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Biologic and pharmacologic effects of harringtonine on human leukemia-lymphoma cells*

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Summary. *Ten human leukemia-lymphoma cell lines were tested for the growth-inhibitory effects of harringtonine (HT). HT was most active against HL-60 acute promyelocytic leukemia cells and least active against DND-41 acute lymphoblastic leukemia cells, with a 70-fold differential activity. Sensitivity of the cell lines is, in decreasing order: HL-60 > RPMI-8402 > DND-39A ≃ ML-2 ≃ MOLT-3 ≃ KG-1 > Daudi ≃ NALL-1 > BALM-2 > DND-41. The cell lines with rapid cell growth tended to be more sensitive to HT. To further elucidate the selectivity of the differential sensitivity, uptake and release of HT were compared in HL-60 and DND-41 cells. Uptake of [^{3}H]HT into HL-60 and DND-41 cells showed no difference; however, the binding of [^{3}H]HT to cellular components was > 16-fold higher in HL-60 cells than DND-41 cells. There were also minor, but significant differences in the inhibition of [^{3}H]leucine incorporation into proteins of these two cell lines in the presence of 1 μg/ml HT. To test whether the biological effects of HT are related to the concentration of, or exposure time to, HT, KG-1 cells were exposed to HT for different periods of time and the growth-inhibitory effects were compared. Increasing exposure time from 1 h to 3 h resulted in a 100-fold decrease in concentration × exposure time (c × t) at ID_{50}; from 3 h to 6 h, in a 20-fold decrease at ID_{70}; and from 6 h to 24 h, in a 16-fold decrease at ID_{90}. HT was not inactivated by cells up to 24 h. These results indicate that (a) the sensitivity of different cell lines to HT may be related to the degree of HT binding; and (b) the effects of HT are more dependent on exposure time than concentration. Continuous infusion is thus rational for clinical trials of this drug, and the degree of HT binding to leukemic cells may be predictive of clinical response.*

Introduction

Harringtonine (HT) is an antitumor cephalotaxine alkaloid isolated from an evergreen tree, *Cephalotaxus fortunei* Hook f., which is indigenous to southern and northeastern China. The structures of cephalotaxine and related alkaloids have been determined [26, 27]. HT and homoharringtonine are the major components among these alkaloids, which have been shown to be active in murine experimental tumor systems [7]. HT inhibits protein synthesis in HeLa cells at the level of chain initiation, resulting in secondary inhibition of DNA synthesis [12].

Clinical trials carried out in China revealed therapeutic activity of HT in patients with leukemia and lymphoma [3, 4, 11]. Phase I and II studies are in progress in the United States [9, 14, 22].

In this report we studied the effects of HT on ten different human leukemia-lymphoma cell lines to determine whether there were any differential sensitivity against HT. Attempts were made to explain HT's biological effects with reference to pharmacological factors. We also determined whether the biological effects were related to the concentration of, or exposure time to, HT.

Materials and methods

Drug and radiomaterials. HT powder of more than 95% purity was prepared by Dr Han Jui, Chinese Academy of Medical Sciences, Beijing, China [6, 25] and was used for all experiments. Stock HT solution 10^{-3} *M* was prepared by dissolving in 0.1 *N* HCl. The stock solution was stable for at least 6 months at −20° C [25]. After sterilization by passing the HT solution through a 0.22 μm filter (Millipore, Bedford, Mass), it was further diluted with Dulbecco's phosphate buffer saline (PBS, GIBCO, Grand Island, NY) to the desired concentration. [^{3}H]HT (0.92 Ci/mmol) was synthesized in the Institute of Materia Medica, Chinese Academy of Medical Science, Beijing, China and purified by high-pressure liquid chromatography on a model 8000 Spectra-Physics instrument and a model SP 8400 variable wavelength detector (Spectra-Physics, Piscataway, NJ) using an ODS-3, 5-μm column with formate-methanol as solvent system [6]. L-[4,5-^{3}H]leucine (51.6 Ci/mmol) was purchased from New England Nuclear, Boston, Mass.

Cell lines. Three AML cell lines (HL-60, ML-2, and KG-1), five ALL cell lines (RPMI-8402, MOLT-3, NALL-1, BALM-2, and DND-41), and two lymphoma cell lines (DND-39A, Daudi) were used. The origin and doubling time of each of these cell lines are summarized in Table 1. Further details as to the properties of these cell lines have already been described [8, 13, 16–21, 23, 24]. The cells were maintained in RPMI-1640 medium (GIBCO) with 10% (v/v) heat-inactivated fetal bovine serum (FBS, GIBCO) and fed with fresh medium three times a week. The cells in the exponential growth phase were used.

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Table 1. Summary of leukemia-lymphoma cell lines used, their origin and doubling time

Cell line	Origin	Population-doubling time (h)	Reference
HL-60	Acute promyelocytic leukemia	19	[8]
RPMI-8402	Acute lymphoblastic leukemia (T cell)	23	[20]
DND-39A	Lymphoma (B cell)	19	[23]
ML-2	Acute myelocytic leukemia	24	[18]
MOLT-3	Acute lymphoblastic leukemia (T cell)	22	[16]
KG-1	Erythroleukemia	27	[13]
Daudi	Lymphoma (B cell)	27	[21]
NALL-1	Acute lymphoblastic leukemia (null cell)	27	[19]
BALM-2	Acute lymphoblastic leukemia (B cell)	30	[17]
DND-41	Acute lymphoblastic leukemia (T cell)	41	[24]

Cell growth inhibitory effects of HT on various leukemia-lymphoma cell lines. For the cell growth-inhibition assay, 4.5-ml aliquots of the cell suspension, each containing 1×10^5 viable cells/ml, were placed in culture tubes (Falcon 3033, Becton-Dickinson, Oxnard, Calif) and 0.5 ml HT solution at 10 times the desired concentration was added to each. For the control experiments PBS was added instead of HT solution. After thorough mixing, 1 ml cell suspension was placed in a cell well (Corning 25820, Corning Glass Works, Corning, NY) and was incubated at 37° C in humidified 5% CO_2/95% air for 3 days in the continuous presence of HT. At the end of the incubation period, viable cells were enumerated by the trypan blue dye exclusion method. The drug concentration-cell growth inhibition curves (dose-response curves) were drawn by plotting the number of viable cells as a percentage of the control against drug concentrations.

Uptake and binding of [^{3}H]HT and inhibition of L-[4,5-^{3}H] leucine incorporation by HT. The Vortex-Finnpipette procedure [6] was used to measure the initial uptake of [^{3}H]HT into HL-60 and DND-41 cells, which were the most and least sensitive to HT, respectively. Aliquots of 0.2 ml [^{3}H]HT (0.4 μCi, 0.22 μg/ml) were added to 1.8-ml volumes of cell suspension (4.5 to 7.4 × 10^6 viable cells/ml) in Basal Medium Eagle (BME, GIBCO) and incubated at 22° C or 2° C. At specified time intervals, 0.5 ml cell – [^{3}H]HT mixture was removed and superimposed on the mixture of mineral oil (Sigma Chemical, St Louis, MO) and silicon oil (J. T. Baker Chemical, Phillipsburg, NJ), the final density of which was adjusted to 1,032 g/ml. Cells were separated from supernatant under the oil layer with 30 s centrifugation in an Eppendorf model 5412 microcentrifuge. After removal of the supernatant and most of the oil layer, radioactivity in the cell pellet was measured by liquid scintillation spectrometer (Packard Tri-Carb model 3775) using Liquiscint scintillation fluid (National Diagnostics, Somerville, NJ). Initial uptake of [^{3}H]HT into the cells was calculated by subtracting the radioactivity at 2° C from the 22° C value for each incubation period.

The binding of [^{3}H]HT to cellular components was measured as radioactivity of cells after repeated washing of [^{3}H]HT-treated cells [6]. Cells ($1.24-1.64 \times 10^7$/ml) in 2.5 ml BME were incubated with 0.5 ml [^{3}H]HT (0.4 μCi, 0.22 μg/ml) at 37° C for 30 min and then centrifuged to yield a cell pellet. The supernatant was substituted with 3 ml fresh PBS, cells were resuspended, and 0.3 ml of cell suspension was removed for the measurement of radioactivity (1st washing). The remainder was incubated for 3 min at 37° C, and centrifuged at 650 *g* for 7 min at room temperature to yield a cell pellet (2nd washing). After the 3rd washing, the cell suspension was again centrifuged and the radioactivity of the final cell pellet was counted.

The inhibiton of L-[4,5-^{3}H]leucine incorporation into cellular protein by HT was determined by the method previously described [5]. Cells were suspended in 1 ml Hank's balanced salt solution (without leucine, GIBCO) containing 0.1 ml (2.58 μCi/ml) [^{3}H]leucine and 1 μg/ml HT or normal saline and incubated for 0, 5, 10, or 60 min at 37° C in 5% CO_2/95% O_2. Incubation was stopped by adding 2 ml ice-cooled 10% perchloric acid, and then cells were homogenized with Polytron (Brinkmann Instruments, Westbury, NY). The radioactivity of the precipitate after washing twice with cold 10% trichloroacetic acid and twice with 100% ethanol was measured as the amount of radioactive leucine incorporated into the protein fraction.

Concentration × exposure time studies. KG-1 cells were adjusted to the viable cell concentration of 1×10^5 cells/ml in RPMI-1640 medium plus 10% FBS. Aliquots of 2.7 ml cell suspension were placed in culture tubes (Falcon 3033) and 0.3 ml HT solution at 10 times the desired concentration of HT was added to each tube. The cell suspensions were then incubated at 37° C for 1, 3, 6, and 24 h in the presence of HT. After each incubation period the cells were washed twice with RPMI-1640 medium and were resuspended in RPMI-1640 medium containing 10% FBS. Cell suspension (1 ml) was placed in each well of the 24-well culture plate and incubated at 37° C in a humidified 5% CO_2/95% air atmosphere for 72 h. Viable cell growth was then determined with the trypan blue dye exclusion method.

For determination of the persistence of cell growth-inhibitory effect of HT, 5×10^{-7} *M* HT was incubated at 37° C in the presence or absence of 1×10^5 KG-1 cells/ml for up to 24 h. After the incubation supernatants were collected by centrifugation and 0.5 ml of each was added to 4.5 ml cell suspension (1×10^5 cells/ml). The cells were then incubated in a humidified 5% CO_2/95% air atmosphere at 37° C for 72 h, and viable cell growth was compared.

Results

Effects of HT on various cell lines

Figure 1 shows the growth-inhibitory effects of HT on ten human leukemia-lymphoma cell lines tested. Based on ID_{90}

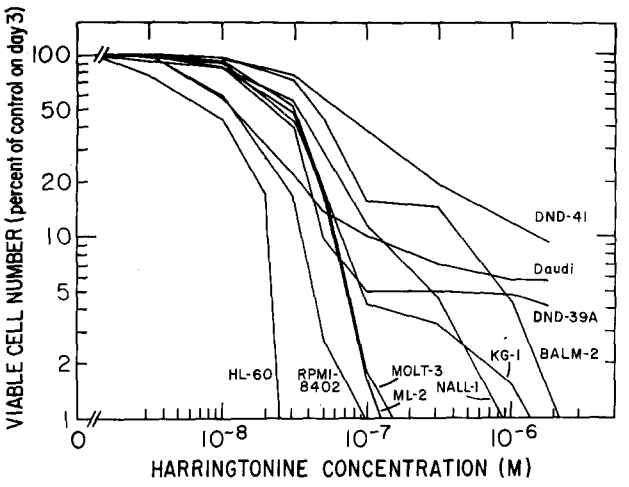

Fig. 1. Cell growth-inhibitory effects of harringtonine on various leukemia-lymphoma cell lines

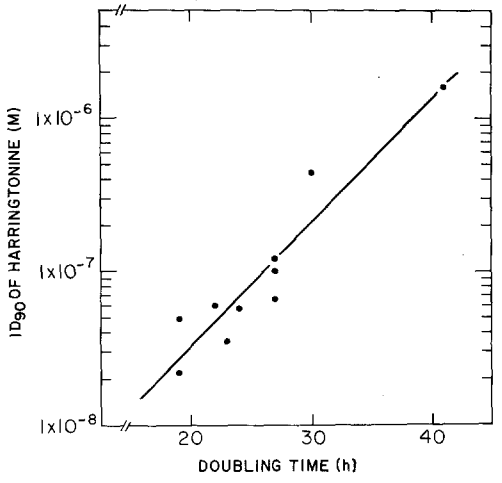

Fig. 2. Relationship between ID_{90} and population doubling time

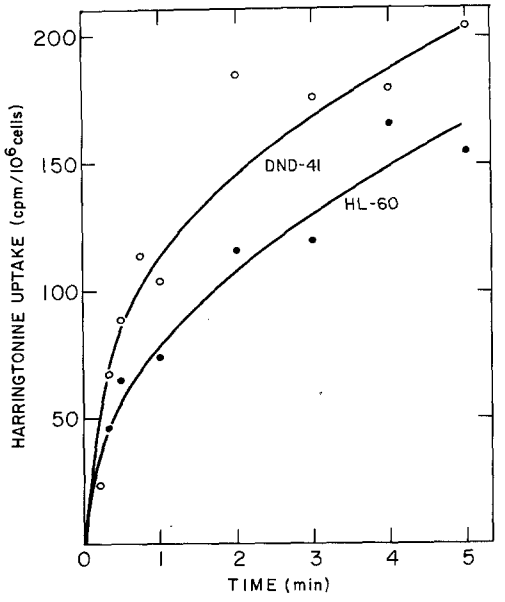

Fig. 3. [^{3}H]Harringtonine uptake into HL-60 (*filled circle*) and DND-41 cells (*open circle*)

(drug concentration that produced 90% inhibition of cell growth compared with control cell growth without drug) on day 3, HT was most active against HL-60 cells and least active against DND-41 cells, with approximately 70-fold differential activity. The sensitivity of the cell lines was, in decreasing order: HL-60 > RPMI-8402 > DND-39A ≃ ML-2 ≃ MOLT-3 ≃ KG-1 > Daudi ≃ NALL-1 > BALM-2 > DND-41. There was a considerable variety in the dose-response curve among the cell lines tested. Thus, in such cell lines as Daudi and DND-39A, the tail of the dose-response curve became flattened and crossed over those with more resistant cell lines. The relationship between ID_{90} and the population-doubling time of these cell lines is shown in Fig. 2. The cell lines with rapid cell growth tended to be more sensitive to HT.

Uptake and binding of HT to HL-60 and DND-41 cells

To elucidate the mechanism of the differential sensitivity, uptake and release of [^{3}H]HT were compared in HL-60 and DND-41 cells, the most and the least sensitive cells, respectively, to HT. The initial uptake of [^{3}H]HT into HL-60 and DND-41 cells is illustrated in Fig. 3. The uptake of [^{3}H]HT into DND-41 cells was, if not similar, slightly higher than that into HL-60 cells, indicating that the sensitivity of HL-60 cells to HT cannot be explained by uptake kinetics. In contrast, the binding of [^{3}H]HT to the cellular components was shown to be much higher for HL-60 cells (Table 2). Thus, after the 3rd washing HL-60 cells retained approximately 5 ~ 7-fold more activity than DND-41 cells. The HL-60 cell pellet after repeated washing retained > 16-fold higher radioactivity than DND-41 cells.

Table 2. Binding of [^{3}H]harringtonine to cellular components (cpm/10^7 cells)

	1st washing	2nd washing	3rd washing	Final pellet
Expt. 1				
HL-60 (A)	11,870	2,730	977	504
DND-41 (B)	11,740	1,108	138	31
A/B	1.0	2.5	7.1	16.5
Expt. 2				
HL-60 (A)	18,425	1,693	568	50
DND-41 (B)	30,707	927	101	0
A/B	0.6	1.8	5.6	> 50

Table 3. Inhibition of [^{3}H]leucine incorporation into acid-insoluble fractions by harringtonine (1 μg/ml)

Cell lines	Incubation time		
	5 min	10 min	60 min
HL-60	46.5 ± 2.0*	58.6 ± 3.1**	68.9 ± 3.9***
DND-41	36.3 ± 3.9*	50.2 ± 0.9**	58.8 ± 9.8***

Values are expressed as percentages of control, means ± SD from three to four measurements
* $P < 0.01$; ** $P < 0.025$; *** $P > 0.05$; for differences between HL-60 and DND-41 cells

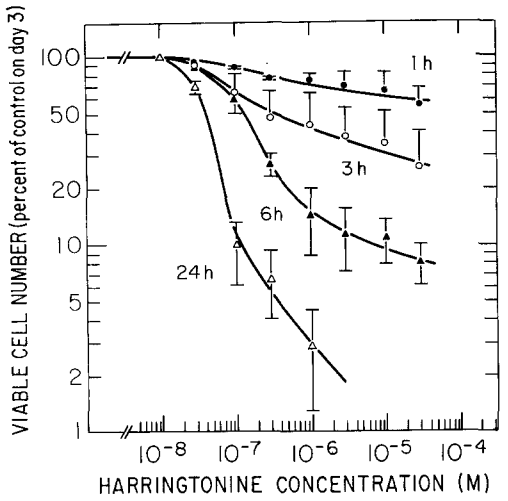

Fig. 4. Concentration of harringtonine × exposure time (c × t) studies using KG-1 cells with exposure time of 1, 3, 6 and 24 h

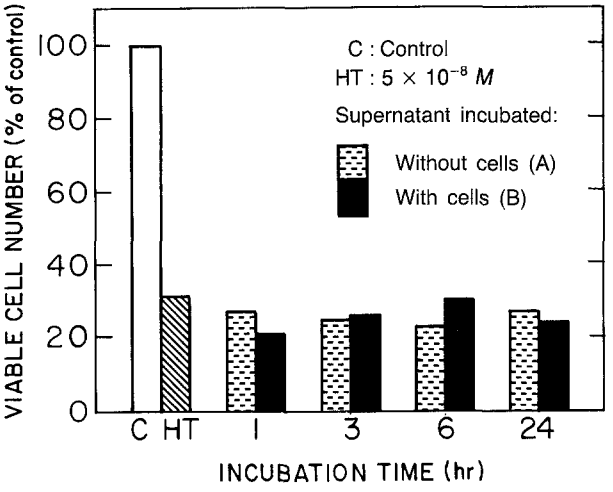

Fig. 5. Stability of cell growth-inhibitory effect of harringtonine. *C*, control; *HT*, inhibition of cell growth in the 5×10^{-8} *M* harringtonine. **A** inhibition of cell growth in the medium containing 1/10th of the supernatant obtained after exposure of medium alone to 5×10^{-7} *M* harringtonine for a specified time period at 37° C; **B** inhibition of cell growth in the medium containing 1/10th of the supernatant obtained after exposure of cell suspension to 5×10^{-7} *M* of harringtonine for a specified time period at 37° C

Inhibition of leucine incorporation into HL-60 and DND-41 cells by HT

Inhibition of [^{3}H]leucine incorporation into the acid-insoluble fractions of HL-60 and DND-41 cells by HT is shown in Table 3. Although HT produced significantly higher inhibition of protein synthesis of HL-60 cells than of DND-41 cells, the differences were minor and by 60 min the difference became less clear.

Concentration × exposure time studies

The relationship between HT concentration and exposure time is illustrated in Fig. 4. Increases in exposure time resulted in progressive shifting of the dose-response curve to the left, or more effective cell growth inhibition. Thus, based on concentration × exposure time (c × t) a 3-fold increase in exposure time from 1 h to 3 h resulted in a more than 100-fold decrease at ID_{50}; a 2-fold increase in exposure time from 3 h to 6 h resulted in a 20-fold decrease at ID_{70}; and a 4-fold increase from 6 h to 24 h resulted in a 16-fold decrease at ID_{90}.

In separate experiments using the cell growth inhibition assay supernatants (initial concentration 5×10^{-7} *M* HT, final concentration in assay mixture 5×10^{-8} *M*) obtained after 1, 3, 6, and 24 h of incubation with or without cells were as effective in inhibiting cell growth as 5×10^{-8} *M* HT without incubation (Fig. 5). These results show that HT's biological activity was stable and was not influenced by the presence of cells for at least 24 h at 37° C.

Discussion

The present study shows that there is a considerable variety in the sensitivity among human leukemia-lymphoma cell lines against HT. We found that there was some correlation between HT sensitivity and population-doubling time, indicating that the higher the rate of cell multiplication the more the cells became susceptible. Close examination of the dose-response curves of these human leukemia-lymphoma cell lines also shows that while differential sensitivity at ID_{50} and ID_{90} is essentially in the same ranking order, there is a considerable variety in the tail end of each curve. This is especially true with two lymphoma cell lines, Daudi and DND-39A, where there was a progressive plateau in the curve which then crossed over those of less sensitive cell lines. The exact nature of this phenomenon is not clear. Daudi and DND-39A cell lines might have a subpopulation of cells with a very slow doubling time or with intrinsic resistance to HT.

Chou et al. [6] studied the [^{3}H]HT uptake and binding and the [^{3}H]leucine incorporation into a variety of L1210 lines with acquired drug resistance. They reported that the chemotherapeutic effect of HT was correlated with the degree of [^{3}H]HT uptake. In [^{3}H]HT-preloaded cells, parent L1210 cells retained significantly more radioactivity than did the subline resistant to vincristine and HT. The radioactivity appeared to be predominantly bound to the microsomal fractions. In the L1210 sublines the degree of inhibition of protein synthesis by HT also correlated with the therapeutic effect. The present results with HL-60 and DND-41 cells show that HT's biological effects were best correlated with their capacity to retain [^{3}H]HT. There was also a minor difference in [^{3}H]leucine incorporation into acid-insoluble fractions of these two lines. The initial inhibition of [^{3}H]leucine by HT during the 5- and 10-min periods was significantly higher in HL-60 than in DND-41 cells. However, the differences between the two cell lines became obscured at 1 h. It seems difficult to explain a 70-fold difference in HT's biological effect by the inhibition of leucine incorporation alone, and probably other factors may be involved. We have identified population-doubling time as one of such factors. The difference between observation made earlier with L1210 cells [6] and in the present study is most likely related to the fact that the earlier work with the animal cells involved acquired drug resistance, whereas the present one dealt with natural resistance to HT. Decreased drug uptake (or decreased membrane transport of the drug) as a main cause of pleiotropic drug resistance has been recorded in tumor cells made resistant to plant alkaloids and anthracyclines [2, 10]. It is possible that HT uptake may correlate with acquired drug resistance, whereas the affinity of HT to cellular components may relate to natural resistance to the drug. Increased HT binding may be predictive of clinical response of

previously untreated leukemia and lymphoma. While further studies of [^{3}H]HT retention and release in other cell lines were desired, the amount of radioactive HT available was limited, so that further examination was not possible.

Clinical trials carried out in China suggested the optimum regimen of 0.2–0.3 mg HT/kg body weight administered by slow IV infusion for 5–7 days followed by a 7- to 15-day rest period [4]. The slow IV infusion was to avoid cardiovascular complications (hypotension and/or arrythmias) observed following IV bolus. Recent phase I trials of homoharringtonine in the United States [9, 14, 22] in solid tumors have also led to the recommendation of a continuous infusion schedule over 5 or 10 days. The optimum administration of the drug based on the biological rationale has not yet been proposed, however. Our $c \times t$ data clearly indicate that HT's biological effects are better expressed by exposure time than by concentration. It has been reported that the magnitude of an anticancer drug's cellular lethality is directly related to its $c \times t$ [1, 15]. This concept is not applicable with HT, where increasing exposure time resulted in more effective cell growth inhibition. While in vitro data on tumor cells alone are insufficient to allow extrapolation to man, our data support the proposition that HT's biological effects are best exploited by giving the drug for an extended period of time, thus favoring clinical trials of this compound administered according to a continuous infusion schedule.

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