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Comparative studies between the effects of mitozolomide and two novel tetrazepinones PYRCL and QUINCL on NIH:OVCAR-3 cells

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Abstract Cytotoxicity, reduction of macromolecule synthesis and cell cycle perturbations by two novel 3-(2-chloroethyl)-tetrazepinones, PYRCL and QUINCL were compared with those produced by the structurally related 3-(2-chloroethyl)-tetrazinone, mitozolomide, in the OVCAR-3 cell line. *Methods*: Macromolecule synthesis was determined by incorporation of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine into acid-precipitable fractions of OVCAR-3 cell extracts. Maxam-Gilbert sequencing was used to compare the DNA alkylating sites induced by the tetrazepinones, with those created by mitozolomide. Alkaline sucrose-density sedimentation was employed to detect genomic DNA damage. Also, the effects of the tetrazepinones on the cell cycle were determined by univariate flow cytometry. *Results*: At 3 h post-treatment, mitozolomide appeared as a selective inhibitor of DNA synthesis, while both tetrazepinones inhibited the synthesis of all three macromolecules. At 24 h post-treatment, the inhibition of DNA synthesis was observed to increase in cells treated with mitozolomide, while it decreased in those previously exposed to the tetrazepinones. Also at 24 h post-treatment, mitozolomide induced accumulation of cells in S(late)/G_2M at low concentrations and in S-middle at high concentrations. In contrast, at the same recovery time, cells treated with the tetrazepinones accumulated specifically in G_2M, the strength of the block being dose-dependent. At an equimolar concentration, the tetrazepinones induced weaker guanine N-7 alkylation than mitozolomide. By 24 h after treatment, cells exposed to the tetrazepinones showed significantly greater DNA fragmentation than those previously treated with mitozolomide. *Conclusion*: In summary, based on (a) their effects on DNA, RNA, protein synthesis and on the cell cycle, (b) their alkylating power and (c) their interactions with DNA, the 3-(2-chloroethyl)tetrazepinones appeared to kill tumor cells by a novel mechanism which may significantly differ from that of their 3-(2-chloroethyl)-tetrazinone counterpart, mitozolomide.

Key words Tetrazepinones · Cell cycle arrest · DNA damage · OVCAR-3 cells

Introduction

The bi- and tricyclic tetrazepinones [14, 15] are a new class of compounds structurally related to temozolomide (**1**, $R = CH_3$) and mitozolomide (**1**, $R = CH_2CH_2Cl$), the most representative members of the imidazotetrazinone class [25]. The tetrazepinones **2** (QUINCL) and **3** (PYRCL) present, like the imidazotetrazinones, an aromatic moiety fused with a cyclic ureido triazene system: the 1,2,3,5-tetrazepin-4-one. The cyclic ureido-triazene is six-membered in the imidazotetrazinones, whereas in the 1,2,3,5-tetrazepinones **2** and **3**, it is seven-membered. This structural difference gives the tetrazepinones novel chemical characteristics that markedly distinguish them from the imidazotetrazinones (Scheme 1).

Tetrazinones **1** decompose in aqueous media to open-chain triazenes of type **4** which further decompose to a methyldiazonium species that alkylates DNA [3]. The tetrazepinones mostly decompose via the loss of nitrogen to give metabolites of type **5** [17, 28, 29]. Structure-activity studies have shown the imidazotetrazinone class to be similar to their parent open-chain 3-alkyl-1,2,3-triazene class in that compounds bearing a CH_2CH_2Cl and CH_3 are active, while $R = CH_2CH_3$ and higher homologues are inactive [11, 20]. The cytotoxicity of mitozolomide is attributed to its ability to induce DNA interstrand cross-links following alkylation of the O-6 position of guanine [12, 13, 26]. Temozolomide, the

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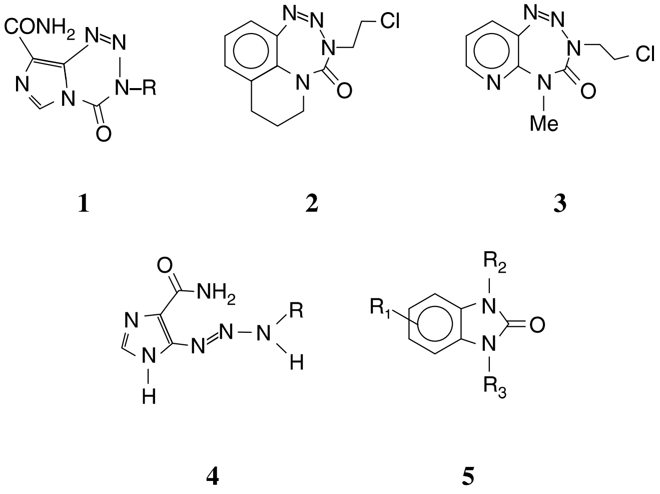


Scheme 1

methyl congener of mitozolomide, does not create interstrand crosslinks, but induces the cytotoxic O-6-methylguanine adduct [1, 2, 5, 10]. Cells capable of repairing the latter adduct are resistant to both temozolomide and mitozolomide.

In recent studies, we have compared the in vitro action of the tetrazepinones with that of temozolomide and mitozolomide [16]. It appears that the activity of the tetrazepinones is independent of the group at N-3. Compounds with $R = CH_2CH_2Cl$ are equitoxic with those containing a methyl or an ethyl group. Moreover, they are equally active against alkylating-agent-resistant (Mer^+) and -sensitive (Mer^-) human brain and colon tumor cell lines [19]. For example, the tetrazepinone QUINCL is equally active against SF-126 (a BCNU-sensitive brain tumor cell line) and SF-188 (a BCNU-resistant brain tumor cell line) [19]. The tetrazepinones show high potency against human ovarian, breast and prostate cancer cell lines.

The significant structural similarities between our novel tetrazepinones QUINCL or PYRCL and mitozolomide prompted us to examine their action against the human ovarian tumor cell line OVCAR-3. The choice of an ovarian cancer cell line was inspired by a previous study by Erba et al. [4] who reported a significant lack of sensitivity of ovarian carcinoma cells to mitozolomide. The latter drug was capable of inducing cell cycle perturbation in only 1 out of 16 primary cultures of human ovarian tumour cells. Moreover, the high myelotoxicity of this compound in the clinic has irreversibly compromised its development. These results stimulated our interest in determining whether our novel tetrazepinones which contain, like mitozolomide, a chloroethyltriazene moiety would show cytotoxic activities similar to or different from those previously observed for classical chloroethylating agents.

In this study, we describe the effect of two tetrazepinones, QUINCL and PYRCL, on the human ovarian tumor cell line OVCAR-3. We found that in contrast to mitozolomide, the 3-(2-chloroethyl)-tetrazepinones are weak alkylators that do not preferentially inhibit DNA synthesis but induce significant levels of single strand breaks in OVCAR-3 cells. The mechanism of action of the 3-(2-chloroethyl)tetrazepinones may therefore differ from that of the bifunctional tetrazinone mitozolomide.

Materials and methods

Drug treatment

Mitozolomide was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. The tetrazepinones were synthesized in our laboratories according to previously published methodology [14]. In all assays, the drugs were dissolved in DMSO and diluted in sterile RPMI medium immediately prior to treatment of cell cultures. The concentration of DMSO never exceeded 1% (v/v). The cells were treated with drugs for 2 h, and treatments were terminated by aspiration of the drug-containing medium and replacement with fresh RPMI-1640 medium. The cells were continuously exposed to the drug for the specified time periods.

Cell culture

The OVCAR-3 cell line [8] was maintained as a monolayer culture at 37 °C in a humidified atmosphere of 5% CO_2/95% air in RPMI-1640 supplemented with 10% fetal bovine serum, 2 m*M* L-glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin. Cells were maintained in logarithmic growth by harvesting with a trypsin-EDTA solution containing 0.5 mg/ml trypsin and 0.2 mg/ml EDTA and reseeding before cells reached confluency. Growth studies showed a doubling time of approximately 30 h. In all assays, the cells were plated for 24 h prior to drug administration.

Cytotoxicity

Cell monolayers were incubated with various amounts of mitozolomide and the tetrazepinones for different time periods and cytotoxicity evaluated by the sulforhodamine B assay [22]. Briefly, the cells were fixed by the addition of 50 µl 50% cold trichloroacetic acid (TCA) at 4 °C for 60 min. The wells were stained with sulforhodamine B dissolved at 0.4% in 1% acetic acid. The resulting colored residue was dissolved in 200 µl 10 m*M* Tris base. The optical density (OD) was measured at 540 nm with a BioRad Microplate Reader (model 3550). The results presented are the average of two independent experiments run in triplicate.

Clonogenic assay

Mitozolomide, PYRCL and QUINCL cytotoxicities were also evaluated using the clonogenic assay, as previously described [18]. Following drug treatment, the cells were incubated under routine conditions in drug-free medium for 11 days and the colonies were counted with an Artek Omnicon 880 counter.

Macromolecule synthesis

DNA, RNA, and protein synthesis were determined as previously described [9]. Briefly, following drug treatment, the radioactive precursors [methyl-^{3}H]-thymidine (specific activity, 20 mCi/mmol), [5-^{3}H]-uridine (specific activity, 20 mCi/mmol) or L-[4,5-^{3}H]-leucine (specific activity, 120–190 Ci/mmol) were added to the cell culture medium for 3 h. [Methyl-^{3}H]-thymidine, [5-^{3}H]-uridine (obtained from New England Nuclear) were added at 0.5 mCi/ml and L-[4,5-^{3}H]-leucine at 1 mCi/ml. At the end of radioisotope incubation, cells were trypsinized and a 0.1-ml aliquot was collected for cell counting in a ZM Coulter counter before centrifugation. The supernatant was removed and replaced with 1 ml cold 10% TCA. The resulting precipitate was collected by centrifugation and digested in 0.1 *N* NaOH. The mixture was neutralized, transferred to a scintillation vial and dissolved in 3 ml liquid scintillation fluid (Universol). The associated radioactivity was counted in a beta scintillation spectrometer. The level of incorporation is expressed as counts per minute per cell and the percent of control calculated as: (cpm/10^5 cells treated)/(cpm/10^5 control cells). Each point represents the average and standard error resulting from at least two independent experiments run in duplicate.

Flow cytometry

The effect of a 2-h exposure to PYRCL and QUINCL on the OVCAR-3 cell cycle was evaluated after recovery times of 24, 48 and 72 h by flow cytometry, as previously described. Briefly, the cells were harvested by trypsinization at the appropriate time points. After fixation in ethanol (70% v/v), cells were stained with an aqueous propidium iodide (Sigma Chemicals) solution. The fluorescence was read in the spectral range between 580 and 750 nm. Each cytometric analysis was performed using a Becton Dickinson FACScan instrument on 1–3 × 10^5 cells. The percentage of cells in each cell cycle phase was estimated using Lysis II software (Becton Dickinson).

Alkaline sucrose gradient

Cells were labeled for 24 h using a medium supplemented with 0.1 mCi/ml [^{3}H]- or ^{14}C-thymidine for internal control cells [30]. Postlabeling (18–24 h) was done before drug treatment. The drug was added for 2 h and the cells were allowed to grow in fresh medium for 24 h. They were then washed twice with PBS and dislodged by gentle scraping. To the ^{3}H-labeled cells was added an aliquot of untreated ^{14}C-labeled cells to serve as an internal control during sedimentation analysis. The cells were then lysed in the dark at 0 °C (lysis buffer: 0.55 *N* NaOH, 0.45 *M* NaCl, 10 m*M* Na_2EDTA, 0.015% sarcosyl) and layered on the top of 5–30% sucrose gradients containing 0.3 *N* NaOH, 0.7 *M* NaCl, and 10 m*M* Na_2EDTA. Sedimentation was carried out at 4 °C in a SW41 rotor, usually at 16 000 rpm overnight. Gradients were fractionated by upward displacement with 1 ml of a dye dissolved in 40% sucrose and the samples collected with a BioRad fraction collector. The 0.3-ml fractions were analyzed by single-phase liquid scintillation counting (dual-label settings).

Sequence specificity of guanine N-7 alkylation

Plasmid pRGM21, which was used to determine guanine specific N-7 alkylation, has been described in detail [21]. Briefly, the wild-type SV40 origin of replication from the Hind III to Sph I site (200 bp) was inserted between the Hind III and Sph I sites of pBR322. Using PCR, the SV40 region of the plasmid was amplified with the following primers: 5′-GGCCATCCAGCCTCG-3′ and 5′-GTATCACGAGGCCCT-3′. The latter primer was end-labeled (T4 polynucleotide kinase, Gibco BRL) with γP^{32}-ATP (ICN Biomedical). The amplified labeled DNA (700 bp) was exposed to the different drugs in 25 m*M* triethanolamine/1 m*M* EDTA, pH 7.2, at 37 °C for 2 h. After precipitation and washing, the DNA was treated with 1 *M* piperidine for 15 min at 90 °C to produce breaks specifically at the sites of N-7 guanine alkylation. DNA fragments were separated on a 0.4-mm 6% polyacrylamide gel in a solution containing 7 *M* urea and a Tris/boric acid/EDTA buffer system.

Results

Cytotoxicity

The sulforhodamine B assay yielded IC_{50} values of 67 μ*M* for mitozolomide, 16.5 μ*M* for PYRCL and 13 μ*M* for QUINCL 3 days post-treatment (Fig. 1A). The dose-response profile obtained for mitozolomide was markedly distinct from those for PYRCL and QUINCL. At the concentration range 10–50 μ*M*, PYRCL and QUINCL showed a rapid dose-dependent decrease in survival, whereas mitozolomide, within the same range, gave a less steep decrease with a saturation portion between 100 and 1000 μ*M*. A colony-forming assay in which cells were allowed to form colonies in drug-free medium for 11 days after drug treatment, yielded nearly identical dose-response curves for all three compounds (IC_{50} values ≈15 μ*M*, Fig. 1B).

In order to quantify cell loss during our studies on macromolecule synthesis, we examined cell survival at 3 and 24 h post-treatment by counting detached cells following trypsinization. At 3 h post-treatment, no cell

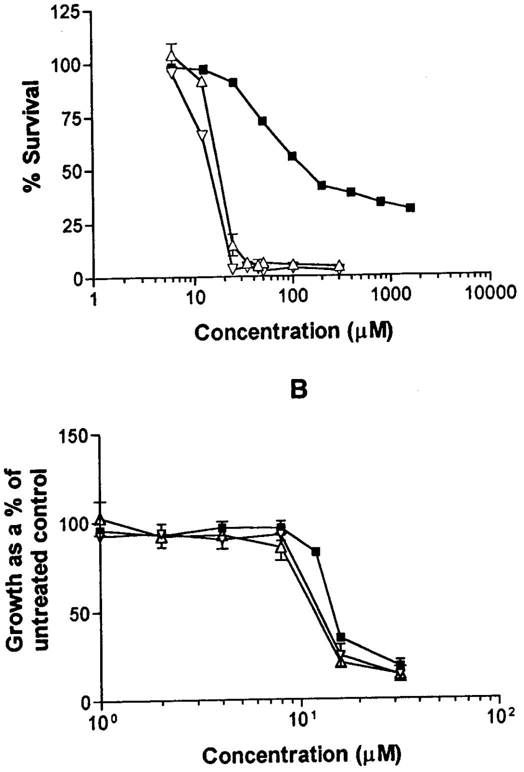


Fig. 1A,B Dose-survival profiles of OVCAR-3 cells after treatment with various concentrations of mitozolomide (■) PYRCL (△) and QUINCL (▽) using the sylforhodamine B assay (**A**) and the clonogenic assay (**B**). **A** Cells were exposed to different concentrations for 2 h followed by a 72-h incubation in drug-free medium. Data are means and SE from two independent experiments run in triplicate. **B** Cells were treated with various concentrations of drugs for 2 h and allowed to form colonies in drug-free medium for 11 days. Data represent means and SE of the number of colonies formed from two independent experiments run in duplicate

Table 1 Inhibition of macromolecule synthesis (DNA, RNA and protein) by mitozolomide, PYRCL, QUINCL 3 and 24 h after treatment. Data are IC_{50} (μM) for reduction of macromolecule synthesis (DNA, RNA and protein) or cell growth (survival)

Drug	3 h post-treatment (IC_{50}, μM)				24 h post-treatment (IC_{50}, μM)			
	Survival	DNA	RNA	Protein	Survival	DNA	RNA	Protein
Mitozolomide	> 200	71 ± 1	> 200	> 200	> 200	10 ± 2	> 200	> 200
PYRCL	> 200	52 ± 8	65 ± 10	56 ± 4	59 ± 4	> 200	> 200	168 ± 61
QUINCL	> 200	58 ± 3	89 ± 16	42 ± 1	97 ± 2	> 200	153 ± 37	65 ± 13

loss was observed over the entire dose range for all three compounds (Table 1, Fig. 2A,B). However, at 24 h post-treatment, a marked difference appeared between the dose-response curves induced by mitozolomide and those induced by the tetrazepinones. A curve presenting a saturation portion in the 50–200 μM range was observed for mitozolomide, whereas the decrease in cell survival induced by PYRCL and QUINCL was more dose-dependent (50–80% kill attained in the concentration range 80–200 M).

Macromolecular synthesis

The IC_{50} values for the inhibition of macromolecule synthesis by mitozolomide, PYRCL and QUINCL, and the IC_{50} values for cell survival at the time of the analysis of macromolecule synthesis (3 and 24 h post-treatment), are shown in Table 1. Typical graphs from which these values were derived are shown in Fig. 2. Mitozolomide at 3 h post-treatment showed significant inhibition of DNA synthesis (IC_{50} 71 μM, Fig. 2C). Accompanying this was an enhancement in RNA synthesis and no change in protein synthesis (Table 1). At 24 h post-treatment, inhibition of DNA synthesis was enhanced (IC_{50} 10 μM). At almost all the mitozolomide concentrations tested, the levels of RNA synthesis were 10–50% above those observed in nontreated cells. Levels of protein synthesis remained unchanged.

In contrast, PYRCL and QUINCL 3 h after drug exposure induced a nonselective dose-dependent inhibition of the synthesis of all three macromolecules (DNA, RNA and proteins). The IC_{50} values were in the 42–90 μM range. At 24 h post-treatment, the inhibition of macromolecule synthesis was released, giving rise to curves with only 10–25% inhibition of DNA synthesis (IC_{50} values > 200 μM, Table 1). Paradoxically, the decrease in the inhibition of macromolecule synthesis in the 100–200 μM range was accompanied by 70–80% cell kill 24 h after treatment (Fig. 2B,D).

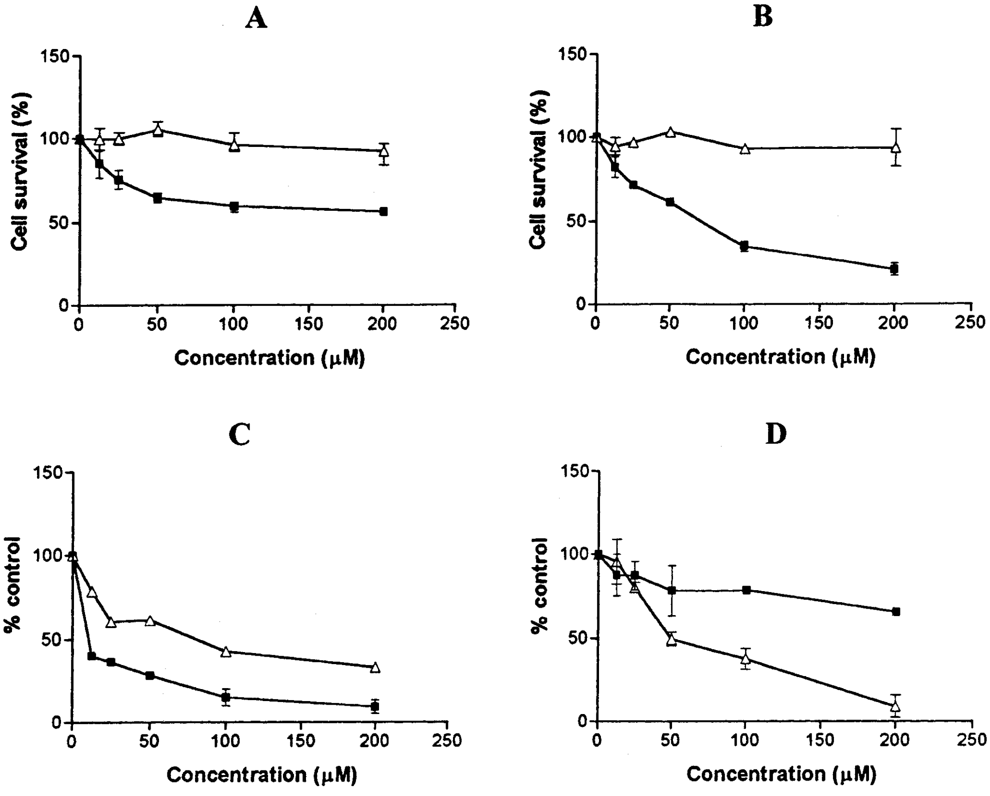


Fig. 2A–D Dose-survival profiles (**A**, **B**) and DNA synthesis (**C**, **D**) of OVCAR-3 cells treated with mitozolomide (**A**, **C**), PYRCL (**B**, **D**) at 3 (△) and 24 h (■) post-treatment

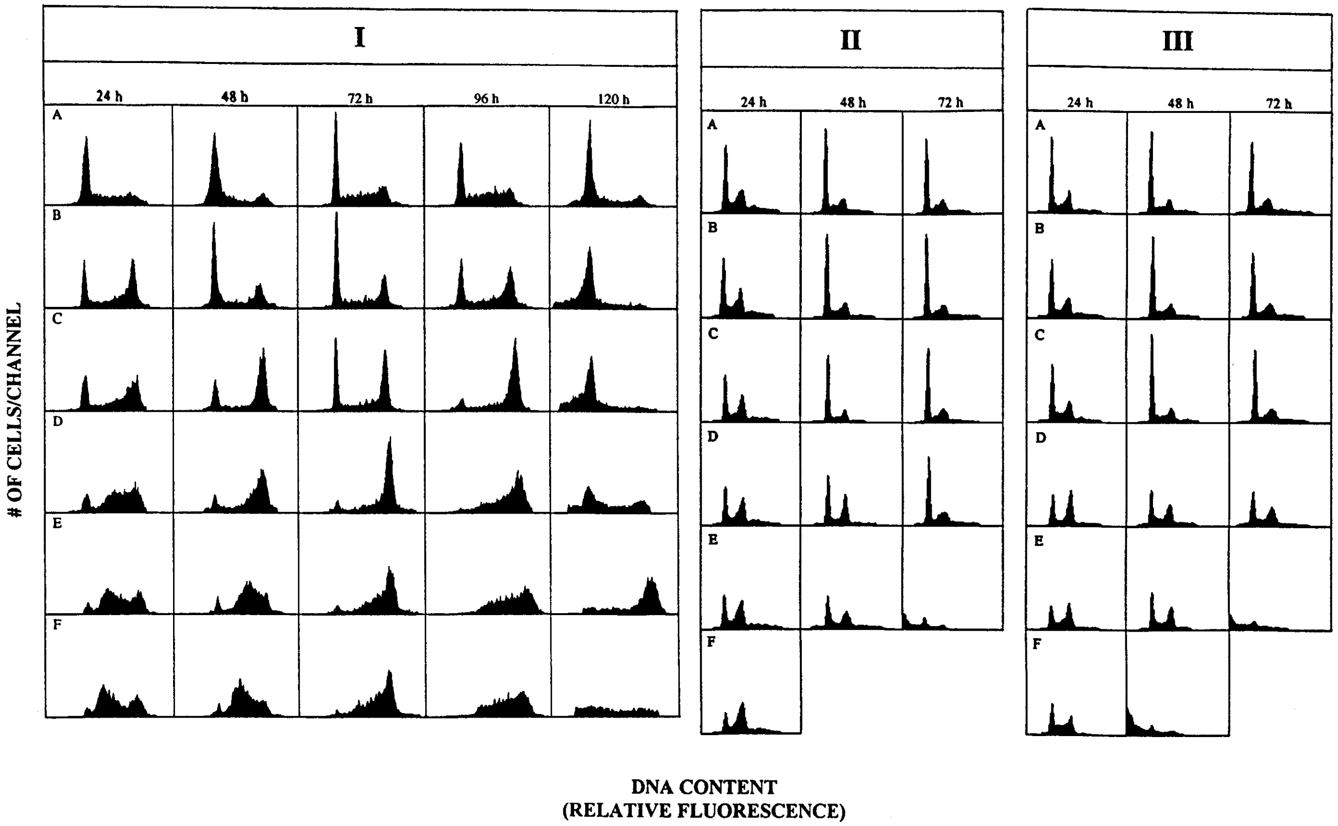


Fig. 3 Effect of various concentrations of mitozolomide (*I*), PYRCL (*II*), and QUINCL (*III*) on OVCAR-3 cell cycle progression. Mitozolomide concentrations: *A* 0 μM, *B* 10 μM, *C* 20 μM, *D* 50 μM, *E* 100 μM, *F* 200 μM. PYRCL concentrations: *A* 0 μM, *B* 10 μM, *C* 20 μM, *D* 40 μM, *E* 80 μM, *F* 160 μM. QUINCL concentrations: *A* 0 μM, *B* 12.5 μM, *C* 25 μM, *D* 50 μM, *E* 100 μM, *F* 200 μM

Flow cytometry

The effect of the three agents on the cell cycle was evaluated by flow cytometry at specified times during the 24–120 h post-treatment time period (Fig. 3). At 24 h recovery, mitozolomide at low concentrations (10–20 μM) showed strong S(late)/G_2M arrest. As the concentrations increased, cells accumulated earlier in the cycle, in the order S-late, S-middle, S-early, with increasing concentrations. These arrests were transient, since after 48 and 72 h recovery, the cells blocked in S-middle and S-early progressed to a later phase of the cell cycle (e.g. S-early to S-middle, S-middle to S-late).

At 24 h post-treatment, the tetrazepinones exhibited a significantly different cell cycle perturbation profile. They induced strong G_2M arrest at high concentrations (Fig. 3, II and III). At concentrations as high as 200 μM, no significant cell cycle arrest was induced in the S phase. The blocks were specifically localized in G_2M regardless of the concentration. At 48 and 72 h post-treatment, in contrast to mitozolomide, the cells exposed to PYRCL and QUINCL were killed at high concentrations, rendering it difficult to undertake satisfactory flow cytometric analysis. More importantly, in cells treated with mitozolomide, the analysis could be pursued at high doses for as long as 5 days post-treatment.

DNA damage

N-7 alkylation

Mitozolomide is known to alkylate ^{32}P-labeled DNA strands at the 7-position of guanine [6, 9]. We compared the sequence selectivity and the alkylating capacity of the tetrazepinones to those of mitozolomide. As shown in Fig. 4, strong N-7 alkylation was observed for mitozolomide at supralethal concentration (1 mM) (Fig. 4, lane C). At equal concentrations, weaker bands were observed for PYRCL and QUINCL (Fig. 4, lanes D and E), indicating that the levels of guanine alkylation at the 7-position were negligible. The overall band intensities for the tetrazepinones were markedly weaker than those observed for mitozolomide at an equimolar concentration.

Alkaline sucrose density-gradient

In addition to the determination of the effects of tetrazepinones on macromolecule synthesis and the cell cycle, we studied the damaging potential of PYRCL and QUINCL on cellular DNA. We chose to analyze the DNA at 24 h post-treatment, since at this time point considerable cell cycle perturbations were observed.

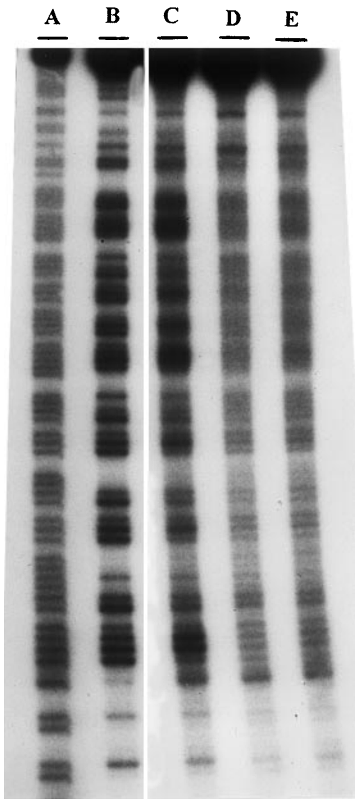


Fig. 4 DNA alkylation produced by mitozolomide, QUINCL and PYRCL at an equimolar concentration (1 m*M*) in the HindIII/SphI sequence of SV40 DNA. *Lane A* control purine-specific cleavage, *lane B* control guanine-specific cleavage, *lane C* mitozolomide-treated DNA, *lane D* PYRCL-treated DNA, *lane E* QUINCL-treated DNA

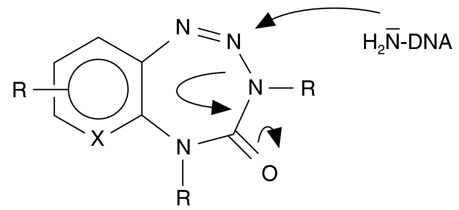
Also, at this time the results were indicative of unrepaired damage, which we believe presents a more significant relationship between DNA damage and cell death.

Interestingly, the tetrazepinones induced significant DNA damage at concentration ranges corresponding to 50–70% cell-killing at 24 h post-treatment. It is important to note that this DNA damage occurred in attached cells (and was therefore not secondary to cell death). A significant shift from the internal control peak was observed towards low molecular weight fractions (Fig. 5C, D). Surprisingly, at this time no such effect was observed in mitozolomide-treated cells. However, when heated in a citrate-phosphate buffer (pH 7.0) followed by exposure to alkaline conditions, single strand breaks could be detected in DNA from cells treated with mitozolomide (Fig. 5B). This suggests that the type of DNA damage induced by mitozolomide and the terazepinones may be different.

Discussion

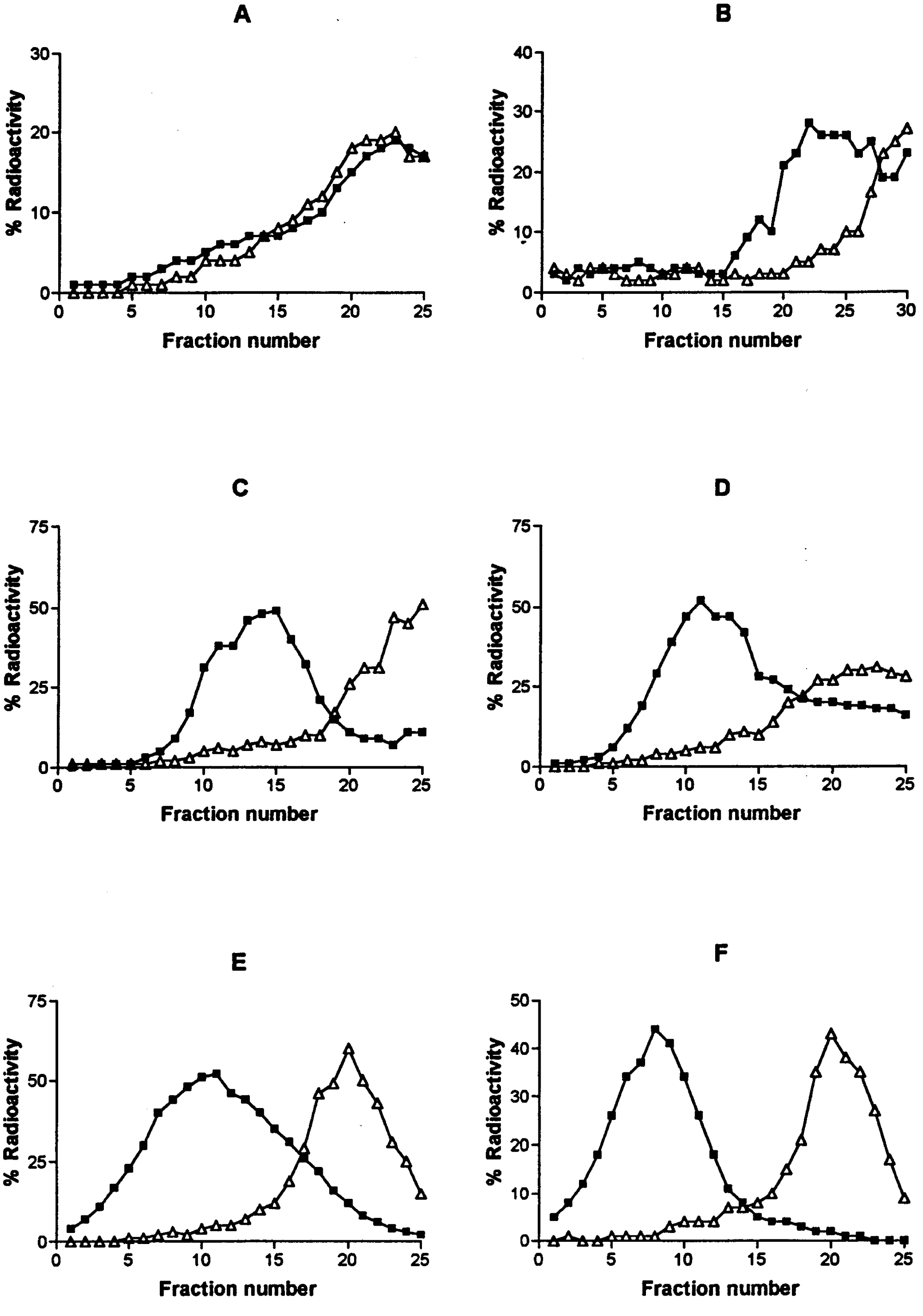
The results obtained thus far give *prima facie* evidence that the 3-(2-chloroethyl)-1,2,3,5-tetrazepinones kill tumor cells by a mechanism of action different from that of the 3-(2-chloroethyl)-1,2,3,5-tetrazinone mitozolomide. Previous studies in our laboratories have shown that, unlike the 1,2,3,5-tetrazin-4-one ring, the 1,2,3,5-tetrazepin-4-one cycle does not decompose via the hydrolysis of the ureido moiety to generate an open-chain triazene [1]. It principally decomposes via a loss of nitrogen to give ring-contracted products (e.g. 2-oxobenzimidazole of type **5**) [17]. This may account for their weak alkylating power when compared with mitozolomide and temozolomide, which stoichiometrically hydrolyzes to the strong alkylating monoalkyltriazene species of type **4** at basic pH.

It is surprising that despite their capacity to induce only weak guanine N-7 alkylation in isolated DNA, the tetrazepinones induce significant DNA damage in OVCAR-3 cells. Furthermore, previous studies have shown that, in contrast to mitozolomide, the 3-(2-chloroethyl)-tetrazepinones create barely detectable levels of DNA interstrand crosslinks [16]. This further indicates that the chemistry of DNA lesions induced by the tetrazepinones may differ from those of the classical alkylating agents such as chloroethylnitrosoureaas and chloroethylaryltriazenes. Based on the anomalous elongation of the N2-N3 bond of the tetrazepinone ring [15, 28, 29], we believe that reactions with the triazene portion of tetrazepinones can occur with amine-containing DNA bases (e.g. adenine), as shown in Scheme 1. This hypothesis is currently being tested in our laboratory.



Mitozolomide selectively inhibited DNA synthesis in the OVCAR-3 cells. This observation is in agreement with previous reports describing its selective inhibition of DNA synthesis in the TLX-5 mouse lymphoma cell line [12] and in the human ovarian carcinoma cell line SW626 [4]. In contrast, the tetrazepinones did not induce selective inhibition of macromolecule synthesis in OVCAR-3 cells: DNA, RNA and proteins were all inhibited to almost the same extent and 24 h following drug treatment, the inhibition of macromolecule synthesis decreased. The apparent decrease in the inhibitions of macromolecule synthesis was probably due to the activation of DNA, RNA and protein synthesis as a response to multiple drug-induced cellular lesions. Paradoxically, this increase in precursor incorporation was accompanied by significant cell death and marked cell cycle arrest in G_2M. Lack of correlation between inhibition of DNA synthesis and inhibition of cell growth has already been reported by Sorenson and Alan [24] who observed that 24, 48 and 72 h after a 2-h treatment, the DNA content of L-1210 cells exposed to cis-diamminedichloroplatinum(II) (cis-DDP) doubles despite a significant growth retardation. Blocks to DNA

Fig. 5A–F Genomic DNA damage produced by mitozolomide, PYRCL and QUINCL, 24 h post-treatment. Strand breaks were determined by alkaline sucrose density centrifugation. Internal control ^{14}C-labeled DNA from untreated cells (△); ^{3}H-thymidine-labeled DNA from treated cells (■). **A** Control, **B** mitozolomide 250 μ*M*, **C** PYRCL 100 μ*M*, **D** PYRCL 200 μ*M*, **E** QUINCL 100 μ*M*, **F** QUINCL 200 μ*M*



synthesis are thought to create gaps in the newly synthesized daughter strands which are repaired by a gap-filling mechanism that requires increased thymidine incorporation. This explanation permits a correlation between DNA damage, increased thymidine incorporation and inhibition of growth. It may also apply to the effect of QUINCL and PYRCL on DNA synthesis, since these drugs induced significant levels of single-strand breaks in the OVCAR-3 cells 24 h post-treatment, while marked increase in thymidine incorporation was observed. Long-patch repair of single strand breaks and gap-filling mechanisms can account for the significant increase in thymidine incorporation observed during cell death.

In contrast to the tetrazepinones, which induced cell cycle arrest only in G_2M, mitozolomide mostly induced cell cycle arrest in S-early and S-middle within the same concentration range. This suggests that the DNA damage induced by this drug may block the initiation and progress of DNA replication. The N-7, O-6-alkylguanine and DNA interstrand crosslink known to be induced by mitozolomide [4, 6, 7] can potentially block

DNA replication. The IC_{50} value for the inhibition of DNA synthesis (18 μM) was lower than those observed for cell survival at 24 h (>200 μM) and 72 h post-treatment (67 μM), and almost equal to the value obtained by a colony-forming assay (15 μM). This suggests that inhibition of DNA synthesis could probably be a cause of cell death induced by mitozolomide. No such correlation can be made for the tetrazepinones, since inhibition of DNA synthesis occurs at concentrations higher than IC_{50} values of cell survival.

The marked difference between cellular responses induced by mitozolomide and the tetrazepinones was also in their kinetics of cell-killing. Both the sulforhodamine B assay and cell counting showed a slow rate of reduction in survival induced by mitozolomide when compared with the tetrazepinones. One and 3 days after treatment, mitozolomide was less potent than the tetrazepinones. However, in a longer 11-day colony-forming assay, all three drugs showed almost equal cytotoxic activity. Delayed cell death induced by mitozolomide is probably caused by the promutagenic O-6-alkylguanine adduct, a lesion which causes the cells to replicate with genetic defects and to die later in subsequent rounds of cell division [23, 27]. By contrast, the type of DNA lesions induced by the chloroethyltetrazepinones cause cell death in less than 24 h post-treatment. These results suggest that cell killing by the tetrazepinones may not be induced principally by a mutagenetic mechanism.

Whereas the cytotoxic activity of mitozolomide can be explained by classical alkylation mechanisms (e.g. alkylation of guanine at the 6 or 7 position), the effects of the tetrazepinones on DNA synthesis and the cell cycle, their alkylating power and the DNA strand breaks that they induce, can best be explained by the chemistry of the 1,2,3,5-tetrazepinone ring degradation. As mentioned above, in cell culture media, while mitozolomide decomposes to a monoalkyltriazene, the tetrazepinones are mostly converted to a 2-oxo-imidazole species following the loss of nitrogen. This should translate into a different mechanism of interactions with DNA. The study of these interactions is the subject of considerable ongoing work in our laboratory.

The clinical testing of mitozolomide was discontinued because of severe hematologic toxicity (thrombocytopenia). Since our new tetrazepinone series is equipotent with mitozolomide, yet kill tumor cells by a novel mechanism of action, we hope that they will afford a better therapeutic index in the clinic.

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