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# Effect of homoharringtonine on the viability of murine leukemia P388 cells resistant to either adriamycin, vincristine, or 1-β-*D*-arabinofuranosylcytosine*

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**Summary.** Cultured murine leukemia P388 cell populations were derived from P388 cells resistant to vincristine (P388/VCR), adriamycin (P388/ADR), and 1-β-*D*-arabinofuranosylcytosine (P388/ARA-C) that were developed in vivo and to the parental drug-sensitive cells (P388/O) that were passaged in vivo. The doubling times of the cultured cell populations (mean ± SD) between cell densities of $5 \times 10^4$ and $1 \times 10^6$ cells/ml were 14.2 ± 2 h (P388/O), 16.5 ± 1.9 h (P388/VCR), 16.9 ± 1.2 h (P388/ADR), and 15.0 ± 1.4 h (P388/ARA-C). Exponentially proliferating cultured cell populations were exposed to selected homoharringtonine (HHT) concentrations for 24 h and the surviving cell fractions were determined by colony formation in semisolid medium. The results, based on differential sensitivity of the cell populations to HHT, indicated that cultured P388/VCR cells were cross-resistant to 0.018–1.8 μg/ml HHT, P388/ADR cells were cross-resistant to 0.058–1.8 μg/ml HHT, and P388/ARA-C cells were collaterally sensitive to 0.09–0.36 μg/ml HHT. The results with the cultured P388/VCR, P388/ADR, P388/ARA-C, and P388/O cell populations were confirmed in animal experiments. $CD2F_1$ mice bearing intraperitoneal (i.p.) implants of $1 \times 10^6$ P388/VCR, P388/ADR, P388/ARA-C, or P388/O leukemia cells were given HHT i.p. qd on days 1–9 postimplantation. Optimal treatment ($\leqslant LD_{10}$) produced in vivo cell kills of 2 to 3 $\log_{10}$ units in P388/O and about 7 $\log_{10}$ units in P388/ARA-C, whereas P388/VCR and P388/ADR cells actually increased by 1–2 $\log_{10}$ units during treatment. The results of this study indicate that cross-resistance (P388/VCR and P388/ADR) or collateral sensitivity to HHT (P388/ARA-C) is a function of the cellular properties of the target tumor cell populations that is independent of host factors.

## Introduction

Homoharringtonine (HHT; NSC 141633) is one of a group of alkaloids unique to the genus *Cephalotaxus* [31]. The alkaloids in this group are derivatives of the major component cephalotaxine. The active antitumor compounds are all cephalotaxine esters: deoxyharringtonine, harringtonine, isoharringtonine, and HHT [8, 9]. The cephalotaxine esters are potent inhibitors of eukaryotic protein synthesis, inhibiting either its initiation [12, 32] or the initial cycles of chain elongation [10].

Clinical studies in China using the cephalotaxine esters have demonstrated that 41 leukemia patients exhibited partial remissions in 63% of the cases and complete remission in 12%. In addition, patients who had become resistant to treatment with other chemotherapeutic drugs responded to treatment with the cephalotaxine esters (cited in [8]). It was also shown that harringtonine was effective as a single agent in treating acute granulocytic leukemia, resulting in 28% complete and 55% partial remissions. Harringtonine was also effective in treating chronic granulocytic leukemia, erythroleukemia, and myelomonocytic leukemia (cited in [6]).

Clinical activity reported from China prompted the National Cancer Institute (NCI) to assess HHT in the United States [22]. HHT was selected because of its antitumor activity, which is equal to that of harringtonine, and its greater abundance among the active cephalotaxines [22]. Phase I studies conducted in patients with carcinomas and sarcomas have confirmed HHT's dose-limiting cardiovascular complications, including severe hypotension from short infusions [7, 15, 18, 20]. Most studies in patients with leukemias are now conducted using a continuous daily infusion level [22]. Warrell et al. [34] have reported that 25 of 28 patients with acute nonlymphocytic leukemia, treated with 5 mg/m$^2$ per 24 h for 9 days or 7 mg/m$^2$ per 24 h for 7 days, achieved complete remissions lasting up to 6 months. Four of these remissions occurred in a subset of ten patients who had previously been resistant to conventional treatment. Neidhart et al. [21] have reported that HHT can be given daily at a dose of 1 mg/m$^2$ by continuous i.v. infusion for up to 30 days; nonhematological toxicities were minimal on this schedule. However, the dose-limiting toxicity, myelosuppression, was severe and prolonged in some patients; therefore, these authors recommended a 2-week rest period after each course of therapy before treatment is reinstituted.

The observation that cross-resistance to currently available antileukemic agents may not be complete [34] may be consistent with HHT's unique structure and mechanism of action [22]. However, Chou et al. [6] have reported that sublines of murine leukemia L1210 resistant to vincristine (VCR; NSC 67574) or adriamycin (ADR; NSC 123127) appeared insensitive to harringtonine in chemo-

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therapeutic studies in vivo; on the other hand, a subline of L1210 cells resistant to 1-β-*D*-arabinofuranosylcytosine (ara-C; NSC 63878) was sensitive to harringtonine. We report that mice bearing murine leukemia P388 tumors resistant to VCR or ADR are cross-resistant to optimal treatment with HHT. Furthermore, mice bearing leukemia P388 tumors resistant to ara-C are collaterally sensitive to optimal treatment with HHT. Proliferating cultured cell populations derived from these tumors showed either significant cross-resistance to HHT (P388/ADR and P388/VCR cell poulations) or collateral sensitivity to this agent (P388/ARA-C cell population). These results indicate that cross-resistance or collateral sensitivity to HHT is primarily a function of the cellular properties of the target P388 cell populations that is independent of host factors.

## Materials and methods

Leukemia P388 cells were supplied by the Tumor Repository of the Developmental Therapeutics Program (DTP), Division of Cancer Treatment (DCT), NCI. The P388/O line was maintained in serial passage as described in the NCI protocols [24]. The development of P388/VCR tumor cell populations in (C57BL ♀ × DBA/2 ♂)$F_1$ ($BDF_1$) mice has previously been described [35].

P388/ADR populations were developed by treatment with ADR of $BDF_1$ mice bearing ascites P388/O over a number of transplant generations [29]. A total of $1 \times 10^7$ P388 cells were implanted i.p. and mice were treated with ADR (1.5 mg/kg body weight) daily for 9 days, beginning on day 4 postimplantation. A mouse that had been implanted with $1 \times 10^7$ cells and treated with ADR developed frank ascites tumor and was sacrificed on day 14; a series of passages was then initiated by the i.p. implantation of $4.5 \times 10^5$ cells into other $BDF_1$ mice. On day 14 postimplantation, sufficient ascites had developed for the serial passage of $1 \times 10^7$ cells in a series of $BDF_1$ mice. These mice were then treated with a single i.p. dose of 12.5 mg/kg ADR on day 5 postimplantation. Passage was done on day 11, and the mice bearing the leukemia were treated with a single i.p. dose of ADR (6 mg/kg) on day 7 postimplantation. On day 16, passage was again accomplished. Passage is now routinely made in (BALB/c ♀ × DBA/2 ♂)$F_1$ ($CD2F_1$) mice on day 7 postimplantation. The resistant line is maintained under treatment in vivo by a single 6 mg/kg i.p. dose of ADR on day 4 postimplantation.

To develop P388/ARA-C tumor cell populations, $BDF_1$ mice were implanted i.p. with $1 \times 10^7$ P388/O cells and treated for 9 days, starting 24 h after the leukemia implant, with a daily i.p. dose of 20 mg/kg ara-C. A single mouse showing frank ascites on day 15 postimplantation was selected and sacrificed. Another series of $BDF_1$ mice were then implanted i.p. with these cells and treated with 20 mg/kg i.p. ara-C daily for 9 days, beginning 24 h postimplantation. Cells resistant to ara-C developed during this course of therapy. The cells were maintained under ara-C therapy for 14 passages; after the 14th passage, ara-C maintenance therapy was discontinued.

P388/ADR, P388/VCR, and P388/ARA-C tumors were stored at −209° C in liquid nitrogen when not in passage. After their removal from storage, the P388/ADR cells were implanted into $CD2F_1$ mice and maintained without treatment for one passage before being treated with 6 mg/kg ADR. The P388/ARA-C and P388/VCR tumors required no drug therapy for the maintenance of drug resistance.

Therapeutic trials to test the in vivo antitumor activity of VCR, ADR, or ara-C were accomplished according to NCI protocols [24]. A titer of each inoculum ($10^7$–$10^2$ cells) was included in every experiment to provide data on tumor stem-cell doubling time. Testing was carried out over a range of doses, from frankly toxic to nontoxic and inactive dose levels, in parallel groups of mice bearing the drug-resistant or parental drug-sensitive leukemias. These groups were treated with the same drug preparation. The $\log_{10}$ change in the tumor burden after the last treatment was estimated from the median survival time of treated mice relative to that of the nontreated control mice, doubling times of the tumor cell populations, and number of drug treatments [28]. The response of the resistant tumor was compared with that of the parental sensitive tumor line at the nontoxic dose that showed optimal activity against the drug-sensitive tumor cells.

Proliferating cultured P388/O cells were derived from sensitive cell populations passaged in vivo in $CD2F_1$ mice, and cultured P388/VCR, P388/ADR, and P388/ARA-C cells were established from resistant cell populations developed in vivo. Stock cultures of these leukemia cell lines were propagated in Dulbecco's modified Eagle's minimum essential medium (Flow Laboratories, Inc., Rockville, Md, USA) [19] supplemented with 10% horse serum (Flow Laboratories) and 5 μ*M* 2-mercaptoethanol [39]. Under our experimental conditions, the leukemia cell populations proliferated exponentially, with doubling times (mean ± SD) of 14.2 ± 2.0 h (P388/O), 16.5 ± 1.9 h (P388/VCR), 16.9 ± 1.2 h (P388/ADR), and 15.0 ± 1.4 h (P388/ARA-C) between cell densities of $5 \times 10^4$ and $1 \times 10^6$ cells/ml. At the time of these studies, the P388/VCR cell line had completed approximately 1600 population doublings in cell culture; the P388/ADR cell line, 1680; and the P388/ARA-C cell line, 690.

The effect of the antitumor agents on the viability of proliferating cultured cell populations was determined using procedures previously described by Wilkoff and Dulmadge [37]. Proliferating cell populations were exposed to selected drug concentrations for 24 h, and the number of surviving cells was determined by colony formation in semisolid medium [37]. Colony-formation efficiencies of control cultures (mean ± SD) were 66% ± 3.1% (P388/O), 82.1% ± 1.7% (P388/VCR), 73% ± 11% (P388/ADR), and 66% ± 6.1% (P388/ARA-C).

In the design of the drug studies, the in vitro concentrations of the antitumor agents were based on the in vivo $LD_{10}$ data to estimate pharmacologically acceptable levels of drug in vivo [27]. The cell-culture equivalent dose (CCED) is defined as 1.3 × $LD_{10}$ (μg/ml is approximately equivalent to mg/kg; see [38]) and is used as an estimate of the maximal drug concentration acceptable in vivo. The CCED (μg/ml) for the antitumor agents were: VCR, 4; ADR, 18; ara-C, 32.5; and HHT, 1.8.

When the sensitivities of cultured P388/ADR, P388/VCR, or P388/ARA-C cell populations to drugs were compared with that of cultured P388/O cell populations to the same agents, the two cell populations were evaluated in parallel in the same experiment. Surviving fractions for each cell population at a specific concentration were compared by using chi-square analysis. The standard errors of the ratios of surviving fractions of

P388/ADR and P388/O cell populations were calculated as previously described by Kendall and Stuart [14].

VCR, ADR, ara-C, and HHT were obtained from the DTP, DCT, NCI. VCR, ADR, and ara-C were dissolved in sterile distilled and deionized water and diluted with cell-culture medium to the appropriate final concentrations. HHT was dissolved in 1 *N* HCl, adjusted to neutrality with 1 *N* NaOH, and diluted to selected concentrations with cell-culture medium.

## Results

Cultured P388/VCR cells are significantly resistant to VCR (Fig. 1), as previously reported [35, 36]. For example, P388/VCR cells are significantly resistant to 5 and 50 μg/ml VCR after exposure periods of 6 or 24 h. These in vitro concentrations are relatively high compared with the CCED of 4 μg/ml. Thus, concentrations of 5 or 50 μg/ml probably cannot be achieved in vivo. Results based on the differential sensitivity of P388/VCR and P388/O cells to HHT indicate that P388/VCR cells are significantly cross-resistant to 0.018–1.8 μg/ml HHT (Fig. 2). These HHT concentrations are estimated to be pharmacologically acceptable, since the CCED is 1.8 μg/ml.

Cultured P388/ADR cells are significantly resistant to 0.0625–2.0 μg/ml ADR (Table 1). These concentrations are estimated to be pharmacologically attainable in vivo and are comparable with ADR levels in the tissues of pa-

**Table 1.** Sensitivity of cultured P388/ADR and P388/0 cells to ADR, VCR, and HHT after an exposure period of 24 h[a]

| Agent | μg/ml | $S_f$[b] | | $S_f$ P388/ADR: $S_f$ P388/0 | |
|---|---|---|---|---|---|
| | | P388/ADR | P388/0 | ± SE[c] | *P* value[d] |
| ADR | 0.0625 | 400/400 | 193/ 4.000 | 20.7± 6.2 | <0.001 |
| | 0.2 | 400/400 | 0/ 40.000[e] | ∞ | <0.001 |
| | 0.625 | 333/400 | 0/ 40.000[e] | ∞ | <0.001 |
| | 2.0 | 574/40.000 | 0/ 40.000[e] | ∞ | <0.001 |
| VCR | 5.0 | 201/ 4.000 | 0/ 40,000[e] | ∞ | <0.001 |
| | 50.0 | 295/ 40.000 | 0/ 40,000[e] | ∞ | <0.001 |
| HHT | 0.018 | 400/400 | 400/400 | 1.0± 0 | NS |
| | 0.058 | 352/400 | 598/ 4.000 | 5.9± 0.6 | <0.001 |
| | 0.18 | 470/800 | 390/ 8.00 | 12.1± 8.4 | <0.001 |
| | 0.58 | 727/ 40.000 | 11/ 40.000 | 66.1±12.8 | <0.001 |
| | 1.8 | 82/ 30.000 | 0/ 30.000[e] | ∞ | <0.001 |

[a] Proliferating cells were exposed to HHT, and the number of cells surviving drug treatment was estimated by assay in semisolid medium as described in *Materials and methods*

[b] $S_f$ surviving fraction = total number of colonies/total number of cells plated

[c] Standard error of the ratio was estimated as described by Kendall and Stuart [14]

[d] $S_f$s for each exposure period were compared by using chi-square analysis; NS, not significant

[e] No surviving cells (no colony formation) could be detected

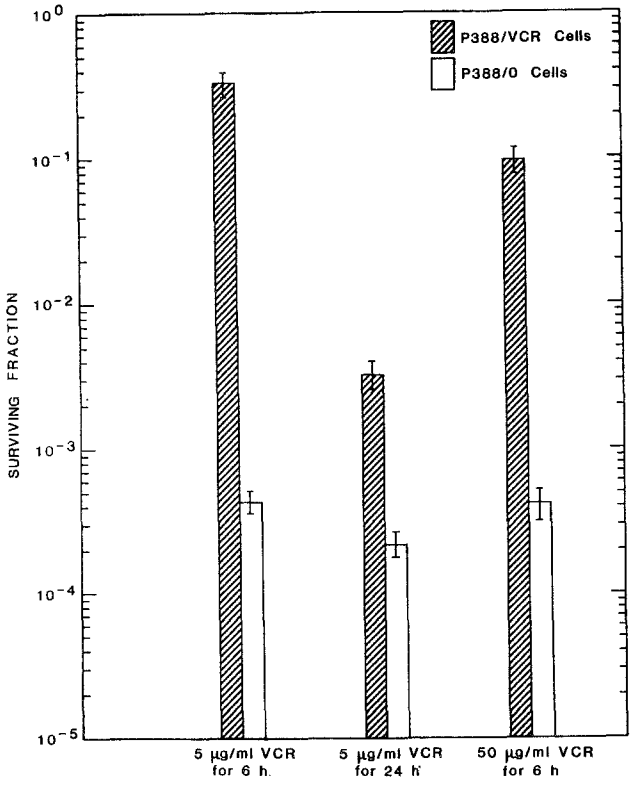


**Fig. 1.** Resistance of cultured P388/VCR cells to VCR. Proliferating cells were exposed to 5 μg/ml VCR for 6 or 24 h and to 50 μg/ml VCR for 6 h, and the number of cells surviving drug treatment was estimated by assay in semisolid medium as described in *Materials and methods*. Surviving fractions for each cell population were compared by using chi-square analysis. Differences in surviving fractions were significant ($P < 0.001$) at concentrations of 5 and 50 μg/ml. *Bars*, 95% confidence limits

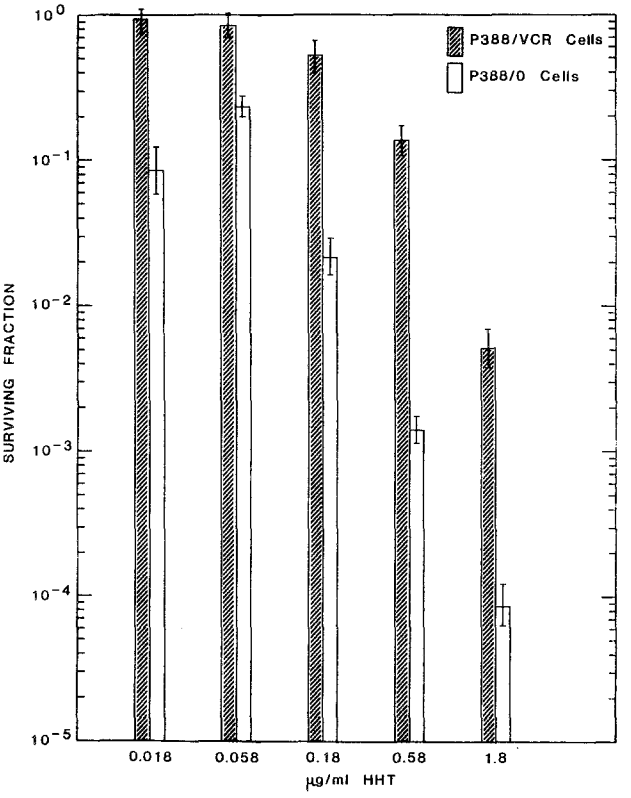


**Fig. 2.** Cross-resistance of cultured P388/VCR cells to HHT. Proliferating cells were exposed to HHT for 24 h, and the number of cells surviving drug treatment was estimated by assay in semisolid medium as described in *Materials and methods*. Surviving fractions for each cell population were compared by using chi-square analysis. Differences in surviving fractions were significant ($P < 0.001$) at concentrations of 0.018–1.8 μg/ml. *Bars*, 95% confidence limits.

tients and experimental animals during therapy [1–3]. The P388/ADR cells are also significantly cross-resistant to 5 and 50 μg/ml VCR as well as to 0.058–1.8 μg/ml HHT (Table 1).

Proliferating cultured P388/ARA-C cell populations are significantly resistant to 3.2 and 10 μg/ml ara-C after an exposure period of 24 h (Fig. 3). Based on the CCED, these concentrations are estimated to be pharmacologically acceptable in vivo. Results of experiments comparing the sensitivities of P388/O and P388/ARA-C cell populations to a series of HHT concentrations for 24 h indicate that P388/ARA-C cells are collaterally sensitive to 0.09–0.36 μg/ml HHT (Fig. 4).

The results with cultured P388/VCR, P388/ADR, P388/ARA-C, and P388/O cell populations have been confirmed in animal experiments (Table 2). $CD2F_1$ mice bearing i.p. implants of $1 \times 10^6$ P388/VCR, P388/ADR, P388/ARA-C, or P388/O leukemia cells were given HHT i.p. qd on days 1–9. Optimal treatment ($\leq LD_{10}$) produced in vivo cell kills of 2–3 $\log_{10}$ units in P388/O and about 7 $\log_{10}$ units in P388/ARA-C, whereas P388/VCR and P388/ADR cells actually increased by 1–2 $\log_{10}$ units during treatment.

**Table 2.** Collateral sensitivity of P388/ARA-C and cross-resistance of P388/VCR and P388/ADR leukemias to HHT

| Experiment no. | Tumor | Therapeutic response of leukemic $CD2F_1$ mice to HHT (i.p. 1.3 mg/kg qd on days 1–9 optimal dose ($< LD_{10}$) from dose-response studies | | | |
|---|---|---|---|---|---|
| | | % ILS[a] | Approx. tumor burden at start of treatment[a] | Approx. no. of cells alive at end of treatment | Approx. $\log_{10}$ change in tumor burden after last treatment |
| 1 | P388/0 | 110 | $4.7 \times 10^6$ | $4.5 \times 10^4$ | −2.0 |
| | P388/ARA-C | 133 | $8.0 \times 10^6$ | ca. $1.0 \times 10^0$ | −6.9 |
| | P388/VCR | 65 | $4.4 \times 10^6$ | $4.1 \times 10^7$ | +1.0 |
| | P388/ADR | 0 | $3.0 \times 10^6$ | $6.1 \times 10^8$ | +2.3 |
| 2 | P388/0 | 130 | $4.8 \times 10^6$ | $1.9 \times 10^3$ | −3.4 |
| | P388/ARA-C | 163 | $4.9 \times 10^6$ | ca. $1.0 \times 10^0$ | −6.7 |
| | P388/VCR | 55 | $4.2 \times 10^6$ | $1.1 \times 10^8$ | +1.4 |
| | P388/ADR | 9 | $4.0 \times 10^6$ | $4.2 \times 10^8$ | +2.0 |

[a] Based on median day of death (there were no cures)

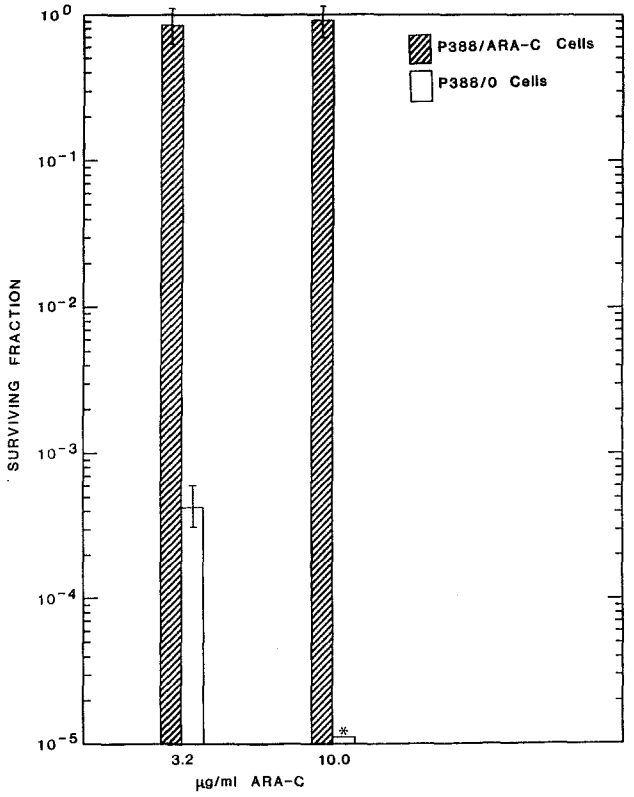


**Fig. 3.** Sensitivity of cultured P388/ARA-C and P388/O cells to ara-C after an exposure period of 24 h. Proliferating cells were exposed to ara-C, and the number of cells surviving drug treatment was estimated by assay in semisolid medium as described in *Materials and methods*. Surviving fractions for each cell population were compared by using chi-square analysis. Differences in surviving fractions were significant ($P < 0.001$) at concentrations of 3.2 and 10 μg/ml. *Bars*, 95% confidence limits. The asterisk (*) designates that no surviving cells (no colony formation) could be detected

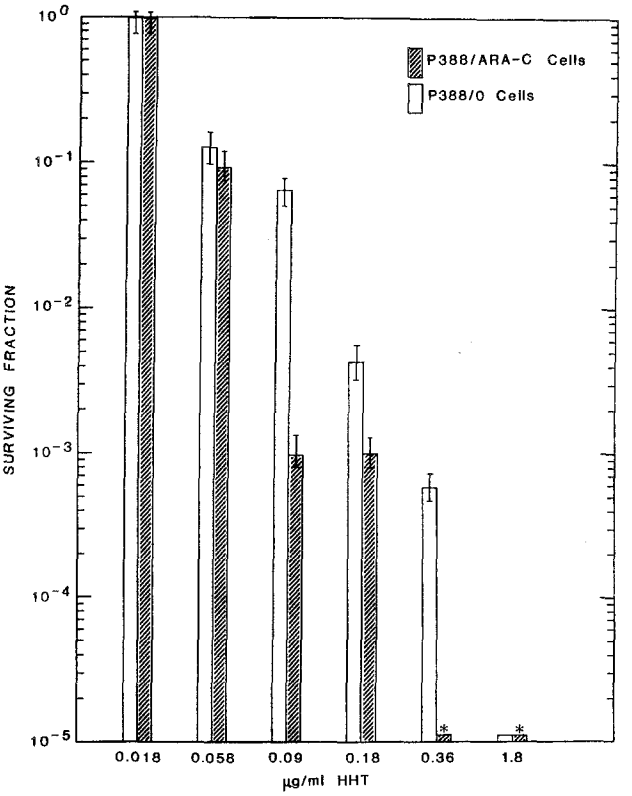


**Fig. 4.** Collateral sensitivity of cultured P388/ARA-C cell populations to HHT. Proliferating cells were exposed to HHT for 24 h , and the number of cells surviving drug treatment was estimated by assay in semisolid medium as described in *Materials and methods*. Surviving fractions for each cell population were compared by using chi-square analysis. Differences in surviving fractions were significant ($P < 0.001$) at concentrations of 0.09–0.36 μg/ml. *Bars*, 95% confidence limits. Asterisks (*) designate that no surviving cells (no colony formation) could be detected

## Discussion

The results of the present study indicate that leukemia P388 cell populations resistant to either ADR or VCR are significantly cross-resistant to treatment with HHT in either cell culture or $CD2F_1$ mice bearing the leukemias. In another study, Chou et al. [6] have reported that [$^3$H]harringtonine was rapidly taken up by L1210/0 cells and retained, with a slow rate of limited release to the medium. Cells resistant to VCR (L1010/VCR) showed impaired uptake of harringtonine at 20° C. The initial uptake of harringtonine in L1210 sublines in vitro occurred in the following order: L1210/0 > L1210/cyclophosphamide, L1210/ARA-C, L1210/6-mercaptopurine, L1210/5-fluorouracil, L1210/ADR > L1210/VCR. In cells preloaded with [$^3$H]harringtonine, L1210/0 cells retained more labeled drug than L1210/VCR cells after repeated washings with fresh medium at 37° C; the radioactivity bound predominantly to the microsomal fractions. The relative capacity of harringtonine to inhibit in vitro [$^3$H]leucine incorporation in the sublines followed a rank order similar to that for their rates of uptake of harringtonine. The efficacy of harringtonine (2.4 or 3.6 mg/kg i.p.) in increasing the life span of $BDF_1$ mice bearing the sublines of leukemic cells showed the following rank order: L1210/0 > L1210/cyclophosphamide, L1210/6-mercaptopurine > L1210/-ARA-C, L1210/5-fluorouracil > L1210/ADR, L1210/VCR. In our study, the leukemia P388 cells resistant to ara-C were extremely sensitive to HHT in both cell culture and $CD2F_1$ mice bearing the ara-C-resistant P388 tumor. Based on our data and the results described by Chou et al. [6], it appears that harringtonine and HHT have similar biological properties.

Takemura et al. [32] have tested the growth-inhibitory effects of harringtonine against ten human leukemia-lymphoma cell lines. Harringtonine was most active against HL-60 acute promyelocytic leukemia cells and least active against DND-41 acute lymphoblastic leukemia cells (a 70-fold differential in activity). Cell lines with rapid cell growth tended to be more sensitive to harringtonine. These investigators could detect no difference in the uptake of [$^3$]harringtonine by HL-60 and DND-41 cells; however, the binding of [$^3$H]harringtonine to cellular components was > 16-fold higher in HL-60 cells than in DND-41 cells. Boyed and Sullivan [5] have reported that harringtonine induces changes compatible with myelomonocytic differentiation in HL-60 cells; these authors concluded that a possible mode of action for this drug may be the induction of irreversible differentiation of leukemic cells into a nonproliferative state.

Harringtonine, its analog HHT, and VCR are plant alkaloids and ADR is an antibiotic, and their major common feature is that they are relatively large hydrophobic molecules with heterocyclic structures. This type of drug resistance appears to develop in tumor cell populations treated with natural products that have these structural features [4, 16, 33]. VCR blocks the organization and polymerization of microtubules [26], ADR forms complexes with DNA [1], and HHT and harringtonine inhibit protein synthesis [10, 12, 33]. Presumably, these interactions are responsible for most of the biological effects of these agents. It has been suggested that multidrug resistance is due to a decreased intracellular drug accumulation [4, 17] caused by alterations in the plasma membrane [13]. Multidrug-resistant cells often contain double minute chromosomes or homogeneously staining chromosomal regions, suggesting that gene amplification is at least partly the basis for multidrug resistance [4]. Specific DNA sequences amplified in multidrug-resistant cells have recently been isolated and characterized [11, 23, 25]. The gene product of these amplified sequences is the P-glycoprotein [23], which is postulated to function as an efflux "pump" protein in cells expressing the multidrug phenotype [23]. The P388/ADR subline used in the present study is a multidrug-resistant line with the P-glycoprotein marker [30]. Whether this mechanism functions in cross-resistance to HHT remains to be elucidated. In addition, the mechanism underlying the collateral sensitivity of P388/ARA-C cells to HHT needs to be defined.

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