were given about 2 h before CPM, resulting in P values of 70.0% and 51.3%, respectively (Table 1). The same pattern of CPM potentiation by TPZ, but much smaller, has been observed previously against KHT tumors [35]. In another study in

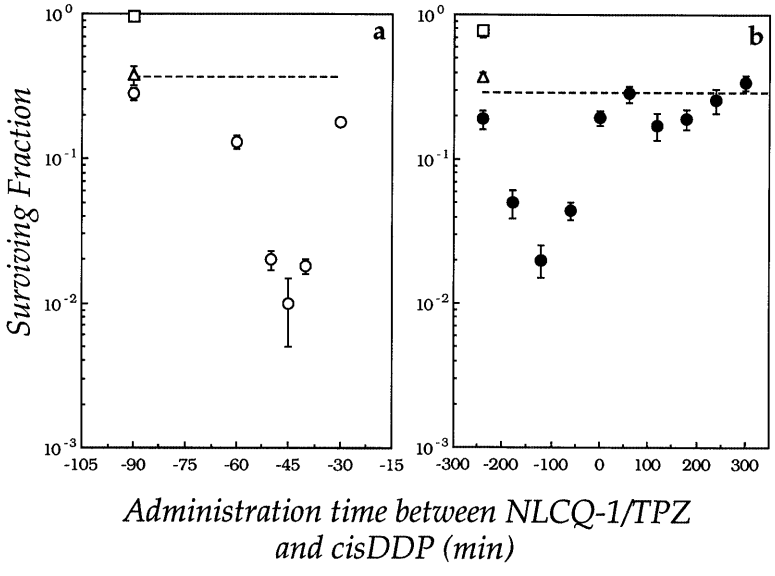

Fig. 2a, b Schedule-dependent potentiation of the antitumor effect of 8 mg/kg cisDDP by (**a**) NLCQ-1 (0.027 mmol/kg) (data from reference 26), or (**b**) TPZ (0.168 mmol/kg) in BALB/c mice bearing EMT6 tumors(❑ NLCQ-1/TPZ alone; Δ cisDDP alone; O, ● combined drugs). The *dashed line* represents the additive effect (calculated as the product of the surviving fractions obtained with the two independent agents). Four tumors per point were used. The single cell suspensions obtained after each tumor dissociation were combined and plated in quadruplicate to form colonies. *Bars* indicate SD of quadruplicate measurements

Table 1 Potentiation of chemotherapeutic agents by NLCQ-1/TPZ in EMT6 tumors obtained using the optimal administration schedule

Chemotherapeutic drug	NLCQ-1			TPZ		
	SFa[a]	SFc[b]	Degree of potentiation[c]	SFa[a]	SFc[b]	Degree of potentiation[c]
cisDDP	0.361	0.010	35.1	0.285	0.020	26.5
L-PAM	0.765	0.080	68.5	0.420	0.120	30.0
CPM	0.710	0.010	70.0	0.517	0.004	51.3
5-FU	0.755	0.200	55.5	0.600	0.070	53.0
Doxo	0.587	0.380	20.7	0.451	0.360	9.1
VP-16	0.382	0.200	18.2	0.400	0.210	19.0
Taxol	0.672	0.080	59.2	0.575	0.280	29.5

[a]Surviving fraction calculated for the additive effect (see Materials and methods)
[b]Actual surviving fraction obtained with the combination treatment
[c]Percentage of cells killed due to pure potentiation by the bioreductive compound (see Materials and methods)

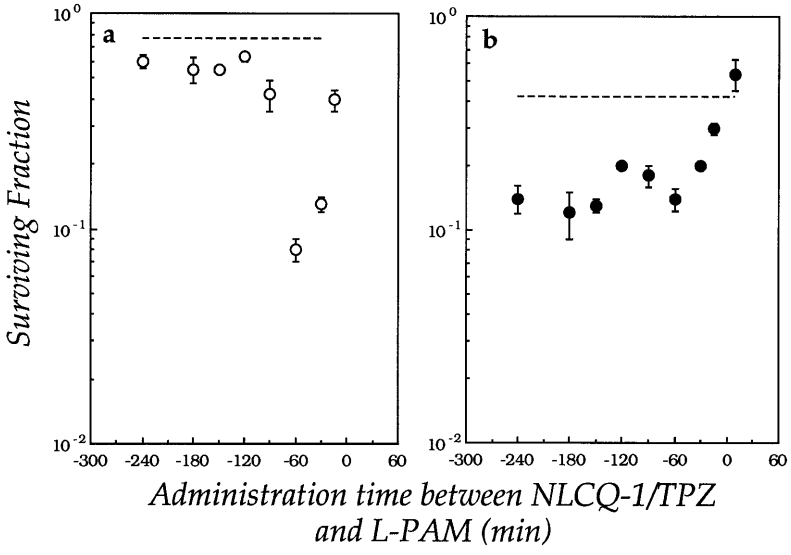

Fig. 3a, b Schedule-dependent potentiation of the antitumor effect of 5 mg/kg L-PAM by (**a**) NLCQ-1 (0.027 mmol/kg) (data from reference 26) or (**b**) TPZ (0.168 mmol/kg) in BALB/c mice bearing EMT6 tumors (O, ● combined drugs; NLCQ-1/TPZ alone not shown). The *dashed line* represents the additive effect (calculated as the product of the surviving fractions obtained with the two independent agents). Four tumors per point were used as described for Fig. 2. *Bars* indicate SD of quadruplicate measurements

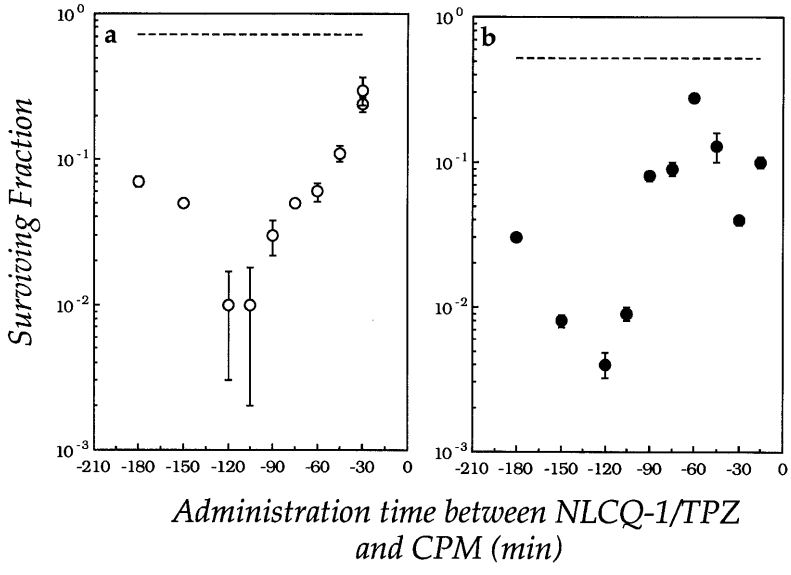

Fig. 4a, b Schedule-dependent potentiation of the antitumor effect of 100 mg/kg CPM by (**a**) NLCQ-1 (0.027 mmol/kg) (data from reference 26) or (**b**) TPZ (0.168 mmol/kg) in BALB/c mice bearing EMT6 tumors (O, ● combined drugs; NLCQ-1/TPZ alone not shown). The *dashed line* represents the additive effect (calculated as the product of the surviving fractions obtained with the two independent agents). Four tumors per point were used as described for Fig. 2. *Bars* indicate SD of quadruplicate measurements

which RIF-1 tumors were used, TPZ maximally enhanced the antitumor effect of CPM beyond additivity when it was given 24 h before CPM [2]. Only about 15% tumor cell killing was due to pure potentiation, despite the fact that a larger dose of TPZ (0.27 mmol/kg) was combined with 100 mg/kg CPM in the study against RIF tumors. However, the smaller potentiation by TPZ in this case could have been the result of the antitumor effect of CPM alone, which was significantly larger (second log) against RIF-1 tumors than that observed by us against EMT6 tumors (first log). In the current study we did not examine interactions at times beyond those shown in Fig. 4. Even though the same total killing effect was observed from both combination treatments, the P values showed clearly that a greater synergistic effect was obtained with NLCQ-1 than with TPZ. Therefore, 51% of the killing effect in the combination-treated group with TPZ was due to potentiation by TPZ and 49% due to additive toxicity of TPZ and CPM, whereas in the case of the group treated with NLCQ-1 plus CPM, 70% of the killing effect was due to potentiation by NLCQ-1 and 30% due to additivity.

Figure 5 shows interactions between NLCQ-1/TPZ and the thymidylate synthase inhibitor 5-FU, given at 150 mg/kg. In this case, potentiation patterns were different for each hypoxia-selective cytotoxin. Thus, for NLCQ-1, optimal potentiation occurred when NLCQ-1 was clearly administered after 5-FU, whereas in the case of TPZ, potentiation occurred when TPZ was administered 3 h before or 1 h after 5-FU, as has been seen before in RIF-1 tumors [2]. However, in our recent in vitro studies with V79 cells, potentiation of 5-FU by both NLCQ-1/TPZ occurred only when each bioreductive drug was given to the cells after 5-FU [29]. This may imply that schedule-dependent potentiation is also tumor-specific. Similar P values of 55.5% and 53.0% were

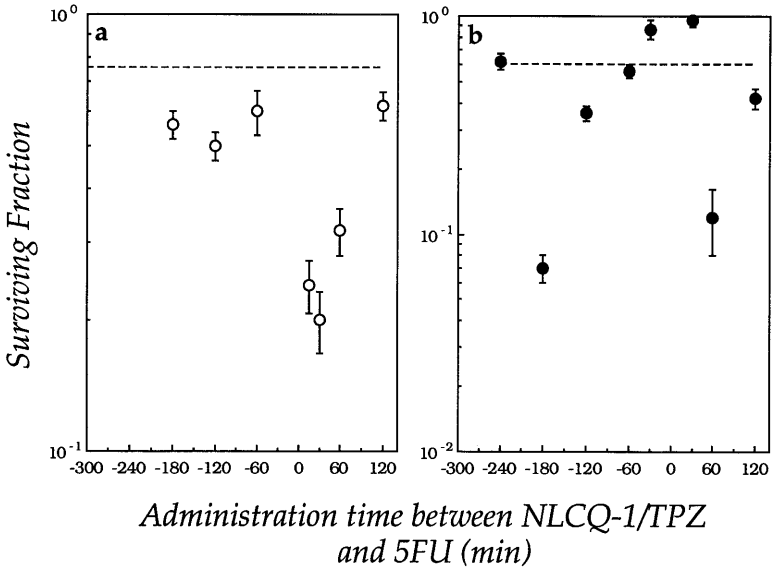

Fig. 5a, b Schedule-dependent potentiation of the antitumor effect of 150 mg/kg 5-FU by (**a**) NLCQ-1 (0.027 mmol/kg) or (**b**) TPZ (0.168 mmol/kg) in BALB/c mice bearing EMT6 tumors (O, ● combined drugs; NLCQ-1/TPZ alone not shown). The *dashed line* represents the additive effect (calculated as the product of the surviving fractions obtained with the two independent agents). Four tumors per point were used as described for Fig. 2. *Bars* indicate SD of quadruplicate measurements

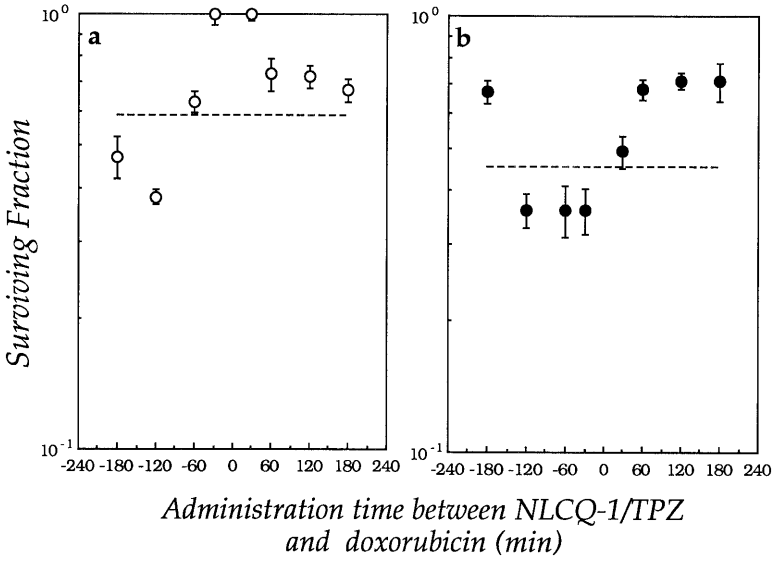

Fig. 6a, b Schedule-dependent potentiation of the antitumor effect of 12 mg/kg Doxo by (**a**) NLCQ-1 (0.027 mmol/kg) or (**b**) TPZ (0.168 mmol/kg) in BALB/c mice bearing EMT6 tumors (O, ● combined drugs; NLCQ-1/TPZ alone not shown). The *dashed line* represents the additive effect (calculated as the product of the surviving fractions obtained with the two independent agents). Four tumors per point were used as described for Fig. 2. *Bars* indicate SD of quadruplicate measurements

calculated for NLCQ-1 and TPZ, respectively, at the optimal administration intervals (Table 1). Some antagonistic interaction was observed when TPZ was administered very close to 5-FU. No more than an additive antitumor effect was observed for both combination treatments when NLCQ-1/TPZ was administered 12 h before 5-FU (data not shown).

The antitumor effect of 12 mg/kg Doxo was only slightly potentiated by NLCQ-1 or TPZ, also in a schedule-dependent manner (Fig. 6). Thus, a P value of 20.7% was obtained when NLCQ-1 was given 2 h before Doxo whereas a P value of 9.1% was obtained when TPZ was given 0.5–2 h before Doxo (Table 1). A significant antagonistic effect was observed when NLCQ-1 was given at the same time as or up to 3 h later than Doxo. Similarly, significant antagonism was observed when TPZ was given after Doxo or 3 h before Doxo. In the study using RIF tumors, potentiation occurred (P value about 9%) when TPZ was given only 24 h before a similar dose of 12 mg/kg Doxo [2]. However, no antagonism was observed in that study.

A slight synergistic interaction was observed also between NLCQ-1/TPZ and 35 mg/kg VP-16 (Fig. 7). In this case P values of 18.2% and 19.0% were calculated for NLCQ-1 and TPZ, respectively (Table 1). Optimal potentiation was obtained when NLCQ-1 was given 1 h before VP-16, and for TPZ, 3 h after VP-16. Antagonistic interactions were observed with both bioreductive drugs, with NLCQ-1 when it was administered at the same time as or shortly after VP-16, and with TPZ when it was administered at the same time as or shortly before or after VP-16. When similar interactions were investigated between TPZ and VP-16 against RIF-1 tumors, antagonism was observed at almost all the examined time intervals between the two drugs and slight

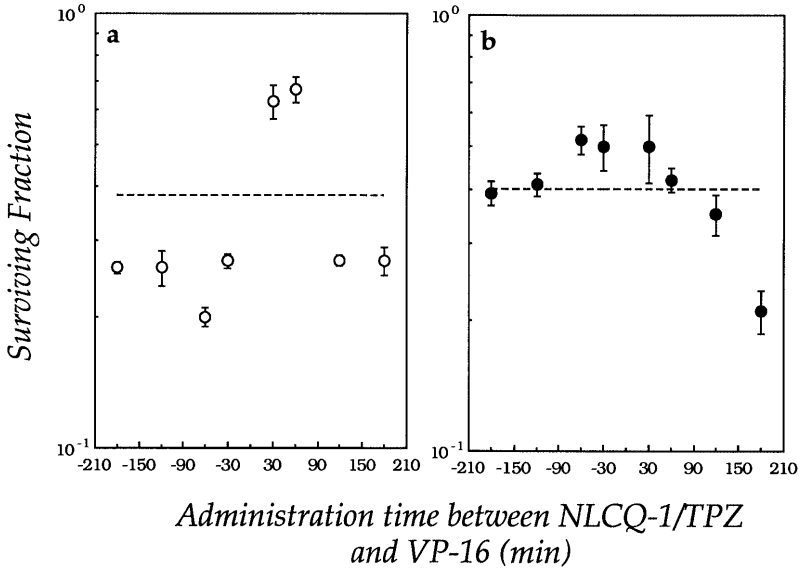

Fig. 7a, b Schedule-dependent potentiation of the antitumor effect of 35 mg/kg VP-16 by (**a**) NLCQ-1 (0.027 mmol/kg) or (**b**) TPZ (0.168 mmol/kg) in BALB/c mice bearing EMT6 tumors (O, ● combined drugs; NLCQ-1/TPZ alone not shown). The *dashed line* represents the additive effect (calculated as the product of the surviving fractions obtained with the two independent agents). Four tumors per point were used as described for Fig. 2. *Bars* indicate SD of quadruplicate measurements

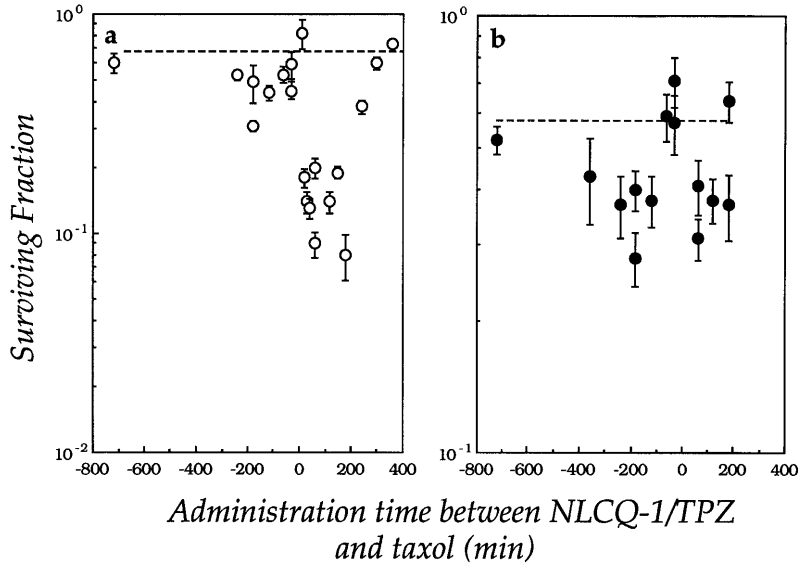

Fig. 8a, b Schedule-dependent potentiation of the antitumor effect of 20 mg/kg Taxol by (**a**) NLCQ-1 (0.027 mmol/kg) or (**b**) TPZ (0.168 mmol/kg) in BALB/c mice bearing EMT6 tumors (O, ● combined drugs; NLCQ-1/TPZ alone not shown). The *dashed line* represents the additive effect (calculated as the product of the surviving fractions obtained with the two independent agents). The results of two experiments are combined in the plots presented. Four tumors per point were used as described for Fig. 2. *Bars* indicate SD of quadruplicate measurements

potentiation (P value about 6.5%) was seen only when TPZ was given 24 h before VP-16 [2].

Finally, interactions between NLCQ-1/TPZ and 20 mg/kg of Taxol were investigated. Because Taxol is a widely used chemotherapeutic agent, two independent experiments were conducted. The results of these two experiments are combined in the plots presented in Fig. 8. A strong potentiation was observed when NLCQ-1 was administered 1–3 h after Taxol, resulting in a P value of 59.2%. Potentiation was also observed when TPZ was administered about 3 h before or 1–3 h after Taxol, resulting in a maximal P value of 29.5% (Table 1). However, when we investigated schedule-dependent interactions between NLCQ-1/TPZ and Taxol in vitro using V79 cells potentiation was obtained by both bioreductive compounds only when they were given to the cells 2–3 h after Taxol and never when they were given before [29]. No potentiation was seen when TPZ (or NLCQ-1) was given 12 h before Taxol, in contrast with the results reported for RIF-1 tumors, where TPZ yielded a P value of about 21.0% when it was administered 24 h before Taxol [2]. Also, our results using EMT6 tumors differ from those reported for RIF-1 tumors, where no potentiation of Taxol was seen when TPZ was administered after Taxol.

Discussion

As mentioned above, NLCQ-1 has been recently tested at the NCI against human tumor xenografts in athymic nude mice in combination with various commonly used chemotherapeutic agents and has shown significant synergism in the antitumor effect but not the systemic toxicity of chemotherapy (personal communication). Thus, Taxol alone, given i.v. at 8 mg/kg daily for 5 days

resulted in 11.7 days growth delay (compared to untreated control) in PC-3 prostate tumors, whereas Taxol in combination with NLCQ-1 at 10 mg/kg given i.p. 90 min before each dose of Taxol (which is not the optimal administration time) resulted in 18 days of tumor growth delay. Moreover, CPM given i.p. at the minimally active dose of 36 mg/kg daily for 4 days resulted in 5.2 days of tumor growth delay in PC-3 tumors, whereas CPM in combination with NLCQ-1 at 10 mg/kg given i.p. 90 min before each dose of CPM resulted in 12.8 days of tumor growth delay. In view of these promising results, NLCQ-1 is under consideration for phase I clinical testing. Therefore, it would be very significant to know the potential of NLCQ-1 as an adjuvant to chemotherapy with agents exhibiting various mechanisms of action, and to determine whether the administration schedule, the chemotherapeutic agent, the bioreductive agent itself, or all of these are important for potentiation. This was the reason for the inclusion of the leading bioreductive agent TPZ in the present study. Another factor (not examined here) that may be of importance for potentiation and is currently being investigated is the type of tumor itself [28].

The data reported here indicate some evidence that a schedule-dependent synergistic interaction occurred between NLCQ-1 and all of the examined chemotherapeutic agents. However, significant potentiation ($P > 25.0\%$) was observed only with cisDDP, L-PAM, CPM, 5-FU and Taxol, whereas there was some ambiguity with regard to potentiation of Doxo and VP-16. Similarly, cisDDP, L-PAM, CPM, 5-FU and Taxol were potentiated by TPZ in a schedule-dependent manner and with a P value $> 25.0\%$. Potentiation by NLCQ-1 was in general greater than that observed by TPZ.

Little schedule-dependency was observed in the potentiation of CPM by NLCQ-1 or in the potentiation of both CPM and L-PAM by TPZ since significant synergism was observed for a relatively broad range of time intervals (Figs. 3b and 4), with the proviso that NLCQ-1 or TPZ had to be administered before the alkylating agent. The same proviso was important for the potentiation of the crosslinking agent cisDDP by both NLCQ-1 and TPZ (Fig. 2).

A marked schedule dependency was observed for the potentiation of cisDDP, L-PAM, 5-FU and Taxol by NLCQ-1 and (with the exception of L-PAM) TPZ. However, some differences were observed in the behavior of the two bioreductive agents. Thus, optimal potentiation occurred when NLCQ-1 was administered clearly after 5-FU/Taxol whereas TPZ could be administered before or after 5-FU/Taxol. Also, minimal potentiation occurred when NLCQ-1 was administered before or after VP-16, and TPZ clearly after VP-16. The differences between our results with regard to TPZ and those reported previously [2] probably reflect differences in the tumor model. Such differences have been observed by us in our ongoing studies using SCCVII tumors in C3H mice (unpublished results). The more insensitive chemotherapeutic agents for both bioreductive drugs were Doxo and VP-16, and the most unfavorable administration schedule was simultaneous delivery with the chemotherapy. The latter might be the result of blood flow changes caused by NLCQ-1/TPZ, and indeed such changes caused by TPZ have been reported for some tumors [4]. Another reason could be that since synergistic interaction occurs between the bioreductive drug metabolites (rather than the intact drug) and chemotherapy, some time is probably needed for activation of the bioreductive drug before delivery of chemotherapy. This obviously makes sense in the case of the alkylating agents, where NLCQ-1/TPZ had to precede chemotherapy for potentiation to occur. For example, the plasma elimination half-lives of NLCQ-1 and L-PAM after i.p. administration to mice are similar (18.5 min [39] and 17.5–25 min [10], respectively), suggesting a similar drug absorption rate in the tumor, if no alteration occurs in the pharmacokinetics of L-PAM by NLCQ-1.

In the case of Doxo, whose activity is mainly due to DNA intercalation and indirect inhibition of topoisomerase II [42], competition for the same DNA sites may be the reason for the serious antagonism observed when the weak DNA-intercalating agent NLCQ-1 [22, 30] is given at the same time or after Doxo. Recent studies suggest that TPZ is also a hypoxia-activated topoisomerase II inhibitor [31] and thus competition for the same DNA sites might explain the antagonism observed with Doxo or the topoisomerase II inhibitor VP-16. However, the schedule dependency may be more complicated and may involve pharmacokinetic as well as mechanistic issues with regard to each chemotherapeutic agent. However, such issues are beyond the scope of this investigation.

With regard to mechanisms of potentiation, at least in the case of the alkylating drugs, the mechanism could involve an enhancement in the formation of DNA interstrand crosslinks or DNA-protein crosslinks induced by the alkylating agent and/or inhibition of DNA damage repair as has been demonstrated before with other hypoxia-selective cytotoxins related to NLCQ-1 or TPZ [12, 13, 25, 27]. Potentiation of Taxol or 5-FU in vitro is mediated by NLCQ-1 via a clearly synergistic enhancement in the apoptotic mechanisms induced by each chemotherapeutic agent alone, and a slightly synergistic and persistent inhibition in DNA, RNA and protein synthesis [29]. Since the same schedule-dependent potentiation seen in vivo also exists in vitro, it is quite possible that the above mechanisms account, at least in part, for the potentiation of Taxol/5-FU in vivo.

Because our present study was meant to only examine schedule dependency in the interaction between NLCQ-1/TPZ and chemotherapy in EMT6 tumors, toxicity issues with the various combination treatments were not directly addressed. However, no signs of toxicity (lethality or neurotoxicity) were observed up to the point of tumor excision (18 h after treatment). Previous studies with NLCQ-1 and alkylating agents have demonstrated that, indeed, there is no systemic or bone marrow toxicity at the combined doses used in the present study. Thus, we can conclude that a therapeutic

benefit could be achieved in the clinic by combining NLCQ-1 with at least alkylating agents [26]. Therapeutic benefits have been already demonstrated from combination treatments with TPZ and cisDDP in the clinic [16, 40, 41]. With regard to the other chemotherapeutic agents, doses that were not toxic in combination with TPZ in C3H/Km mice have been used [2]. In our study, however, we used BALB/c mice and therefore it is not guaranteed that toxicity issues were not involved. The potentiation seen with TPZ, particularly in the case of 5-FU, may not provide a therapeutic advantage since TPZ has been shown to significantly decrease the LD_{50} value of 5-FU in C3H/Km mice [2]. Further investigation is underway in our laboratory to clarify toxicity issues in combination chemotherapy with NLCQ-1/TPZ. It is clear, however, that both NLCQ-1 and TPZ have considerable promise as adjuvants to chemotherapy.

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