Comparative *in vitro* cytotoxicity of cyclophosphamide, its major active metabolites and the new oxazaphosphorine ASTA Z 7557 (INN mafosfamide)

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Summary

Cyclophosphamide (CPA), the most commonly used alkylating agent in the treatment of a wide variety of hematologic and solid tumors, requires oxidation by hepatic microsomal enzymes to its active alkylating species. A number of alternative methods exist to simulate the *in vitro* cytotoxicity of CPA against animal and human tumors, including the co-incubation of CPA with the S-9 fraction of rat liver homogenates (S-9) and the use of either 4-hydroperoxy CPA (a stabilized form of a major blood-borne metabolite of CPA), phosphoramide mustard (PM, considered to be the ultimate intracellular alkylating metabolite of CPA), or ASTA Z 7557 [4-(2-sulfonatoethylthio)-CPA, a new oxazaphosphorine compound which after dissolution undergoes rapid spontaneous hydrolysis *in vitro* with liberation of 4-hydroxy-CPA]. Using a human tumor clonogenic assay (HTCA) we have quantitated the median molar inhibitory dose 50 (IC₅₀) concentrations of S-9 activated-CPA, 4-hydroperoxy-CPA, PM, and ASTA Z 7557 against 107 previously untreated tumors, as well as determining the *in vitro* biological stability of the former three CPA metabolite preparations. 4-Hydroperoxy-CPA proved the most consistently cytotoxic (median molar IC₅₀ = 5.7 × 10⁻⁵ M) compound, followed by ASTA Z 7557, S-9 activated-CPA and PM in that order. Of additional interest S-9 activated CPA and PM proved relatively unstable biologically when frozen at −120°C, whereas 4-hydroperoxy-CPA lost none of its cytotoxicity over a 36 day period during freezing. On the basis of these data 4-hydroperoxy-CPA appears the compound of choice for use *in vitro* to evaluate the activity that CPA is likely to express clinically against solid tumors. Since 4-hydroperoxy-CPA is not available for clinical use, ASTA Z 7557, which was slightly less cytotoxic to ovarian cancers and a wide variety of other tumors in the HTCA, appears an attractive agent to develop further clinically, especially for regional chemotherapy (e.g., intraperitoneal and intra-arterial treatment) of solid tumors.

Introduction

Cyclophosphamide (CPA) is the most commonly used alkylating agent in the treatment of a wide variety of hematologic and solid tumors. This potent anticancer drug differs from other clinically useful bis(chlorethyl)amines by its requirement for oxidation to active alkylating species by hepatic microsomal enzymes.
microsomes. This metabolism can be stimulated in vitro with rat liver homogenates containing microsomes (1–3). 4-Hydroxy-CPA is believed by most investigators (4–7) to be the primary extracellular mediator of CPA antitumor activity. Others (8, 9) believe that phosphoramidate mustard performs this role both extra- and intracellularly.

ASTA Z 7557 (4-(2-sulfonatoethylthio)-CPA) is a new oxazaphosphorine compound which after dissolution undergoes rapid spontaneous hydrolysis in vitro and in vivo with liberation of 4-hydroxy-CPA (10). This promising new anticancer agent has shown a high degree of activity in vivo against several rat and mouse tumors (10) and has recently been entered into phase I clinical trials in Europe.

Several standard anticancer agents (e.g., CPA, DTIC [dimethyltriazenoimidazole carboxamide] and hexamethylmelamine) require bioactivation in vitro by microsomal enzymes to express their cytotoxicity in vitro. Lieber et al. (11) and more recently Alberts et al. (12, 13) have adapted a soft agar human tumor clonogenic assay (14, 15) for use with the “S-9 fraction” of rat liver homogenates to evaluate the in vitro activity of CPA against fresh human tumors. In addition, others have developed CPA bioactivation systems for drug sensitivity testing, which do not utilize soft-agar cell culture methods (16–18).

The human tumor clonogenic assay (HTCA) has proven to be a useful system for screening new chemical compounds for anticancer activity (19, 20), for testing new clinical agents for phase II activity (21–23), and for predicting clinical response to cytotoxic therapy in cancer patients (15, 23–27). We are now reporting the results of an in vitro trial in the HTCA of the relative cytotoxicities against fresh human tumors and human tumor cell lines of CPA activated by the “S-9 rat liver fraction” (S-9 fraction), 4-hydroxy-CPA, 4-hydroperoxy-CPA (a stabilized form of 4-hydroxy-CPA), phosphoramidate mustard, and the new Asta-Werke compound, ASTA Z 7557.

Materials and methods

Human tumor cell line

The WiDR human colon cancer cell line (obtained from the American Type Culture Collection, Rockville, MD) was used to evaluate the biological stability of S-9 fraction activated CPA, 4-hydroxy-CPA, 4-hydroperoxy-CPA and phosphoramidate mustard. WiDR cells were cultured as a monolayer in RPMI 1640 plus 10% fetal calf serum (FCS), penicillin (100 units/ml)/streptomycin (100 µg/ml) and glutamine (2 mM) at 37°C in a 95% humidity, 5% CO₂ room air incubator and passed serially as needed. The cells were harvested when they were undergoing exponential growth. Harvesting was done by incubation of the phosphate-buffered saline washed flasks with Trypsin-EDTA, 0.25% (Gibco) for 5 minutes at 37°C. Medium was added and the cells allowed to incubate for 15 minutes. The cells were then shaken off the bottom of the flask and washed twice in McCoy’s 5A medium and centrifuged at 150 g for 7 minutes. Adequate dilutions of single cell suspensions were prepared and plated at a final concentration in agar of 10,000 cells/petri dish.

Fresh human tumors

All biopsies were obtained in accord with guidelines and informed consent forms as approved by the Human Subjects committee of the Arizona Health Sciences Center. Tumor specimens obtained during the course of routine diagnostic surgical procedures and bone marrows from patients were transferred promptly and under aseptic conditions to the laboratory in tissue culture medium. Malignant effusions and bone marrow aspirates were collected in preservative-free heparin. Solid tumors were disaggregated mechanically (28) or with a combination of collagenase and DNase (29, 30). Cell suspensions from all tumor types were plated in 35 mm plastic petri dishes in the double-layer HTCA as described by Hamburger and Salmon (14) and incubated in the presence of 5% CO₂ in air.