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Mechanisms Involved in the Potentiation of Paclitaxel or 5-FU by the Hypoxic Cytotoxin NLCQ-1 (NSC 709257) *In Vitro*

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Abstract. *Background: 4-[3-(2-Nitro-1-imidazolyl)-propylamino]-7-chloroquinoline hydrochloride (NLCQ-1) is a novel, weakly DNA-intercalating hypoxia selective cytotoxin, which significantly potentiates the antitumor effect, but not the systemic toxicity, of commonly used chemotherapeutic agents. In the present study, we investigated if the same schedule-dependent synergism seen in vivo exists in vitro as well, between NLCQ-1 and paclitaxel or 5-fluorouracil (5FU), and some of the mechanisms involved in such interactions. Materials and Methods: V79 cells, while in monolayers, were exposed to paclitaxel or 5FU under aerobic conditions at various time-intervals before or after their hypoxic exposure to NLCQ-1, while in suspension. Survival was assessed by the clonogenic assay and synergistic interactions were evaluated by using the fractional product analysis. Cells treated as above were examined for apoptosis, DNA damage and repair, as well as DNA, RNA and protein syntheses inhibition. Results: A schedule-dependent synergistic interaction existed between NLCQ-1 and paclitaxel/5FU, similar to that seen in transplanted EMT6 murine tumors. Optimal potentiation was observed when NLCQ-1 was administered 2 or 4 h after the antimitotic paclitaxel or the thymidylate synthase inhibitor 5FU. Apoptosis induced by paclitaxel or 5FU alone was enhanced by NLCQ-1 up to 2.5- and 1.7-fold, respectively. DNA damage was detected as single-strand breaks (ssbs) in the combination treatments and was unrepairable at least up to 24 h post treatment. DNA, RNA and protein syntheses were inhibited by paclitaxel/5FU alone ca. 50% immediately post treatment and this inhibition was persistent up to 24 h post treatment. In combination with NLCQ-1, a slight synergistic and persistent inhibition was observed in all three syntheses. Conclusion: Enhancement in apoptosis, unrepairable DNA damage and inhibition of DNA, RNA and protein syntheses are some of the mechanisms involved in the potentiation of paclitaxel or 5FU by NLCQ-1.*

Tumor-associated hypoxia has been traditionally considered a potential therapeutic problem because it renders solid tumors more resistant to ionizing radiation (1). Recent studies suggest that sustained hypoxia in growing tumors may cause cellular changes that can result in a more clinically aggressive phenotype, an increased potential for invasiveness and resistance to both radiation and chemotherapeutic agents (2). However, it was proven in preclinical studies that hypoxia-activated bioreductive drugs can reverse this hypoxia-associated resistance, not only by supplementing radiation or chemotherapy (which primarily attack aerobic, proliferating cells), but often by interacting in a synergistic way with them. Thus, a therapeutic benefit has been achieved in preclinical studies with a number of bioreductive drugs (3-10). Recently, a therapeutic benefit was also achieved in the clinic with the bioreductive compound tirapazamine (TPZ) in combination with radiation or cisplatin (11-14).

Recently, we have developed a new strategy of targeting hypoxia-selective cytotoxins to DNA through weak intercalation, which ensures DNA affinity high enough to produce toxicity, yet low enough to permit efficient extravascular diffusion and penetration to hypoxic tumor tissue (15). This strategy has been exemplified by our lead compound 4-[3-(2-nitro-1-imidazolyl)-propylamino]-7-chloroquinoline hydrochloride, (NLCQ-1, NSC 709257) (16). NLCQ-1 demonstrates significant hypoxic selectivity *in vitro* (17) upon reduction by cytochrome P450 and b_5 reductases (18) and synergistically enhances the effect of radiation against tumor cells *in vitro* and murine tumors or human xenografts *in vivo* (19, 20). In addition, NLCQ-1 optimizes the effect of radioimmunotherapy (RAIT) against human colonic xenografts when administered 14 days post RAIT (21). Furthermore, NLCQ-1 interacts synergistically with a variety of chemotherapeutic agents with different mechanisms of action, including alkylating agents (22, 23),

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the antimitotic paclitaxel (24, 25) and the thymidylate synthase inhibitor 5-fluorouracil (5FU) (26). Synergistic interaction with each chemotherapeutic agent was schedule-dependent and it was not accompanied with a concomitant enhancement in systemic toxicity (16, 27).

In the present study, we tried to further elucidate the mechanisms involved in the synergistic interaction seen *in vivo* between NLCQ-1 and paclitaxel or 5FU, by using *in vitro* techniques. Since the enhancement in activity of each chemotherapeutic agent by NLCQ-1, *in vivo*, was schedule-dependent (27), similar administration protocols were adopted *in vitro*. In order to better mimic the *in vivo* conditions, cells were treated under hypoxic conditions with NLCQ-1 and under normoxic conditions with each chemotherapeutic agent. Such treated cells were examined for apoptosis, DNA damage and repair, as well as DNA, RNA and protein syntheses inhibition.

Materials and Methods

Chemicals. NLCQ-1 was synthesized in our laboratory (17) and prepared as an aqueous solution at 5.16 mM. Paclitaxel (Bristol-Myers Squibb Co., Princeton, NJ, USA; 6 mg/ml) and 5-fluorouracil (5FU; Pharmacia, Kalamazoo, MI, USA; 50 mg/ml) were purchased as formulated solutions from the companies and diluted appropriately with tissue culture medium. [^{3}H]-thymidine, [^{3}H]-uridine and [^{3}H]-leucine were purchased from Amersham Life Science (Elk Grove, IL, USA).

Cells. V79 exponentially-growing cells as monolayer cultures in RPMI 1640 medium supplemented with 10% bovine calf serum were trypsinized, centrifuged (750 xg) for 5 min, harvested and suspended in 25-ml glass Erlenmeyer flasks fitted with rubber caps, usually at 5x10^5 cells/ml (5 ml) for hypoxic exposure to NLCQ-1 or seeded in 25-ml Costar flasks and allowed to attach and grow for 12 h for subsequent aerobic exposure to paclitaxel/5FU, as monolayers, in a humidified incubator (37˚C). Hypoxia was obtained by gassing the shaken glass flasks (100 rpm) with a humidified mixture of 95% N_2 plus 5% CO_2 for 1 h (17).

Drug treatments. V79 cells, while in monolayers, were exposed to 1 μM paclitaxel or 25 μM 5FU (12 h at 37˚C) under normoxia (95% air, 5% CO_2). Then the monolayers were washed free of drugs and fresh medium was added. At various time-intervals, cells were harvested and transferred into conical glass flasks for their hypoxic exposure to 20 μM NLCQ-1 (1 h, 37˚C) while in suspension. Hypoxia was induced for 1 h prior to addition of NLCQ-1. Similarly, suspended cells that had been first exposed under hypoxia to NLCQ-1 were washed free of drug and then seeded in flasks to be exposed under normoxia to the chemotherapeutic drug, as monolayers, for 12 h, at various time-intervals post NLCQ-1 treatment. Survival was assessed by the clonogenic assay and synergistic interactions were evaluated by using the fractional product analysis, which can be applied in instances of independent action of drugs (28). The plating efficiency of untreated cells was 61.5-72.6%.

Cells treated as above were examined for apoptosis (caspase-3 activation and nucleosome formation), DNA damage and repair (comet assay), and DNA, RNA and protein syntheses inhibition (incorporation of [^{3}H]-thymidine, [^{3}H]-uridine and [^{3}H]-leucine, respectively) at various time-intervals post treatment. The schedule for optimal interaction between NLCQ-1 and each chemotherapeutic agent was followed in these experiments.

Apoptosis. Caspase-3 activation was examined at 0, 12, 16, 20 and 30 h post treatment by using a fluorogenic assay (29). At least 2x10^6 cells per sample were used. Samples of cells were pelleted and washed free of drugs and lysed at 37˚C for 10 min after vortexing with a lysing buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, pH 7.4; 100 μl/10^6 cells) in which digitonin was added immediately before use (1 μM final concentration). The lysates were then centrifuged (15000 rpm) for 5 min at 0-5˚C. Supernatants of 100 μl in quadruplicates were added in a black V-96 propylene microwell plate (Nalge Nunc International, Rochester, NY, USA) followed by 100 μl of Ac-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; BD Biosciences, San Jose, CA, USA), a fluorogenic substrate in lysing buffer, to a final concentration of 1 μM. In half of the wells, a caspase-3 inhibitor (Ac-DEVD-CHO; BD Biosciences, San Jose, CA, USA) was added together with the substrate at a final concentration of 100 nM. Fluorescence was measured and recorded after 30 min, at 30˚C on a Microplate Fluorometer (Packard Instrument, Downers Grove, IL, USA; Model 7625) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Nucleosome formation was examined at 0, 12, 24 and 36 h post treatment by using an ELISA^{plus} kit (Boehringer Mannheim, Indianapolis, IN, USA), according to the instructions of the company. The assay is based on a quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows for the specific determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) in cell lysates. Briefly, cell lysates were placed into a streptavidin-coated microtiter plate. Subsequently, a mixture of anti-histone biotin-labelled and anti-DNA peroxidase-conjugated monoclonal antibodies were added and incubated for 2 h. During the incubation period, the anti-histone antibody binds to the histone component of the nucleosomes and simultaneously fixes the immunocomplex to the streptavidin-coated microtiter plate *via* its biotinylation. Additionally, the anti-DNA peroxidase-conjugated antibody reacts with the DNA component of the nucleosomes. After removal of unbound antibodies by a washing step, the amount of nucleosomes was quantified photometrically by the peroxidase retained in the immunocomplex, using ABTS [2,2'-azino-di(3-ethylbenzthiazolin-sulfonat] as substrate.

Alkaline comet assay. DNA damage and repair was measured by using the alkaline comet assay (30), which is a single-cell gel electrophoresis method, immediately or 24 h post treatment. In the second case, cells were washed free of drugs, seeded in Costar flasks with fresh medium and allowed to repair their DNA damage for 24 h before being processed for the comet assay. Samples of untreated and treated cells (washed free of drugs) were suspended in 0.5 ml PBS on ice (10^4 cells/sample) and processed, as described previously (30). Samples were viewed using a 20 x objective with a Zeiss epifluorescence microscope attached to an intensified solid-state charge-coupled device (CCD) camera and imaging analysis system (AlphaImager™ 2000, Alpha Innotech Corp. San Leandro, CA, USA). Cells were illuminated with 546 nm light excitation from a 100 W mercury light. Emission was monitored using a 580-nm

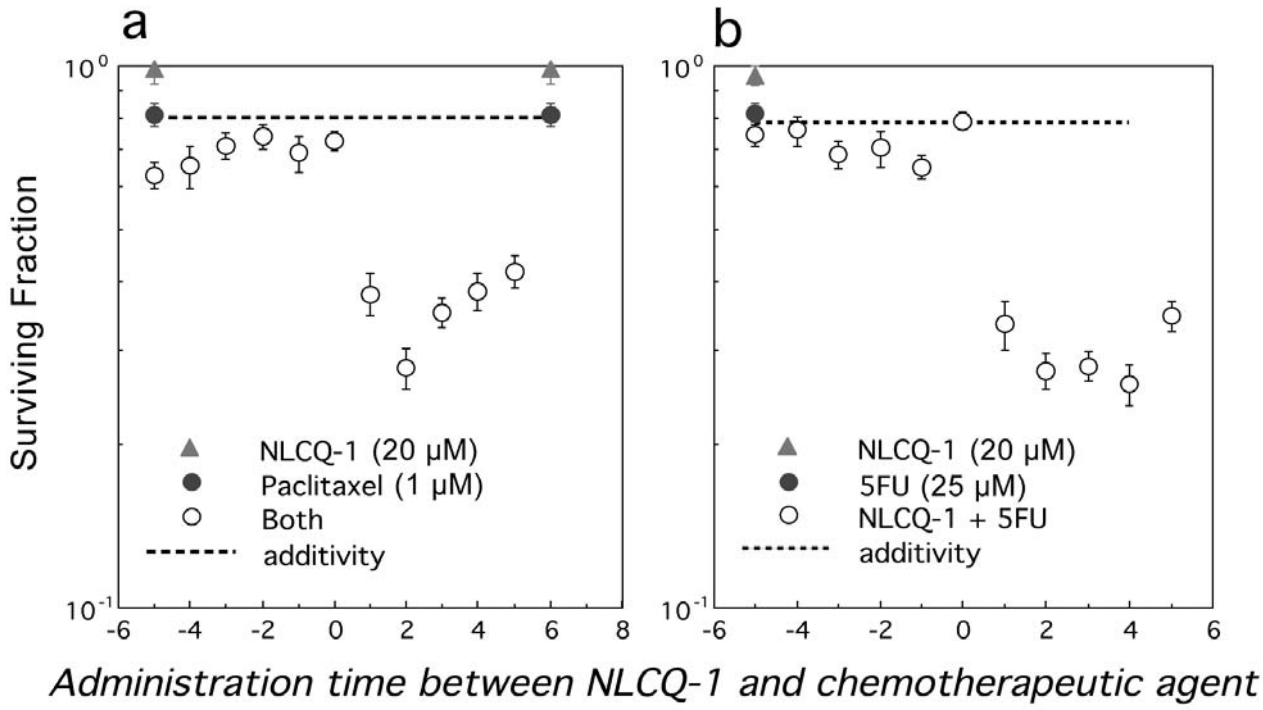

Figure 1. *Schedule-dependent interaction between (a) paclitaxel or (b) 5FU and NLCQ-1 in V79 cells. NLCQ-1 (20 μM) was administered to the cells under hypoxic conditions (1 h, 37˚C) at various time-intervals before or after paclitaxel/5FU treatment (12 h, 37˚C, aerobic conditions); dashed lines represent the additive effect (calculated as the product of the mean surviving fractions obtained with the two independent agents). Bars indicate SE of three experiments.*

reflector and a 590 bandpass filter. At least 100 comets per sample were analyzed and tail moment histograms generated. The "tail moment" was defined as the product of the percentage of DNA in the comet tail multiplied by the distance between the means of the head and tail distributions (30).

Inhibition of DNA, RNA and protein synthesis. DNA, RNA and protein synthesis was examined at 0, 12 and 24 h post treatment, by monitoring the incorporation of [^{3}H]-thymidine, [^{3}H]-uridine and [^{3}H]-leucine, respectively (31). Briefly, cells were plated in Costar flasks at 4x10^4 cells/flask with 4 ml medium containing [^{3}H]-thymidine, [^{3}H]-uridine or [^{3}H]-leucine (1 μl/ml, 0.5 Ci/ml) and incubated for 24 h to allow for attachment and growth. The medium was then removed and the cells were trypsinized, counted, adjusted to the same number (usually 2-3.5x10^4 cells) and pelleted; the pellets were washed twice with a cold solution of saline containing the corresponding unlabelled reagent (1 mM, 2 ml) followed by HBSS. Then, 2 ml of 1 M NaOH was added to each pellet and the cells were digested overnight. Finally, 2 ml of 1 M HCl solution was added to each lysate for neutralization. Each lysate was transferred in three individual vials (1 ml/vial) and 3 ml of a scintillation cocktail was added per vial, for the radioactivity to be measured in triplicate.

Statistical analysis. A two-tailed Student's *t*-test was used for statistical analysis between groups.

Results

Interaction with NLCQ-1. A schedule-dependent synergistic interaction existed between NLCQ-1 and paclitaxel/5FU, similar to that seen in transplanted EMT6 murine tumors (27). Thus, optimal potentiation was observed when NLCQ-1 was administered 2 and 4 h after the antimitotic paclitaxel and the thymidylate synthase inhibitor 5FU, respectively (Figure 1). Cells exposed to NLCQ-1 (20 μM) alone for 1 h under hypoxia demonstrated a surviving fraction of 0.960 to 0.980, slightly greater than seen before in V79 cells (19). The surviving fraction of V79 cells after aerobic exposure to 1 μM paclitaxel for 12 h was 0.811±0.040, whereas the corresponding surviving fraction after aerobic exposure to 25 μM 5FU was 0.820±0.030. The additive effect from combination treatments was calculated using the fractional product analysis (28) and gave surviving fractions of 0.799±0.038 for paclitaxel plus NLCQ-1 and 0.787±0.027 for 5FU plus NLCQ-1 (dotted lines on the plots). The minimum surviving fraction (maximal effect) obtained upon paclitaxel plus NLCQ-1 treatment was 0.277±0.024 and was observed when NLCQ-1 was administered to the cells 2 h after

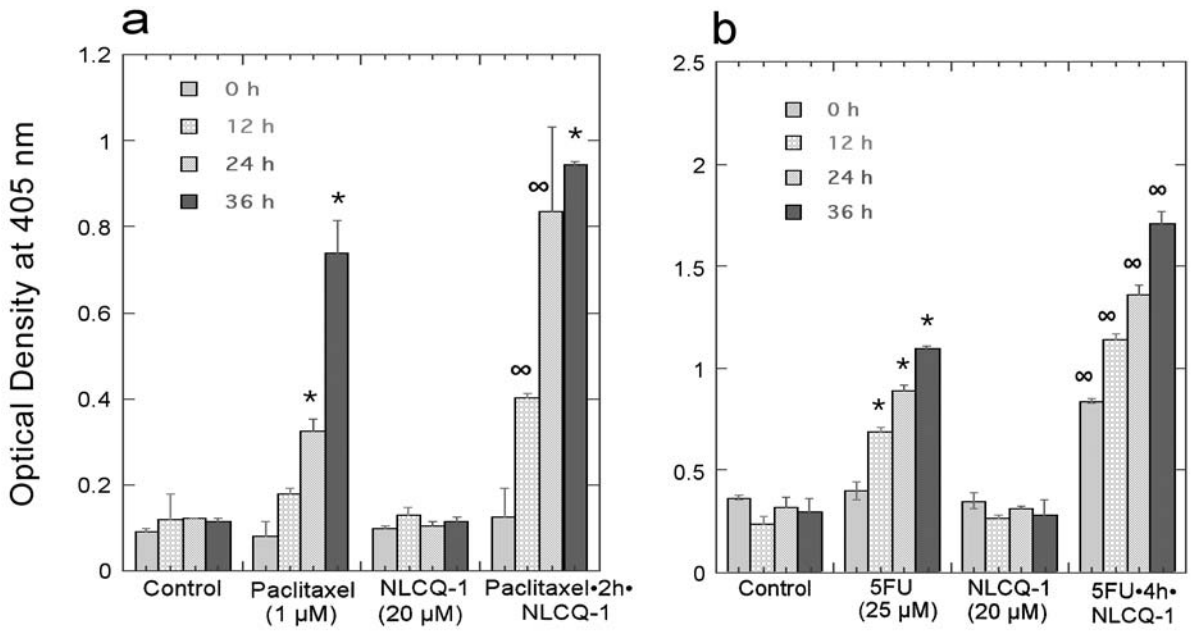

Figure 2. *Nucleosome formation in V79 cells treated with paclitaxel/5FU±NLCQ-1 at various time-intervals post treatment (reproduced with permission from Clin Cancer Res 9: 5714-57820, 2003). NLCQ-1 was given to the cells 2 and 4 h after paclitaxel and 5FU, respectively. For details see Materials and Methods. Bars represent SD of three experiments. * Significantly different from control. ∞ Significantly different from paclitaxel/5FU alone.*

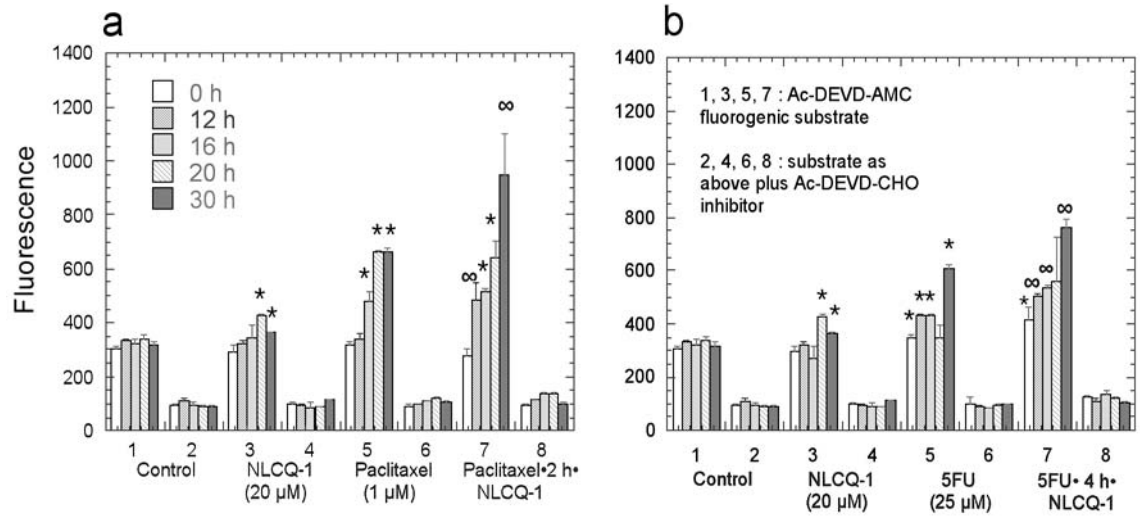

Figure 3. *Caspase-3 activation in V79 cells with paclitaxel/5FU±NLCQ-1 at various time-intervals post treatment. NLCQ-1 was given to the cells 2 and 4 h after paclitaxel and 5FU, respectively. For details see Materials and Methods. Bars represent SD of three experiments. * Significantly different from control. ∞ Significantly different from paclitaxel/5FU alone.*

paclitaxel. The minimum surviving fraction (maximal effect) obtained upon 5FU plus NLCQ-1 treatment was 0.259±0.022 and was observed when NLCQ-1 was administered to the cells 4 h after 5FU (Figure 1). These time-intervals were used for the detection of apoptosis, DNA damage/repair and DNA, RNA or protein syntheses inhibition.

Apoptosis. Apoptosis was observed after NLCQ-1 plus paclitaxel/5FU treatments as nucleosome formation (Figure 2). Nucleosome formation induced by paclitaxel alone 24 h and particularly 36 h post treatment was enhanced by NLCQ-1 by a factor of 2.6 and 1.3, respectively. In addition, nucleosome formation was also increased in the

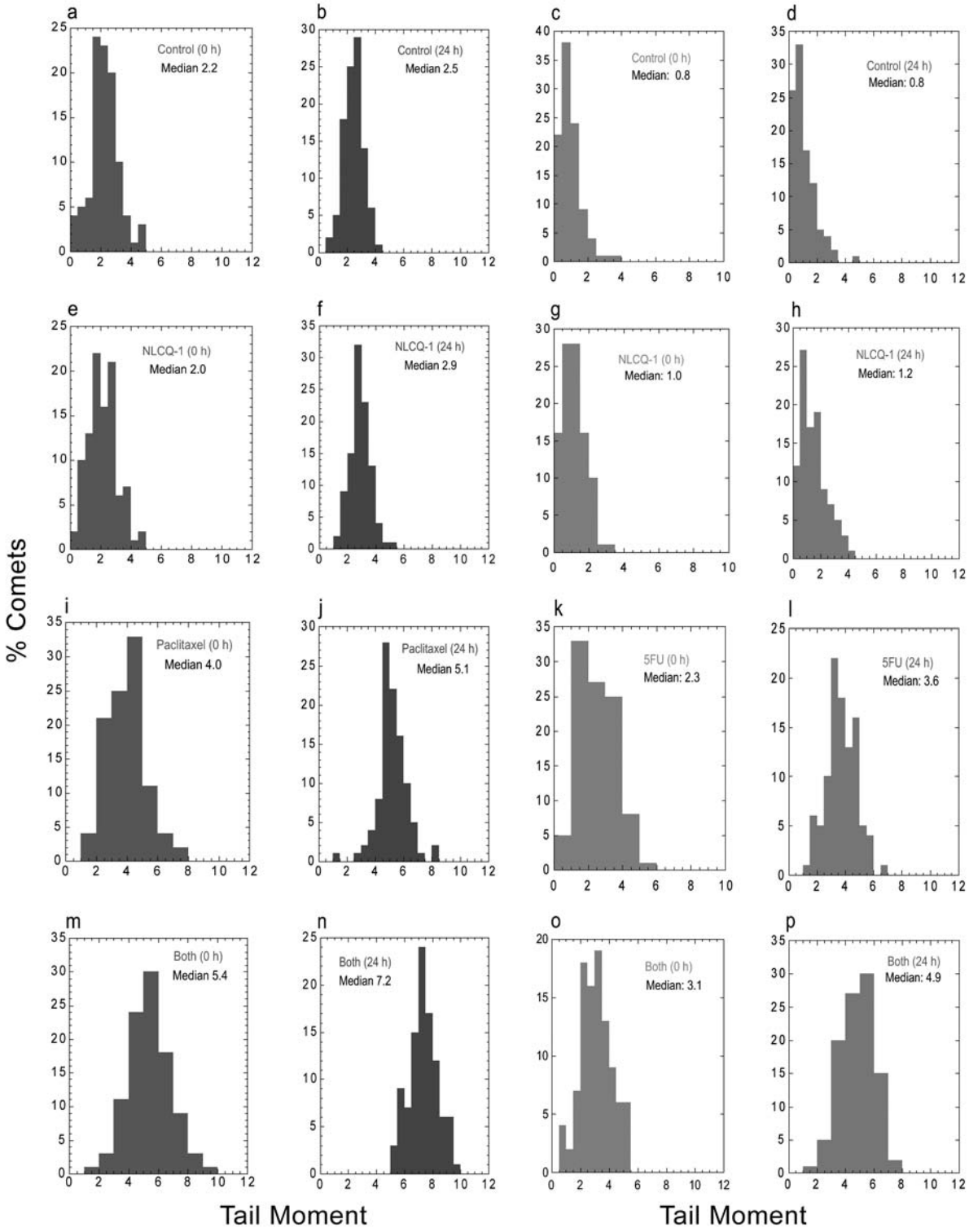

Figure 4. *DNA damage induced in V79 cells as ssbs, at various time-intervals post treatment with paclitaxel/5FU±NLCQ-1. NLCQ-1 was given to the cells 2 and 4 h after paclitaxel and 5FU, respectively. Histograms derived by analyzing 100 cells per group with the alkaline comet assay (Materials and Methods).*

Table I. *Comet analysis data in the paclitaxel±NLCQ-1 experiment. One hundred comets per group were analyzed 0 and 24 h post treatment. p values were calculated by using the Student's t-test.*

Treatment	Median tail moment	Mean tail moment±SD	*p* value
Control (0 h)	2.2	2.28±0.91	
Control (24 h)	2.5	2.48±0.67	= 0.0678 *vs* control at 0 h
NLCQ-1 (20 μM; 0 h)	2.0	2.13±0.97	NS
NLCQ-1 (24 h)	2.9	2.91±0.72	< 0.0001 *vs* NLCQ-1 at 0 h and *p*=control at 24 h
Paclitaxel (1 μM; 0 h)	4.0	3.93±1.25	< 0.0001 *vs* control at 0 h
Paclitaxel (24 h)	5.1	5.19±1.00	< 0.0001 *vs* paclitaxel at 0 h
Paclitaxel • 2 h • NLCQ-1 (0 h)	5.4	5.40±1.40	< 0.0001 *vs* paclitaxel at 0 h
Paclitaxel • 2 h • NLCQ-1 (24 h)	7.2	7.33±1.01	< 0.0001 *vs* combination treatment at 0 h and *vs* paclitaxel at 24 h

Table II. *Comet analysis data in the 5FU±NLCQ-1 experiment. One hundred comets per group were analyzed 0 and 24 h post treatment. p values were calculated by using the Student's t-test.*

Treatment	Median tail moment	Mean tail moment±SD	*p* value
Control (0 h)	0.8	0.94±0.64	
Control (24 h)	0.8	1.03±0.82	= 0.4213 *vs* control at 0 h
NLCQ-1 (20 μM; 0 h)	1.0	1.12±0.62	= 0.0442 *vs* control at 0 h
NLCQ-1 (24 h)	1.2	1.44±0.93	= 0.0054 *vs* NLCQ-1 at 0 h and = 0.0011 *vs* control at 24 h
5FU (25 μM; 0 h)	2.3	2.46±1.12	< 0.0001 *vs* control at 0 h
5FU (24 h)	3.6	3.69±1.07	< 0.0001 *vs* 5FU at 0 h
5FU • 4 h • NLCQ-1 (0 h)	3.1	3.08±1.11	< 0.0001 *vs* 5FU at 0 h
5FU • 4 h • NLCQ-1 (24 h)	4.9	4.79±1.15	< 0.0001 *vs* combination treatment at 0 h and *vs* 5FU at 24 h

combination treated group 12 h post treatment by factors of 2.3 and 3.4 *versus* paclitaxel-treated and control groups, respectively. All increases were statistically significant (Figure 2a). Nucleosome formation induced by 5FU alone 12, 24 and 36 h post treatment was enhanced by NLCQ-1 1.7-, 1.5- and 1.6-fold, respectively, whereas significant enhancement (2.1- fold) was also observed immediately post combination treatment (Figure 2b). NLCQ-1 alone did not increase the nucleosome formation compared to the control at all examined time-intervals.

Apoptosis was also confirmed by detection of caspase-3 activity at earlier time-intervals than nucleosome formation, particularly 30 h post combination treatment, with either paclitaxel or 5FU (Figure 3). In the presence of the caspase-3 inhibitor Ac-DEVD-CHO, the activity of caspase-3 was totally suppressed. NLCQ-1 alone, at 20 μM, did cause a slight activation of caspase-3 at 20 and 30 h post treatment.

DNA damage and repair. Since apoptosis was present after NLCQ-1 plus paclitaxel/5FU treatments, DNA damage should be detectable. DNA damage and repair was detected as single-strand breaks (ssbs) by the alkaline comet assay, as described in Materials and Methods. The results are demonstrated in Figure 4 and Tables I and II. Tail moment histograms are shown for control, single agent-treated and combination-treated cells immediately and 24 h post treatment. The tail moment, in the alkaline comet assay, is an indicator of the number of DNA ssbs (30). Comets of drug-untreated control cells, in the paclitaxel±NLCQ-1 experiment, examined at 0 h post treatment (Figure 4a), demonstrated a median tail moment of 2.2, indicative of the formation of some ssbs after exposure of these cells to normoxia for 25 h followed by hypoxia for 2 h, in the absence of any drug. However, comets of control cells in the 5FU±NLCQ-1 experiment demonstrated a median tail moment of only 0.8, implying that very few, if any, ssbs were formed in these cells immediately post treatment (Figure 4c). The difference in ssbs formation between the control cells in the two separate experiments most probably reflects differences in the cell cycle status, since chromatin structure affects the behavior of DNA during comet formation in the assay (32). Control cells from both experiments did not show statistical difference in the mean tail moment 24 h post treatment (p=0.0678 and 0.4213, respectively),

Table III. *Inhibition of DNA, RNA and protein syntheses by paclitaxel ± NLCQ-1 in V79 cells, at various time-intervals post treatment.*

	%DNA			%RNA			%Protein		
Hours	NLCQ-1	Paclitaxel	Both	NLCQ-1	Paclitaxel	Both	NLCQ-1	Paclitaxel	Both
0	97.9±2.9	52.8±2.2	41.1±5.0 $p=0.0207$	97.8±7.1	47.7±2.8	35.0±0.5 $p=0.0015$	96.1±4.8	50.3±2.1	39.8±1.6 $p=0.0023$
12	95.6±2.1	54.4±5.1	44.4±5.7 $p=0.0862$	98.2 ±11.6	53.4±2.5	41.6±4.7 $p=0.0185$	97.6±5.1	52.4±1.8	39.7±2.0 $p=0.0012$
24	99.6 ±11.4	59.7±2.5	46.0±0.3 $p=0.0007$	97.6±5.3	61.4±1.2	47.8±1.5 $p=0.0003$	96.0±3.2	62.6±4.9	49.9 ±1.7 $p=0.0133$

SD from three measurements. *p* values compare statistical difference *versus* the corresponding paclitaxel-alone-treated groups.

Table IV. *Inhibition of DNA, RNA and protein syntheses by 5FU±NLCQ-1 in V79 cells, at various time-intervals post treatment.*

	%DNA			%RNA			%Protein		
Hours	NLCQ-1	5FU	Both	NLCQ-1	5FU	Both	NLCQ-1	5FU	Both
0	98.5±3.0	49.3±0.5	37.3±0.8 $p<0.0001$	97.3±0.3	51.2±4.2	40.9±2.3 $p=0.0204$	101.9 ±4.2	53.1±2.6	41.9±2.0 $p=0.0041$
12	96.6±3.9	49.4±1.4	35.8±0.9 $p=0.0001$	96.7±3.8	51.4±1.4	38.8±0.9 $p=0.0002$	98.9±8.7	54.0±1.9	39.2±1.5 $p=0.0005$
24	97.9±2.2	56.3±0.6	39.7±1.4 $p<0.0001$	97.1±2.0	57.3±1.2	43.1±1.3 $p=0.0002$	98.9±6.2	59.8±4.1	44.7±2.2 $p=0.0049$

SD from three measurements. *p* values compare statistical difference *versus* the corresponding 5FU-alone-treated groups.

confirming the fact that cell cycle status was most probably responsible for the variation in median tail moment values between the two experiments (Tables I and II). In the paclitaxel ± NLCQ-1 experiment, cells treated as in the control group, but in the presence of 20 μM NLCQ-1 (last hour under hypoxic conditions), showed a median tail moment of 2.0, no greater than the corresponding control (Figure 4e). However, comet analysis 24 h post treatment with NLCQ-1 alone provided a median tail moment of 2.9, indicative of ssbs formation. Moreover, there was a statistically significant difference in the mean tail moment of the NLCQ-1 group at 24 h *versus* the NLCQ-1 group at 0 h, and *versus* the corresponding control group at 24 h (Table I). Thus, NLCQ-1 alone did cause DNA damage as ssbs, which, however, became apparent 24 h post treatment (Figure 4f). Similarly, in the 5FU±NLCQ-1 experiment, NLCQ-1 alone caused the formation of some ssbs even at 0 h post treatment, which, however, became more apparent 24 h post treatment (Figure 4g,h and Table II). Cells treated as in the control group but in the presence of 1 μM paclitaxel (first 12 h under normoxia) resulted in a median tail moment of 4.0, indicative of a significantly greater number of ssbs formation compared to the corresponding control (Figure 4i). Indeed, statistical analysis revealed a $p<0.0001$ value (Table I). Once again, when the analysis occurred 24 h post treatment (Figure 4j), a much greater number of ssbs was present ($p<0.0001$ *versus* paclitaxel at 0 h). Finally, paclitaxel treatment under aerobic conditions followed by NLCQ-1 treatment under hypoxic conditions (Figure 4m) provided a median and mean tail moment of 5.4, which was significantly different from that obtained with paclitaxel alone ($p<0.0001$; Table I). The DNA damage that occurred in this combination treatment was further enhanced 24 h post treatment, providing a median tail moment of 7.2 (Figure 4n).

Similar results were obtained from the 5FU±NLCQ-1 experiment (Table II and Figure 4). Cells exposed to 25 μM 5FU for 12 h under normoxia followed 4 h later by exposure to NLCQ-1 under hypoxia (1 h), provided a median tail moment of 3.1, statistically greater than that in the 5FU-alone-treated group, which was further enhanced (4.9) 24 h post treatment (Figure 4o,p).

DNA, RNA and protein syntheses inhibition. DNA, RNA and protein syntheses were monitored by [^{3}H]-thymidine, [^{3}H]-uridine and [^{3}H]-leucine incorporation, respectively, in cells treated with paclitaxel/5FU±NLCQ-1 at 0, 12 and 24 h post treatment. The results from such studies are summarized in Tables III and IV. DNA, RNA and protein syntheses were inhibited *ca.* 50% by paclitaxel alone immediately post treatment and this inhibition was persistent up to 24 h post treatment (*ca*. 40%). In combination with NLCQ-1, a slight, but statistically significant, synergistic and persistent inhibition was observed in all three syntheses. No inhibition was observed in DNA, RNA or protein syntheses by NLCQ-1 alone, at the dose used (Table III). Similar results were obtained in the 5FU±NLCQ-1 experiment (Table IV).

Discussion

Potentiation by nitroimidazole-based bioreductive drugs has been traditionally investigated with alkylating chemotherapeutic agents. However, we have previously shown that enhancement of the tumor response can be obtained in a schedule-dependent manner by combining NLCQ-1 with the antimitotic drug taxol or the thymidylate synthase inhibitor 5-fluorouracil, in EMT6 tumor-bearing mice (27). This is important because both of these chemotherapeutic agents are broadly used, in combination with other modalities, against a variety of cancers including late stage ovarian (33), advanced lung (34), as well as head and neck (35) for paclitaxel and advanced colorectal cancers (36) for 5FU; namely tumors with potential hypoxic regions. Sequencing of drug delivery can also be extremely important in multidrug chemotherapy, especially if one or more of the agents impose cell cycle blocks or exhibit cell cycle-specific cytotoxicity (37). For example, synergy was observed between 5FU and irinotecan in a human colorectal carcinoma cell line only upon a sequential, but not simultaneous, exposure of the cells to these agents (38). Moreover, an antagonistic effect was observed when cisplatin or other alkylating agents were given prior to paclitaxel against human cancer cells (37). Our recent results indicated that the same sequencing, used for an *in vivo* synergistic interaction between paclitaxel/5FU and NLCQ-1, is required for an *in vitro* optimal interaction as well. This suggests that the mechanism of interaction at the molecular level between NLCQ-1 and each chemotherapeutic agent perhaps plays a more important role than any pharmacokinetic parameter.

It is known that paclitaxel causes mitotic arrest and apoptosis, but only apoptosis and not mitotic arrest is correlated with the antitumor effect of paclitaxel (39). However, in our combined treatment, arrest by paclitaxel in the G2/M-phase may have predisposed cells to further damage by hypoxia-activated NLCQ-1, as suggested by the survival results in Figure 1. Furthermore, NLCQ-1 given after paclitaxel triggered apoptosis, presumably in the G2/M-phase, as was evident by nucleosome formation and caspase-3 activation (Figures 2 and 3). Apoptosis was also evidenced by flow cytometry, as a large accumulation of combined-drugs, treated cells in the sub G0/1-phase (data not shown). NLCQ-1 alone, at the used dose, did not cause a measurable amount of apoptosis, at least as nucleosome formation. However, a statistically significant increase in caspase-3 activation was observed by NLCQ-1 alone, at 20 and 30 h post treatment (Figure 3). It is assumed that the level of nucleosome formation by NLCQ-1 alone was too small to be detected by the ELISA method. Indeed, the percentage of NLCQ-1- alone-treated cells in the sub G0/1-phase 24 h post treatment was 14.7% *versus* 8.3 for the corresponding control cells (data not shown).

Similarly, it is known that thymidylate synthase inhibition, the main mechanism of 5FU antitumor activity, is limited to the S-phase of the cell cycle (40), which again may explain the required sequence between 5FU and NLCQ-1 for a synergistic interaction. 5FU also induces apoptosis through caspase-8 and caspase-3 activation (41). Our data confirmed the literature results. Hypoxia-activated NLCQ-1, given after 5FU, potentiated this apoptotic pathway (Figures 2 and 3).

Nitroimidazole-based bioreductive compounds exert their cytotoxicity upon reductive activation under hypoxic conditions, which leads to the formation of unstable metabolites that can cause DNA ssbs and the formation of bulky adducts to cell macromolecules (42). In our study, NLCQ-1 at 20 μM caused a small but significant number of ssbs, which became apparent 24 h post treatment (Tables I and II). However, NLCQ-1 at higher concentrations can cause a large number of ssbs, especially in cells that lack repair mechanisms such as EM9 cells (43). Recently, it has been shown that even the mitotic spindle poison paclitaxel can induce DNA ssbs in cells (44, 45). Our results indicated that V79 cells exposed to 1 μM paclitaxel for 12 h formed a significant number of ssbs, which was further enhanced 24 h post treatment. Paclitaxel-exposed cells, as above, were further sensitized by subsequent hypoxic exposure to NLCQ-1 for 1 h, providing a median tail moment of 7.2 (1.4-fold greater than paclitaxel alone), 24 h post treatment (Table I). For comparison purposes it is worthwhile to mention that 10 Gy of radiation, which is a very good ssbs inducer, provided a mean tail moment of 9.1±3.0 in V79 cells immediately after irradiation (31).

It has been shown in the literature that DNA damage appears to be an important additional determinant of drug effect even among compounds that have been developed as specific and potent thymidylate synthase inhibitors, such as 5FU (46, 47). Thus, 5FU induces concentration-dependent DNA damage as ssbs in colon adenocarcinoma cells (47). Our results confirm that 12-h exposure of V79 cells to 5FU

results in DNA ssbs formation, which is more predominant 24 h post treatment. In addition, this DNA damage is further increased if the 5FU-pretreated cells are exposed for 1 h under hypoxia to NLCQ-1. However, 12 h of aerobic exposure of V79 cells to paclitaxel (1 μM) alone had a greater effect than a similar exposure to 5FU (25 μM) on the formation of DNA ssbs. NLCQ-1 increased to the same degree the paclitaxel/5FU-induced ssbs formation in V79 cells (Tables I, II) and perhaps further inhibited DNA damage repair mechanisms.

The chemosensitivity of tumor cells can be assayed primarily by DNA synthesis inhibition (37) and secondarily by RNA and protein syntheses inhibition. Both paclitaxel and 5FU can inhibit DNA synthesis (48, 36). Our results indicated that these chemotherapeutic drugs inhibited DNA, RNA and protein syntheses to almost the same degree. In addition, this inhibition was persistent up to 24 h post treatment (Tables III, IV). NLCQ-1, on the other hand, which alone did not have any statistically significant effect on DNA, RNA or protein syntheses, enhanced in a synergistic way the effect of either paclitaxel or 5FU.

In conclusion, enhancement in apoptosis, persistent DNA damage and an increase of DNA, RNA and protein syntheses inhibition are some of the mechanisms involved in the potentiation of paclitaxel and 5FU by the hypoxia-activated compound NLCQ-1. In addition, we showed that the drug schedule is an important determinant of the activity of combined modality treatments and that NLCQ-1 must succeed 5FU or paclitaxel treatment for optimal antitumor activity.

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