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Unique sensitivity of nitrogen mustard-resistant human Burkitt lymphoma cells to novobiocin

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Overcoming resistance to chemotherapeutic agents is a major objective of clinical oncology. If the mechanism of resistance to a particular drug is understood, it may be possible to specifically treat the resistant fraction when it arises, or even prevent its development. Recently Frei *et al.* [1] developed Raji-HN2, a human Burkitt lymphoma cell line, that is approximately 10-fold resistant to HN2*. These cells have been characterized in our laboratory as containing approximately 3-fold higher specific activity of extractable topoisomerase II than that of topoisomerase II extracted from the parental Raji cells [2]. This difference in topoisomerase II activity was correlated with the difference between Raji and Raji-HN2 cells in survival and in the ability to repair HN2-induced damage leading to DNA interstrand crosslink formation [2, 3]. In the course of further characterizing Raji-HN2 cells, we found that they were hypersensitive to novobiocin, an inhibitor of eukaryotic topoisomerase II [4]. In this communication, we present our further characterization of the unique hypersensitivity to novobiocin demonstrated by Raji-HN2 cells, evidence suggesting the probable biochemical basis for this phenomenon, and discuss the clinical implications of this observation.

Methods

Cells. Cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum. Raji-HN2 cells were treated weekly with 10 μM HN2 to maintain the resistant phenotype [1, 2]. Treatment of cells with drugs was in serum-free medium. All cells used were in the exponential growth phase [2].

Clonogenic assay. Cell survival was determined by colony formation in soft agarose. After drug treatment, cells were diluted 500-fold into 0.15% low-melting-point agarose (Sea Plaque, FMC Corp., Rockland, ME) prepared in RPMI medium containing 20% fetal bovine serum. After 7–9 days of incubation at 37°, cell colonies were stained with tetrazolium salts and counted in a Biotran counter [2]. Each treatment was assayed in triplicate, and the individual values did not vary by more than 15% from the mean.

Topoisomerase II activity. Enzyme activity contained in the 0.35 M NaCl extracts of isolated nuclei was measured by the P4 DNA unknotting assay as described previously [2]. Specific activity is expressed as units of enzyme per microgram of protein of the nuclear extract. One unit of enzyme activity is that which unknots 200 ng of knotted P4 DNA.

Results and discussion

Raji-HN2 cells are approximately 10-fold more resistant to the cytotoxicity of HN2 and contain 3-fold more extractable topoisomerase II activity than Raji cells [1, 2]. The enzyme is of vital importance to Raji-HN2 cells because of the cytotoxicity achieved in this cell line by a number of topoisomerase II inhibitors, including novobiocin [2, 3]. In a preliminary experiment, Raji-HN2 cells were treated for various lengths of time with 400 μg/ml novobiocin to determine the kinetics of novobiocin-induced cell killing. Novobiocin exerted its cytotoxic effects rapidly; a 30-min treatment resulted in approximately 80% reduction of cell survival (unpublished data). Novobiocin at 800 μg/ml had no effect on the survival of Raji (Burkitt lymphoma), HeLa (cervical carcinoma), K562 (erythroleukemia), and KG1a (bone marrow stem cells) but reduced the survival of Raji-HN2 cells by 90% (data not shown).

Because Raji and Raji-HN2 cells demonstrated different sensitivities to novobiocin or HN2, we were interested in determining the response of these cells to treatment with a combination of novobiocin and HN2. Two types of experiments were performed: (i) pretreatment with a single dose of novobiocin followed by treatment with increasing concentrations of HN2, and (ii) pretreatment with a single dose of HN2 followed by treatment with increasing concentrations of novobiocin. When used in combination with

* Abbreviation: HN2, nitrogen mustard [2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine].

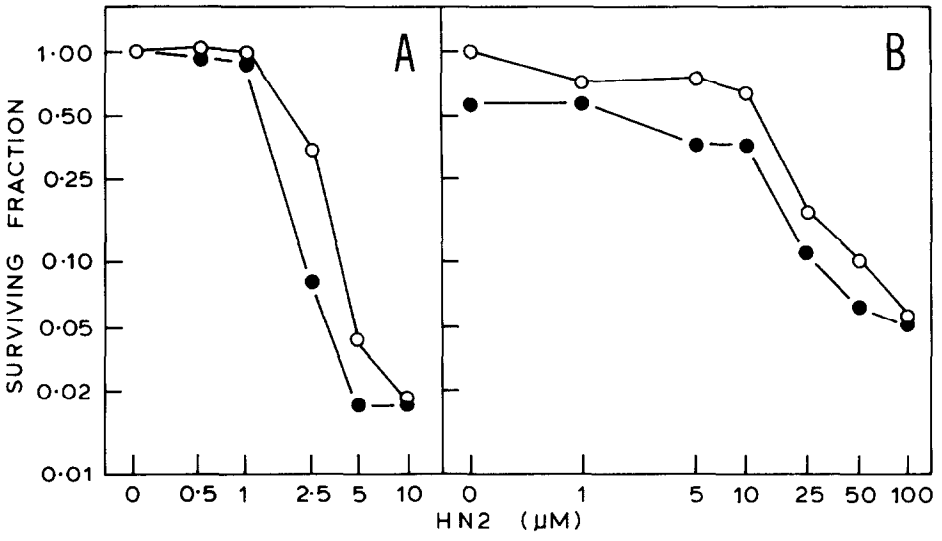

Fig. 1. Effect of novobiocin on HN2 cytotoxicity. Cells pretreated for 30 min with novobiocin (200 μg/ml) were then treated with increasing concentrations of HN2 for 30 min. Panel A: Raji cells not pretreated (○) or pretreated (●) with novobiocin. Panel B: Raji-HN2 cells not pretreated (○) or pretreated with novobiocin (●). Control dishes contained 800–1000 cell colonies each. One representative experiment of four is shown.

HN2, novobiocin potentiated the killing of both Raji and Raji-HN2 cells by HN2 (Fig. 1). Novobiocin alone (200 μg/ml) was moderately cytotoxic to Raji-HN2 cells (see Figs. 1 and 2). The increased cell killing by the combination drug treatment probably represented the additive cytotoxicities of HN2 and novobiocin. A critical observation was the finding of synergistic cytotoxicity for both Raji and Raji-HN2 cells when a non-cytotoxic concentration of HN2 was combined with novobiocin (Fig. 2). Raji cells were not sensitive to novobiocin, but they were rendered sensitive by co-treatment with a noncytotoxic concentration of HN2 (Fig. 2).

Untreated Raji-HN2 cells contain approximately three times the extractable topoisomerase II activity of Raji cells [2]. Novobiocin treatment reduced the topoisomerase II activity in the nuclear extract of Raji-HN2 cells (Fig. 3). This decrease in enzyme activity parallelled the decrease in survival of Raji-HN2 cells in the presence of increasing novobiocin concentrations. The concentration range of 200–400 μg/ml was found to be critical for both inhibition of extractable topoisomerase II activity and cytotoxicity (Figs. 2 and 3). When novobiocin-treated cells were resuspended in fresh medium lacking the drug, both the topo-

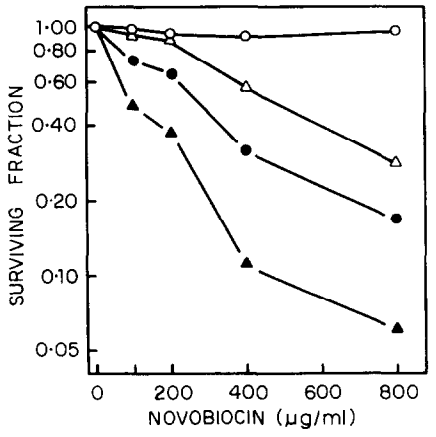

Fig. 2. Potentiation of novobiocin cytotoxicity by HN2. To control cells not treated or cells pretreated for 30 min with increasing concentrations of novobiocin, HN2 was added for an additional 30 min. The HN2 concentrations used (1.5 μM for Raji and 5 μM for Raji-HN2 cells) represent the highest non-cytotoxic concentrations for these cells (see Fig. 1). Key: novobiocin-treated Raji cells without (○) or with (△) HN2; novobiocin-treated Raji-HN2 cells without (●) or with (▲) HN2. Control dishes contained 800–1000 cell colonies each. The values of both cell lines treated with 400 or 800 μg/ml of novobiocin were significantly different from those of cells co-treated with HN2 (Student's *t*-test, $P < 0.005$). One representative experiment of three is shown.

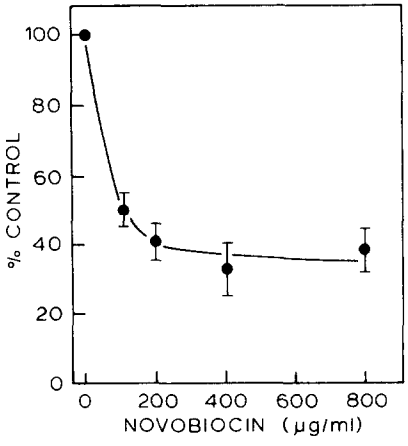

Fig. 3. Effect of novobiocin on topoisomerase II activity of Raji-HN2 cells. Topoisomerase II activity in the nuclear extract of cells treated with novobiocin for 30 min was determined by P4 DNA unknotting assay. Mean and range (maximum and minimum values) of duplicate assays are shown. The control sample contained 50 units of enzyme activity.

isomerase II activity in the nuclear extract and cell survival increased with increasing time after drug removal (data not shown). Novobiocin inhibits eukaryotic topoisomerase II by acting as a competitive inhibitor of ATP, which is required for enzyme activity [5]. It does not damage DNA under the conditions used in our experiments and is regarded as essentially nontoxic for eukaryotic cells. Hypersensitivity of Raji-HN2 (and, perhaps, other resistant tumor) cells to novobiocin is correlated with the inhibition of elevated topoisomerase II activity of these cells. A change in the ATP-binding site of the enzyme may produce an enormous increase in the susceptibility of the enzyme to the inhibitory binding of novobiocin.

The differential sensitivity to novobiocin exhibited by Raji and Raji-HN2 cells provides a clinical rationale for the use of novobiocin, or a novobiocin-like compound, in cancer chemotherapy. Novobiocin can be used under two different clinical settings. In the first instance, in view of the synergistic action of novobiocin when used with HN2 for Raji cells, the use of low concentrations of novobiocin in combination with HN2, or other alkylating agents, could selectively increase the destruction of sensitive tumor cells. Second, the use of novobiocin alone, in high concentrations, could selectively kill tumors that have developed resistance to alkylating agents, and which have elevated topoisomerase II activity. Novobiocin itself would likely have no effect on nonmalignant cells or on the HN2-sensitive fraction.

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Altered expression and transcription of the topoisomerase II gene in nitrogen mustard-resistant human cells

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Topoisomerase II, a target of several classes of anti-tumor drugs, is involved in chromosome segregation and is implicated in gene regulation, structure and function [see reviews in Refs. 1–3]. Recently we reported that Raji-HN2, a Burkitt lymphoma cell line made resistant to HN2*, is hypersensitive to topoisomerase II inhibitors and contains about 3-fold more of extractable topoisomerase II activity than the parental Raji cell line [4, 5]. Although the function of topoisomerase II in Raji-HN2 cells is unknown, the increased extractable enzyme activity in these cells is correlated with resistance to HN2 and increased cell doubling time [5]. In this paper we report, using antiserum and cDNA specific for topoisomerase II as probes, that the synthesis of the enzyme and transcription of the gene were elevated in Raji-HN2 cells when compared with Raji cells.

Materials and methods

Cells. Raji-HN2 cells were treated weekly with 10 μM HN2 to maintain the resistant phenotype [5, 6]. In the experiments described below, both Raji and Raji-HN2 cells were harvested simultaneously at the exponential growth phase.

Protein analysis. Cells were lysed directly in SDS buffer and electrophoresed in a 7.5% acrylamide gel [7]. The fractionated polypeptides were blotted to a nitrocellulose membrane and reacted sequentially with a polyclonal antiserum to purified calf thymus topoisomerase II and ^{125}I-labeled protein A [8].

RNA analysis. RNA prepared from cells lysed with guanidine isothiocyanate was fractionated on an oligo-dT column to yield poly(A) RNA [9]. After electrophoresis in a 1% agarose gel containing 1 M formaldehyde [9], the RNA was blotted directly to a Zetaprobe nylon membrane (Bio-Rad) with 0.2 M NaOH for 1.5 hr. The blot was then neutralized with 2 × SSPE for 10 min.

DNA analysis. DNA extracted from nuclei [9] was digested with restriction enzymes (2 units/μg DNA for 6 hr at 37°), fractionated in a 1% agarose gel, and transferred to a Zetaprobe membrane by blotting with 0.4 M NaOH [10].

cDNA cloning. A Raji-HN2 cDNA library was prepared by linker-ligation at the EcoRI site of bacteriophage λgt11 [11]. The library (2 × 10^5 plaques) was screened, under low stringency conditions [9], with a *Drosophila* topoisomerase II cDNA clone [12], and twenty-six positive clones were obtained. Two partial clones, SP-12 and SP-17, were used in this study.

* Abbreviations: HN2, nitrogen mustard [2-chloro-*N*-(2-chlorethyl)-*N*-methylethanamine]; cDNA, complementary DNA; kb, kilobase; kD, kilodalton; SDS, sodium dodecyl sulfate; and 1 × SSPE = 180 mM NaCl, 10 mM $NaPO_4$, pH 7.7, 1 mM EDTA.