Combined Antitumor Effect of Cyclophosphamide and Bromodeoxyuridine in BDF1 Mice Bearing L1210 Ascites Tumors

Yoshiyuki KAGAWA,*a Ichiro NOGE,a Masamune HIGASHIGAWA,a and Yoshihiro KOMADAB

a Department of Clinical Pharmaceutics, School of Pharmaceutical Sciences, University of Shizuoka; 52–1 Yada, Suruga-ku, Shizuoka, Shizuoka 422–8526, Japan; and b Department of Pediatrics, Mie University School of Medicine; 2–174 Edobashi, Tsu, Mie 514–8507, Japan. Received August 18, 2007; accepted October 30, 2007

We investigated the combined effect of cyclophosphamide (CPA) and 5-bromo-2'-deoxyuridine (BrdUrd) both in mice bearing L1210 ascites tumors and in L1210 leukemic cells in vitro. Administration of BrdUrd (100 mg/kg) for 5 consecutive days before a single dose (80 mg/kg) of CPA significantly extended the survival of mice by 158%, compared with CPA alone. BrdUrd administered at daily doses of 100 or 200 mg/kg for 5 consecutive days did not extend the survival of mice. An in vitro MTT assay revealed that BrdUrd enhanced the cytotoxic effect of 4-hydroxycyclophosphamide, an active form of CPA, in the L1210 cells. These results indicate that BrdUrd enhanced the antitumor effect of CPA both in vivo and in vitro.

Key words 5-bromo-2'-deoxyuridine; cyclophosphamide; antitumor effect; 4-hydroxy-cyclophosphamide

Halogenated pyrimidines are incorporated in place of thymidine in DNA of replicating cells, making the cells more susceptible to the effects of radiation. 5-Bromo-2'-deoxyuridine (BrdUrd) has been clinically used as a radiosensitizer to enhance the tumor killing effect of radiation therapy.1–4 The radiosensitizing effect of halogenated pyrimidines on mammalian cells in vitro is closely associated with the amount of drug incorporated into DNA.5

Alkylating agents, such as cyclophosphamide (CPA), are widely used to treat a broad spectrum of malignancies, including both solid tumors and hematological malignancies.6,7 CPA is converted by hepatic cytochrome P450 metabolic enzymes via two major pathways.8,9 The first involves 4-hydroxylation to the active metabolite, 4-hydroxycyclophosphamide (4-OH-CPA). This conversion is carried out predominantly by cytochrome P450 CYP2B6 in humans.9,10 4-OH-CPA exists in equilibrium with aldotosphamide, which is broken down to form the DNA cross-linking agent phosphoramid mustard and the toxic metabolite acrolein.11–13 The alternative pathway involves a CYP3A4-mediated N-dechloroethylation of CPA to form the inactive metabolite 3-dechloroethylcyclophosphamide (DECP) and the toxic by-product chloroacetaldehyde.14,15 In humans, this pathway is secondary to the activation pathway and accounts for less than 10% of the dose.13

Alkylating agents undergo a transformation to produce highly reactive and positively charged ions. These ions can then form covalent bonds with electron-rich sites on biological molecules, such as nucleic acids, proteins, and amino acids.10 Irradiation may cause the ablation of both single- and double-stranded DNA, denegeneration or loss of bases, and intercalation of DNA and/or chromosomal protein. The actions of alkylating agents appear to have some similarities to those of gamma ray radiation. A previous in vitro report showed that pretreatment with iododeoxyuridine (Iudr) or BrdUrd enhanced the cytotoxicity of various antineoplastic agents, such as melphalan, cisplatin, doxorubicin, and bleomycin.16 However, the mechanism of halopirimidine's chemosensitizing effect is still unclear. There have been few in vivo studies on the combined antitumor effects of halogenated pyrimidines and alkylating agents. We investigated here the combined effect of BrdUrd and CPA, a bifunctional alkylating agent, on life span in L1210 ascites tumor-bearing mice and cytotoxicity in cultured L1210 cells using an MTT assay.

MATERIALS AND METHODS

Chemicals BrdUrd was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CPA and 4-hydroxy-cyclophosphamide (4-OH-CPA), an active form in men, were obtained from Shionogi Pharmaceutical Co., Ltd. (Osaka, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co.

Life Prolongation Study Male BDF1 mice were purchased from Shionogi Pharmaceutical Co., Ltd. and kept at Mie University Animal Center under constant conditions (a 12 h light: 12 h dark regime with Oriental Chool pellet food and water freely available). L1210 leukemia cells were obtained from Shionogi Pharmacological Laboratory, Osaka, Japan and maintained in BDF1 mice by weekly intraperitoneal (i.p.) passage as previously described.18 Experimental mice received an i.p. injection of 1×10⁷ ascites tumor cells in saline on Day 0. Seventy mice were randomly divided into 7 groups. CPA and BrdUrd were prepared just before use by dissolving in saline. Either 100 or 200 mg/kg of BrdUrd was given i.p. to mice 24 h after the tumor inoculation and this was repeated once daily for 5 consecutive days (Day 1 to 5). A single dose of CPA (80 or 160 mg/kg) was given i.p. to mice on Day 5. To evaluate the combined effect of BrdUrd and CPA, 100 mg/kg of BrdUrd was administered i.p. for five consecutive days (Day 1 to Day 5) and a single dose (80 mg/kg) of CPA was administered i.p. just after the BrdUrd dose on Day 5. To investigate the antitumor effect of CPA in the early period, when tumor cells have yet to enter a proliferative phase, one group of mice was given a single i.p. dose (80 mg/kg) of CPA at Day 1. Saline was given to the control groups using the same schedule.

In Vitro Assay L1210 leukemia cells were obtained from Shionogi Pharmacological Laboratory. Cells were fed twice weekly with RPMI 1640 medium supplemented with 10% bovine serum (ICN Biomedicals, Aurora, Ohio, U.S.A.).
0.2% sodium bicarbonate, and 5 μM mercaptoethanol solution, and grown at 37 °C in a humidified incubator with 5% CO₂.

Cells in an exponential phase of growth were used in experiments. An MTT assay was performed to measure cell survival. Cells were seeded in 96-well plates at 1×10⁵ cells/well and incubated for 48 h with or without 0.1 to 0.4 μg/ml of BrdUrd before the addition of 4-OH-CPA. Twenty four hours after the pretreatment with BrdUrd, various concentrations of 4-OH-CPA were added into the wells. Twenty four hours after the treatment with 4-OH-CPA, 10 μl of MTT was added to each well, and the plate was incubated for 4 h at 37 °C. The MTT crystals were solubilized by the addition of 100 μl of acid isopropyl alcohol. The spectrophotometric absorbance of each sample was measured at 570 nm using a DynaTech MR5000 Microplate Reader (Chantilly, VA, U.S.A.). Each datum was expressed as an average of measurements obtained from 6 wells.

To determine whether the two drugs exhibited synergistic cytotoxic effects, the data from the dose–response curves was examined by constructing isobolograms.

Statistical Analysis The statistical analysis was performed using the StatView version 5 program (Hulinks, Tokyo, Japan). Scheffe’s F test was performed to compare the survival times of the mice, and p<0.05 was considered significant.

RESULTS

BDF1 mice bearing L1210 ascites tumors intraperitoneally received two types of a single dose of CPA 5 d after the tumor inoculation with or without a concomitant administration of BrdUrd. Figure 1 shows the life span of the tumor-bearing mice. One hundred and sixty mg/kg of CPA alone significantly extended the life of these mice compared with the control group that received saline (p<0.001), but 80 mg/kg of CPA did not (p=0.58). BrdUrd was administered at daily doses of 100 or 200 mg/kg for 5 consecutive days from 1 d after the tumor inoculation. Neither dose significantly increased life span. The concomitant use of BrdUrd (100 mg/kg, 5 consecutive days) and CPA (80 mg/kg, Day 5) significantly lengthened the life span of mice, compared with the control group or the group treated with 80 mg/kg CPA alone on Day 5 (p<0.001). A single 80 mg/kg dose of CPA administered 1 d (Day 1) after the tumor inoculation significantly extended life span, compared with the control (p<0.05). However, there was no significant difference between the two groups that received 80 mg/kg of CPA at Day 1 and Day 5 (p=0.69). Another experiment showed that 40 mg/kg of CPA (Day 5) and 100 mg/kg of BrdUrd (Day 1 to 5) enhanced the antitumor effect, compared with 40 mg/kg of CPA alone (Day 5) (data not shown).

Mice in the control, BrdUrD 100 mg/kg, and BrdUrD 200 mg/kg groups gradually increased in weight 5 to 7 d after the tumor inoculation, with an increase of more than 10% compared to levels before the tumor inoculation (Fig. 2). Most of the mice died a few days after the increase in weight.
peaked. Groups given the combination of CPA plus BrdUrd and the high dose (160 mg/kg) of CPA showed two peaks of change in weight: their weight increased around 6 d after the tumor inoculation and then decreased to the same level before the inoculation, before increasing again around 2 weeks after the inoculation. They died a few days later. Mice treated with CPA 1 d after the inoculation exhibited a peak at about 10 d, some 4 d later than those in the other group. Swelling of the abdomen was detected when their weight increased.

An MTT assay was performed using the leukemic cell line L1210. Figure 3 shows that 24 h of treatment with 4-OH-CP A killed L1210 cells in a dose-dependent manner, and the IC50 value was 1.27 μg/ml. Three concentrations of BrdUrd were added to the culture medium 24 h before the addition of 4-OH-CP A. In contrast to the result in tumor-bearing mice, the 48-h treatment with BrdUrd alone had an apparent cytotoxic effect in vitro and the IC50 value was 0.41 μg/ml (Fig. 4). BrdUrd at 0.1, 0.2, or 0.4 μg/ml enhanced the cytotoxicity of 4-OH-CP A in a concentration-dependent manner. The isobologram at the IC50 of 4-OH-CP A showed that 0.1 μg/ml of BrdUrd synergistically affected the cytotoxicity of 4-OH-CP A, but 0.2 μg/ml of BrdUrd seemed to have an additive effect (Fig. 5). The isobologram at the IC75 of 4-OH-CP A showed that at 0.1, 0.2, and 0.4 μg/ml, BrdUrd had a synergistic effect on the cytotoxicity of 4-OH-CP A. We also examined cytotoxicity produced by the simultaneous treatment of 4-OH-CP A and BrdUrd for 48 h against the L1210 cells. No enhanced cytotoxicity was produced by the simultaneous treatment of those two drugs (data not shown).

DISCUSSION

Our results showed that oral coadministration of BrdUrd (100 mg/kg) with CPA (80 mg/kg) significantly extended the life span of mice bearing L1210 ascites tumors, compared with the mice given CPA (80 mg/kg) alone or the control group. Although the antitumor effect of 160 mg/kg of CPA alone was comparable to that of CPA (80 mg/kg) coadminis-

Fig. 3. Cytotoxicity of 4-Hydroxycyclophosphamide in L1210 Leukemic Cells with or without Bromodeoxyuridine

L1210 leukemic cells were treated with various concentrations of BrdUrd for 24 h. 4-Hydroxycyclophosphamide (4-OH-CP A) was added to the culture medium for an additional 24 h. Forty eight hours after the addition of bromodeoxyuridine (BrdUrd) and 24 h after the addition of 4-OH-CP A, an MTT assay was performed to assess the cytotoxicity of the drugs. Vertical bars indicate the means±S.D.

Fig. 4. Cytotoxicity of Bromodeoxyuridine in L1210 Leukemic Cells

L1210 leukemic cells were treated with various concentrations of bromodeoxyuridine (BrdUrd) for 48 h. Forty eight hours after the drug treatment, an MTT assay was performed to assess the cytotoxicity of the drugs. Vertical bars indicate the means±S.D.

Fig. 5. Isobologram Depicting the Combined Effect of 4-Hydroxycyclophosphamide and Bromodeoxyuridine on L1210 Leukemic Cells

L1210 leukemic cells were treated with various concentrations of BrdUrd for 24 h. Then 4-hydroxycyclophosphamide (4-OH-CP A) was added to the culture medium for an additional 24 h. Forty eight hours after the addition of bromodeoxyuridine (BrdUrd) and 24 h after the addition of 4-OH-CP A, an MTT assay was performed to assess the cytotoxicity of the drugs. Data from concentration–survival curves generated in Fig. 3 were used to determine the IC50 or IC75 values.
DNA, and tumor cells might be more susceptible to CPA. It was, however, not clear whether the accumulation of BrdUrd into DNA was essential or not for the combined effect because we did not examine the combined effect of a single dose of BrdUrd and CPA. CPA (80 mg/kg) administered 1 d after the tumor inoculation significantly increased life span, while the same dose of CPA administered 5 d after the tumor inoculation did not. It is generally thought that the administration of anticancer drugs is more advantageous to the host before than after the drastic proliferation of tumor cells. Concomitant administration of CPA (80 mg/kg) with BrdUrd (100 mg/kg) extended life more than CPA alone administered 1 d after the tumor inoculation. This result indicated that the combination of CPA and BrdUrd could overcome the disadvantage of being treated after an increase in the number of tumor cells. BrdUrd had been clinically used as a radiosensitizer to enhance the antitumor effect of radiation therapy in Japan. CPA is one of the most popular anticancer agents for the treatment of hematological cancers. Concomitant use of BrdUrd and CPA might be a promising combination for the treatment of human cancers.

The weight of mice after the tumor inoculation showed interesting changes. The mean weight of mice peaked a few days before their death. The weight of mice who received CPA with BrdUrd, or 160 mg/kg of CPA alone, increased 5 to 6 d after the tumor inoculation, and then decreased to the level before the inoculation. It then increased a few days after their death. These results suggested that the increase in body weight that indicated the proliferation of tumor cells might not directly cause death.

To elucidate the enhanced antitumor effect of CPA in combination with BrdUrd, we investigated the influence of BrdUrd on the cytotoxicity of CPA using the L1210 cultured cell line in vitro. Since CPA is a prodrug and is metabolized to an active form, 4-OH-CP A, in the liver after administration, we used 4-OH-CP A in an in vivo assay. While BrdUrd did not show antitumor activity in vivo, 24 h of treatment with BrdUrd in vitro had a concentration-dependent cytotoxic effect in the L1210 cells above a concentration of 0.1 μg/ml. 4-OH-CP A was more cytotoxic in combination with BrdUrd, as shown in the in vivo experiment. Isobolograms of IC50 and IC75 showed that pretreatment with BrdUrd enhanced the cytotoxicity of 4-OH-CP A, consistent with the result of the in vivo assay. In case of IC50 of 4-OH-CP A, however, we cannot conclude whether the combined effect of CPA and BrdUrd was synergistic or additive because of a small number of the data points. These results suggest that the enhanced cell killing contributed to the extended life span of tumor-bearing mice when CPA was used in combination with BrdUrd. To confirm the direct relationship between the tumor cell killing and the extended life span, counting the tumor cell numbers and measuring the ascites volume are needed using tumor-bearing mice.

BrdUrd had a dose-dependent cytotoxic effect on L1210 cells in the MTT assay. Its cytotoxic efficacy (IC50: 0.41 μg/ml) was greater than that of 4-OH-CP A (IC50: 1.27 μg/ml), while the treatment time (48 h) was twice as long as that with 4-OH-CP A (24 h). We did not measure the drug concentration of BrdUrd in mice or the culture medium. The hepatic extraction rate for BrdUrd was reported to be high in dogs,260 BrdUrd administered intraperitoneally to mice was eliminated quickly and only a small amount of the drug reached the tumor cells, while BrdUrd added into the culture medium might remain at a high concentration for several hours. The 5-halogenated analogues of deoxyuridine was reported to induce cytotoxicity against tumor cells in the absence of radiation, and the clinical utilization of those analogues might be limited to specific cancers.239 The difference in the time–concentration relationship or time of drug exposure might cause the different results in vivo versus in vitro. We also could not exclude the possibility that degradation products of BrdUrd in the culture medium influenced the survival of tumor cells. Moreover, CPA was converted not only to 4-OH-CP A, but also to another active metabolite, aldophosphamide.11–13,280 Aldophosphamide generates highly active and toxic compounds, phosphoramido mustard and acrolein. We used only 4-OH-CP A as an active form of CPA in vitro. Aldophosphamide, phosphoramido mustard, and acrolein are highly unstable and we could not examine their cytotoxic effect in an MTT assay. These other metabolites might contribute to the stronger antitumor effect of CPA in vivo.

In the MTT assay, IC50 values of 4-OH-CP A after the pretreatment of BrdUrd seemed to be less than synergistic effect, while the IC75 values showed a synergistic one. Although we did not know the exact reason of the difference, the fact that a significant amount of the tumor cells were killed after the pretreatment of more than 0.1 μg/ml of BrdUrd, might influence the cytotoxicity of 4-OH-CP A. Because for cancer chemotherapeutic agents the drug concentrations that are of most interest are those that produce cell killing, the result of IC75 values is thought to be more important than those of IC50.29

In conclusion, BrdUrd effectively enhanced the antitumor effect of CPA in mice bearing L1210 ascites tumors. MTT assays in vitro partly reproduced the combined effect. These results indicated that concomitant use of CPA and BrdUrd may be promising for cancer chemotherapy. Further study is needed to confirm the combined effect of CPA and BrdUrd using other mice species and other tumor cell lines, and to elucidate how this combination acts.

REFERENCES


