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HL-60 ATP assay for predicting rat oral toxicity study

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Abstract
The ATP assay using HL-60 cells, a promyelocytic leukemia cell line, is known to be a sensitive assay for evaluating the cytotoxicity of compounds. To evaluate the predictive ability of the HL-60 ATP assay to estimate oral systemic toxicity in rats, we examined the cytotoxicity of 88 pharmaceutical candidates produced by Mitsubishi Pharma Corporation retrospectively. Thirty-six of the 88 compounds were lethal at doses of up to 300 mg/kg in the 4-day repeated oral dose toxicity studies in rats. In the HL-60 ATP assay, 32 of 36 lethal compounds had an IC_{50} of less than 100 μM (median = 9.4 μM). Compounds with an IC_{50} < 1 μM showed a minimum lethal dose of 10 mg/kg, and mainly induced hepatotoxicity, gastrointestinal (GI) toxicity, and myelosuppression. A compound group with IC_{50}s ranging from 1 to 30 μM showed a minimum lethal dose of 30 mg/kg, and induced hepatic and GI toxicity. The IC_{50} ranking of the compounds was comparable to the potency of the oral toxicity in rats except for some compounds that showed low bioavailability. These data show that the HL-60 ATP assay may serve as a principal method for predicting systemic toxicity before conducting short-term rodent toxicity studies and contribute to the 3Rs.

Keywords: cytotoxicity, IC_{50}, HL-60, ATP, rat toxicity

Introduction

To reduce animal use in safety evaluations in pharmaceutical research, a reliable cytotoxicity assay for predicting animal toxicity is necessary. Neutral red uptake assays using 3T3 and NHK cells have been validated and are recommended as *in vitro* cytotoxicity test methods for estimating starting doses for acute oral systemic toxicity tests (ICCVAM, 2001, 2006). Also, the ATP assay using HL-60 cells and the MTT assay using Hep G2 and 3T3 cells were ranked the best of 15 assays in terms of correlation with human lethal peak concentrations, with correlation coefficients (R^2) of 0.72, 0.63, and 0.54, respectively (Ekwall, 2000). A preliminary comparison study using the ATP assay in Hep G2 and HL-60 cells showed that 32 of 39 pharmaceutical compounds produced by Mitsubishi Pharma Corporation (MPC) exhibited more potent cytotoxicity to HL-60 cells compared with Hep G2 cells. The remaining 7 compounds showed similar cytotoxicity to the two cell lines. In the present study, we examined the cytotoxicity of 88 compounds, for which a 4-day oral toxicity studies had already been conducted in rats, using the HL-60 ATP assay, and then compared their 50% inhibition concentrations (IC_{50}s) with *in vivo* data. The compounds were pharmaceutical candidates in a discovery phase for brain and nervous system diseases, cardiovascular and metabolic disorders, immunology and inflammatory diseases, and cancers; 36 of the 88 compounds were lethal to rats. Histopathological data was obtained for 63 of the 88 compounds; 61 compounds were toxic to the liver, gastrointestinal (GI) tract, hematopoietic system, kidneys, heart, lungs, and/or other organs, and 2 compounds were non-toxic in rats. We examined whether the approximate lethal dose (ALD), target organs, and toxicity ranking of the 88 compounds could be predicted by IC_{50} in HL-60 cells.

Materials and methods

Chemicals

RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY, USA). CellTiter-Glo™ luminescent cell viability assay kit was purchased from Promega (Madison, WI, USA). Quinidine sulfate and dimethylsulfoxide (DMSO) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Eighty-eight test compounds were synthesized at MPC.

Cells

An HL-60 cell line derived from human promyelocytic leukemia was obtained from DS Pharma Biomedical (Osaka, Japan). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS and incubated at 37°C in a humidified 5% CO_2 air atmosphere.

ATP cell viability assay

The cells were seeded in a 96-well flat-bottom plate at 2×10^4 cells/100 μl/well (n = 3) and treated with each test compound for 24 hours. Representative concentrations of test compounds were set at 1, 3, 10, 30, and 100 μM. DMSO, a solvent control, was used at 0.5% (v/v) or less in the culture medium. Quinidine sulfate, a positive control, was used at 100 μM, which is approximately equivalent to IC_{50}. ATP content was measured in accordance with the protocol of the CellTiter-Glo™ luminescent cell viability assay kit. Briefly, 100 μl of assay reagent was added to the wells and mixed for 2 min at room temperature. After 10 min, intracellular ATP content was measured using a luminescence multilabel counter (ParkinElmer, 1420 $ARVO_{SX}$).

Data analysis

Cell viability was calculated using the following equation:

% Cell viability = [value (test article) – value (blank)] / [value (control) – value (blank)] × 100

IC_{50} was determined from a 4-parameter curve fit (Molecular Devices, SOFTmax Pro).

Rat 4-day orally repeated dose toxicity data

Six-week-old CD (SD) IGS rats were obtained from Japan Charles River (Atsugi, Kanagawa, Japan). All rats used were free of specific pathogens. The rats were kept under controlled conditions and given free access to solid feed (CRF-1 manufactured by Oriental Yeast, Tokyo, Japan) and sterilized tap water. Male and/or female rats (3 or 6 each) per dose were orally administered with a compound for 4 days. Representative doses were 30, 100, and 300 mg/kg, or 10, 30, and 100 mg/kg. The animals were examined for clinical signs, body weight, and food consumption during the dosing period. Animals that showed tolerance to any of the 63 of the compounds were dissected on the day following the last dosing, and underwent hematology, blood chemistry, organ weight, macro- and microscopic examinations. All procedures were approved by the Institutional Animal Care and Use Committee at MPC.

Results and discussion

In the present study, we examined the cytotoxicity of 88 test compounds and the IC_{50} value for each compound was calculated. Cell viability at a dose of 100 μM quinidine sulfate, a positive control, was 49.6 ± 11.7% (mean and standard deviation) in the 38 experiments conducted, and the viability was comparable with the reported IC_{50} for quinidine sulfate of 120 μM (ICCVAM-NICEATM, 2001).

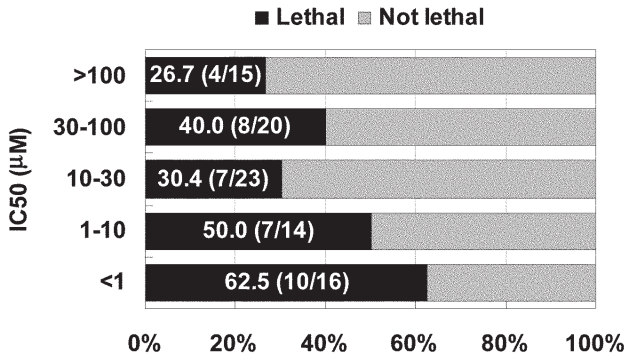

Fig. 1. IC_{50} values from HL-60 ATP assay and lethality in rat 4-day oral toxicity study on 88 pharmaceutical compounds

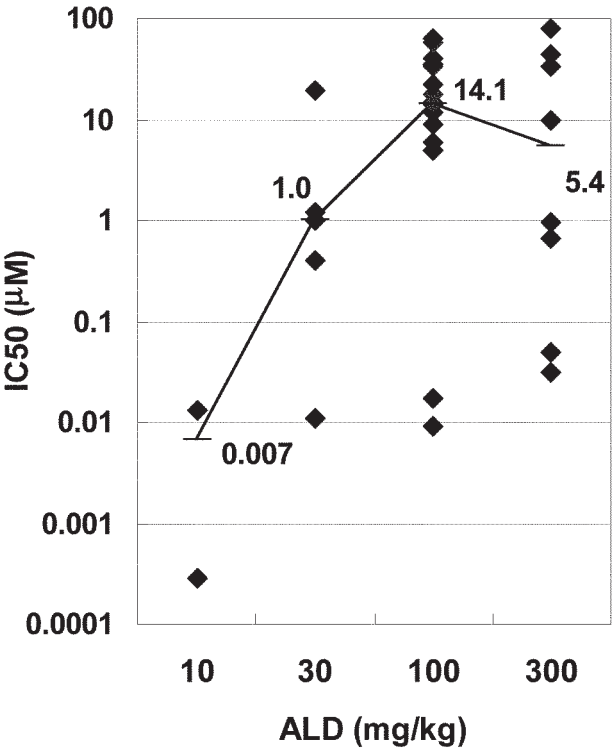

Fig. 2. Median IC_{50} values in HL-60 ATP assay and approximate lethal doses (ALDs) in rat 4-day oral toxicity study on 32 lethal compounds

IC_{50} values and lethality

Eighty-eight compounds were classified by their IC_{50} values in the HL-60 ATP assay as follows; <1 μM (16 compounds), 1 - 10 μM (14 compounds), 10 - 30 μM (23 compounds), 30 - 100 μM (20 compounds), and >100 μM (15 compounds). There appeared to be a correlation between the IC_{50} and rat lethality. When IC_{50} values were below 1 μM, 10 out of the 16 of compounds (62.5%) induced death or moribund sacrifice in rats within 4 days of the administration period (Fig.1). As IC_{50} values were higher, the lethal

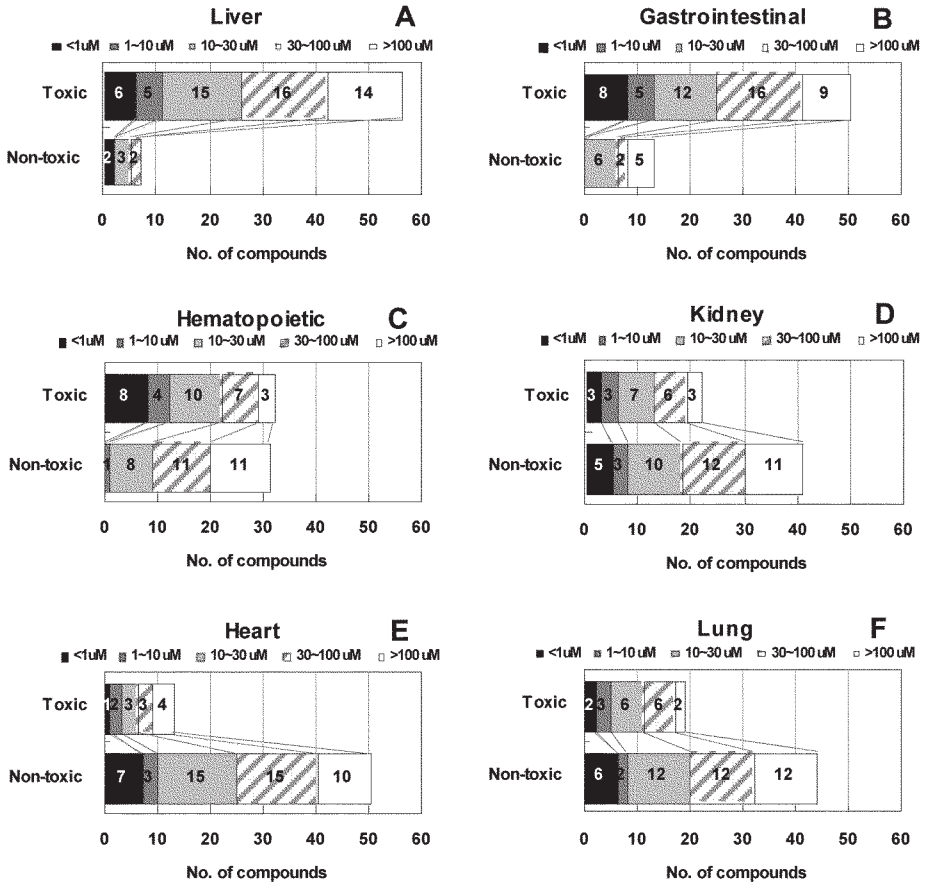

Fig. 3. IC_{50} values in HL-60 ATP assay and target organs for 63 compounds with histopathological findings in rat 4-day oral toxicity studies; 56 toxic and 7 non-toxic compounds in liver (A), 50 toxic and 13 non-toxic compounds in GI tract (B), 32 toxic and 31 non-toxic compounds in hematopoietic system (C), 22 toxic and 41 non-toxic compounds in kidney (D), 13 toxic and 50 non-toxic compounds in heart (E), and 19 toxic and 44 non-toxic compounds lung (F).
The test compounds are classified by IC_{50} values as <1 μM (8 compounds), 1 - 10 μM (5 compounds), 10 - 30 μM (18 compounds), 30 - 100 μM (18 compounds), and >100 μM (14 compounds).

Table 1. IC_{50} values from HL-60 ATP assays and minimum lethal doses on 36 lethal compounds in rat 4-day oral toxicity studies

IC_{50} (μM)	No. of lethal compounds	Minimum lethal dose (mg/kg)	Lethal frequency in rat 4-day oral toxicity study				
			3 mg/kg	10 mg/kg	30 mg/kg	100 mg/kg	300 mg/kg
<1	10	10	0 [a]/1 [b] (0%)	2/5 (40%)	4/9 (44%)	6/9 (67%)	5/5 (100%)
1~10	7	30	0/0 (0%)	0/0 (0%)	2/3 (67%)	6/7 (86%)	4/4 (100%)
10~30	7	30	0/0 (0%)	0/0 (0%)	1/6 (17%)	7/7 (100%)	3/3 (100%)
30~100	8	100	0/0 (0%)	0/1 (0%)	0/7 (0%)	4/7 (57%)	6/6 (100%)
>100	4	100	0/0 (0%)	0/0 (0%)	0/2 (0%)	3/4 (75%)	2/2 (100%)

[a] No. of compounds with lethality per dose level; [b] No. of compounds administered.

frequency gradually decreased. Minimum ALDs associated with the IC_{50} were 10 mg/kg for below 1 μM, 30 mg/kg for 1 - 30 μM, and 100 mg/kg for 30 μM or more (Table 1). The median IC_{50} was 9.4 μM for 32 lethal compounds. Four compounds failed to achieve an inhibition ratio of 50% or more at 100 μM (Fig. 2).

IC_{50} values and target organs

Histopathological findings were detected for 61 of 63 compounds that were subjected to microscopic examination. Main target organs or tissues were the liver, GI tract, hematopoietic system, kidneys, heart, and lungs. Compounds with an IC_{50} <10 μM induced hepatotoxicity, gastrointestinal

Table 2. IC_{50} ranking of compound group A in HL-60 ATP assay and rat 4-day oral toxicity data

Compound	HL-60 ATP assay IC50 (μM)	Rat 4-day oral toxicity study					Pharmacokinetic parameter in rats (single dose)		
		Lethal frequency				Target organ	Cmax (μM) / AUC (μM·h)		BA (%)
		10 mg/kg	30 mg/kg	100 mg/kg	300 mg/kg		30 mg/kg	100 mg/kg	
A1	0.011	0 [a]/6 [b]	2/6	2/6	NE	GI at 10 mg/kg	0.14 / 1.61	NE	9.1
A2	0.030	NE	0/6	0/6	0/6	BM, GI, etc.	0.39 / 4.28	0.34 / 3.25	17.2
A3	0.031	NE	0/6	0/6	6/6	BM, GI, L, etc.	5.43 / 18.7	11. 6 / 73.8	45.7
A4	21.2	NE	0/6	0/6	0/6	BM, GI, L, etc.	4.93 / 31.8	18.2 / 230	57.5

[a] No. of rats with death or moribund sacrifice during a dosing period; [b] No. of rats administered.

NE = not examined; GI = gastrointestinal tract; BM = bone marrow; L = liver.

Table 3. IC_{50} ranking of compound group B in HL-60 ATP assay and lethality in rat 4-day oral toxicity studies

Compound	HL-60 ATP assay IC_{50} (μM)	Lethal frequency in rat 4-day oral toxicity study		
		30 mg/kg	100 mg/kg	300 mg/kg
B1	5	NE	3 [a]/3 [b]	3/3
B2	6	NE	1/3	3/3
B3	12	NE	3/3	3/3
B4	14	0/3	0/3	NE
B5	15	0/3	2/3	NE
B6	21	NE	0/3	0/3
B7	25	NE	0/3	0/3
B8	33	NE	2/3	3/3
B9	35	0/3	3/3	NE
B10	36	0/3	0/3	NE
B11	40	0/3	3/3	3/3
B12	57	0/3	3/3	NE
B13	>100	0/3	1/3	NE
B14	>100	0/3	2/3	NE
B15	>100	NE	0/3	2/3
B16	>100	0/3	0/3	NE
B17	>100	NE	3/3	3/3

[a] No. of rats with death or moribund sacrifice during a dosing period; [b] No. of rats administered.

NE = not examined;

toxicity, and myelosupression (Fig. 3). HL-60 cells, a promyelocytic leukemia cell line, might be specifically sensitive at detecting hematotoxic potential. Two of 63 compounds showed IC_{50} values of 23 and 28 μM in spite of being non-toxic in rats up to doses of 300 mg/kg. As the compounds were anticancer drugs with efficacy *in vitro* at a micromole level, the cytotoxicity of the compounds in HL-60 cells might be linked to their efficacy.

Toxicity ranking of compounds by IC_{50} values

IC_{50} ranking in certain compounds such as group A was generally comparable to the potency of toxicity in rats with one exception; the IC_{50} of compound A2 in HL-60 cells was the same as compound A3, however, there was no lethality in rats because of low bioavailability (Table 2). Potent cytotoxicants of group A induced bone marrow and/or GI toxicity in rats. In compound group B, IC_{50} ranking was not comparable to rat lethality (Table 3). As HL-60 cells have no drug-metabolizing enzyme such as cytochrome P450, a complementary cytotoxicity test using a metabolic activation system is needed to detect metabolite-mediated cytotoxicity.

In conclusion, compounds with an IC_{50} <10 μM in the HL-60 ATP assay were found to represent a high risk in a rodent 4-day toxicity study and IC_{50} ranking was comparable to the potency of the oral toxicity

in rats except for some compounds that showed low bioavailability or metabolite-mediated toxicity. The HL-60 ATP assay is a good throughput method that requires 1 to 10 mg weight of compound and a 2-day experimental period. The HL-60 ATP assay may be a useful method for predicting systemic toxicity in short-term rodent studies and for optimization of lead compounds in the early phase of pharmaceutical research. The principal use of the HL-60 ATP assay before a rodent study may contribute to the 3Rs.

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