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Toxicity of 10 MEIC Chemicals to Mitochondria by Fluorescence Imaging

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Abstract

The *in vitro* toxicity of 10 MEIC chemicals was determined using a fluorescence microscope-video camera-image processor system. Human lung carcinoma cells were loaded with rhodamine 123 which accumulates specifically in mitochondria in living cells. Rhodamine 123 fluorescence in mitochondria relative to that in the cytoplasm of the cells was measured after 3hr treatment with the MEIC chemicals. Results showed that the ED_{50} values of the chemicals determined by this method were highly correlated with the corresponding values determined by the MTT (r=0.949) and lactate dehydrogenase-release assays (r=0.948) after 48 hr. This fluorescence-image processing was faster for measuring changes in mitochondrial function than the MTT assay, and is useful for measuring the direct toxic effects of chemicals on organelle's functions *in situ*.

Key words: mitochondria, fluorescence-image processing, MEIC chemicals, MTT assay, LDH release assay

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Introduction

Most *in vitro* cytotoxicity assays involve colorimetric measurements which are convenient. These include the MTT (1, 2, 3, 4, 5) and neutral red uptake assays (2, 6, 7), crystal violet staining (8, 9), as well as the lactate dehydrogenase (LDH) release assay (4, 10, 11, 12, 13, 14, 15). Another widely used assay is that of colony formation (16, 17). These assays measure overall changes in the proliferative/surviving functions of cell populations and all require 2–14 days except the LDH-release assay of acute toxicity, which requires a 20 min incubation (14). More rapid methods for assay of *in vitro* cytotoxicity of chemicals would be useful. We developed a new assay system with human natural killer (NK) cells which requires a 4 hr incubation in medium containing test chemicals, and found that killing activity was an excellent marker of cytotoxicity not only at the level of the cell population, but also at the level of the individual cell and, if possible, of subcellular organelles in individual living cells, since this would give more precise information on mechanisms of toxicity. Therefore, we tried to develop an image processing system to observe mitochondria *in situ* stained with a fluorescent dye (19–24).

Mitochondria in living cells are intensely stained against a dark cytoplasmic background with fluorescent cations such as rhodamine 123 (Rh123) depending on th membrane potential (19). Rh123 has a relatively stable yellowish-green fluorescence on excitation with blue light. We developed a means of measuring the inhibition of Rh123 excretion from primary cultured natural killer cells by flow cytometry (18), but it measures the fluorescence intensity of a whole cell. As reported by Johnson *et al.* (24), when cells are incubated with some chemicals, much of the dye is released from mitochondria and remains in the cytoplasm for a while. Therefore, the fluorescence intensity of a whole cell will also be affected by the rate of passage of the fluorescent dye through the cytoplasmic membrane.

If a toxic substance directly affects the mitochondrial membrane potential, the fluorescence intensity of Rh123 in mitochondria relative to that in the cytoplasm would change greatly and be a direct indicator of alteration of mitochondrial functions. Therefore, we used fluorescence-image processing to determine the toxicity of 10 of the 50 chemicals recommended for the Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC) by the Scandinavian Society for Cell Toxicology (15, 24). We found very close relationships between the organelle toxicity in individual cells determined by fluorescence-image analysis and the cytotoxicity in mass-cultured cell populations.

Materials and Methods

Cell culture

Human lung carcinoma RERF-LC-AI cells (so called AOI cells, cell line number RCB0444, RIKEN Cell Bank) were used because of their flat morphology and the even distribution of mitochondria in their cytoplasm. They were seeded at a density of 20,000 cells per well into 24-well culture plates on a round coverglass of 15-mm diameter (Matsunami Glass Ind., Ltd., Japan) in 1 ml of MEM containing 10%FBS and cultured for 3 days before experiments.

Test chemicals

Ten of the 50 MEIC chemicals were selected arbitrarily. These were sodium chloride, sodium fluoride, nicotine, lithium sulphate, isoniazid, chloroquine diphosphate, quinidine sulphate, caffeine, atropine sulphate and potassium chloride, and were all purchased from Wako Pure Chemical Industries (Tokyo, Japan). Test concentrations of sodium chloride and potassium chloride are expressed as those above the regular concentrations in MEM-5%FBS.

Cytotoxicity assay

The RERF-LC-AI cells in each well were incubated in a humidified CO_2-incubator in 1 ml of medium containing 1 μg/ml of Rh123 at 37°C for 30 min. They were then washed extensively with fresh culture medium (19, 24, 26, 27). Solutions of the ten chemicals were prepared as described (15) and serially diluted with the culture medium just before use. Rh123-loaded cells were incubated with 1 ml of diluted chemicals at 37°C for 3 hr. Then the cells were washed 3 times with PBS (−) and observed in a fluorescence microscope-video camera-image processor consisting of an inverted Diaphot-TMD microscope attached to a TMD-EF2 fluorescence-producing apparatus (Nikon Co., Japan), an ICCD video camera (C2400–87), an ARGUS-50/VIM2 image processor (C3930–50), a computor (C4468) with an MO disk unit, and a color video monitor (PVM1444Q) connected to a color video printer. The 4 latter pieces of equipment were from Hamamatsu Photonics Co.

Imaging and fluorescence measurement of Rh123-stained cells

After chemical exposure, cells were observed at excitation and emission wavelengths

of 470–490 and 520–560 nm, respectively, under a Diaphot-TMD microscope with a 40×CF Fluor objective lens. The fluorescence of Rh123 was excited at 10 mV by a 100-W mercury lamp attached to a TMD-EF2. Imaging was conducted by exposing fluorescent cells to the video camera for 10 seconds. Six images were taken of each sample in 6 randomly selected fields in different cell populations. Usually, one image consisted of 4–10 cells. All the images were saved in an MO disk with ARGUS-50 software for further analysis of the fluorescence intensity in living cells.

The fluorescence intensity inside and outside mitochondria was measured with an image processor. Since the equipment did not magnify the images sufficiently to clearly identify an individual mitochondrion in a cell, the fluorescence intensity inside and outside mitochondria was represented as the fluorescence intensity of 5×5-pixel squares (4.67 μm×4.67 μm) in a mitochondria-rich area and that in an adjacent mtochondria-free cytoplasmic area, respectively (see Fig. 1). When possible at most, 5 pairs of 5×5-pixel squares of mitochondria-rich and adjacent mitochondria-free cytoplasmic areas in a cell were collected. From 6 images, it was possible to accumulate 12–15 values of the ratio to calculate the mean and standard deviation.

MTT assay

RERF-LC-AI cells were cultured in 100 μl MEM-10%FBS in 96-well plates for 24 hr, then incubated with the test chemicals for 48

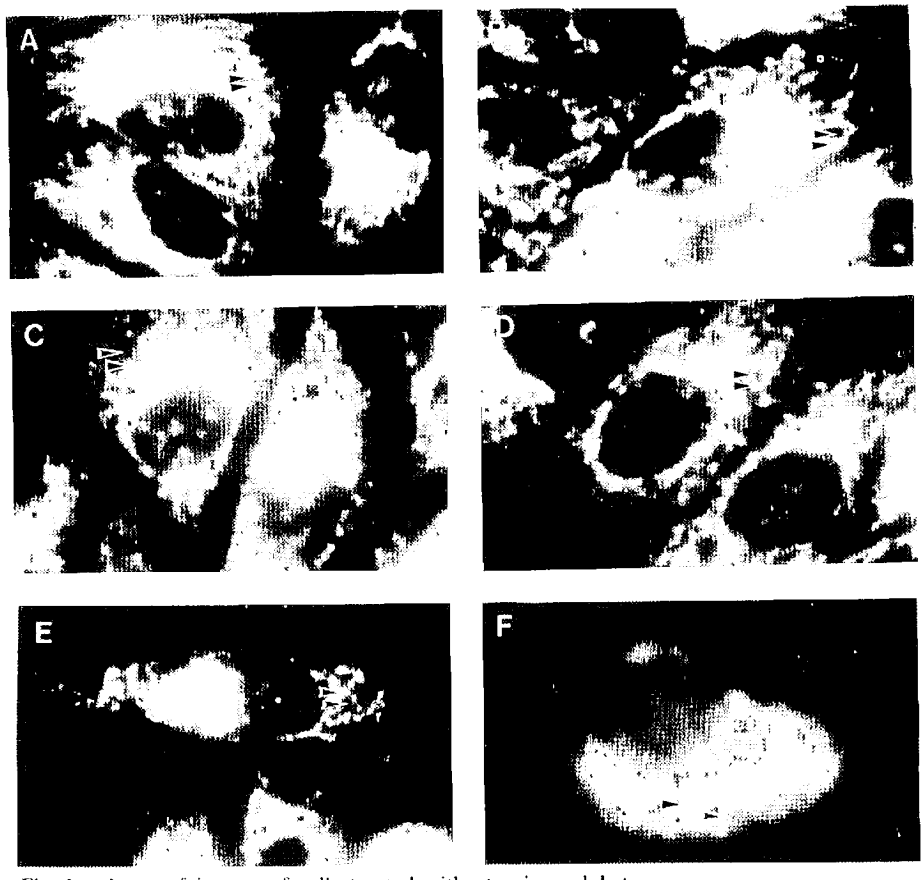

Fig. 1. A set of images of cells treated with atropine sulphate.

Human lung carcinoma RERF-LC-AI cells (AOI cells) were incubated with atropine sulphate for 3 hr. Control cells (A); cells treated with (B) 0.74 mM, (C) 1.48 mM, (D) 2.96 mM, (E) 4.43 mM, and (F) 5.91 mM atropine sulphate. Tips of arrow heads indicate observation points. Bright and adjacent dark regions correspond to mitochondrial and cytoplamic areas, respectively.

hr. Thereafter, 10 μl of MTT (5.5 mg/ml) was added for 4 hr (1). The supernatant was discarded and 200 μl of isopropanol-0.04N HCl was added to each well to extract and dissolve blue-purple formazan crystals. The optical density was determined at 590 nm and ED_{50} values were calculated as reported (1, 3, 5).

Results

Fluorescence images of cells

We screened various cell lines to determine which had the highest fluoescence intensity and the largest individual cells so that their mitochondria could be conveniently detected with the fluorescence microscope-video camera-image processor. As well as the lines of human carcinoma cells [RERF-LC-AI, SQ-5, HeLa S3 (mer^-) and Hep G2], glioma cells (U-87MG and KG-1-C), normal diploid fibroblasts (WI-38), lymphoblastoids (Daudi), primary cultured natural killer cells, and monkey kidney cells (Vero) were also examined. RERF-LC-AI cells emitted the highest fluorescence intensity after loading with rhodamine-123 (1 μg/ml) for 30 min and their monolayer cultures were sufficiently flat to allow measurement of the fluorescence of mitochondria and the adjacent cytoplasm. Figure 1A shows that mitochondria occupied a substantial fraction of the cytoplasm and were concentrated in perinuclear area. On incubating the cells with increasing concentrations of atropine sulphate for 3 hr, the fluorescence intensity of the mitochondria-rich area decreased and the thread-like mitochondria became dot-like and dim (Fig. 1B-F), while the fluorescence intensity of the cytoplasm increased or changed slightly.

Dose-response curves and ratios of fluorescence intensity in mitochondria and cytoplasmic areas

The fluorescence intensity inside and outside mitochondria was determined in digitzed fluorescence images of the cells by area analysis with an image processor. We measured the fluorescence intensity of small areas (5×5-pixel square, corresponding to 4.67 μm×4.67 μm) of peripherally located mitochondria and their adjacent cytoplasm in a cell. In each picture in Fig. 1, the two arrowheads indicate a pair of mitochondria- and adjacent cytoplasmic-areas on which the fluorescence intensity was determined. The fluorescence intensity was read with a computer and the ratio of the intensity was recorded. The mean ratio in untreated control cells varied from 5.2 to 6.8 in different experiments. When cells were incubated with 2.95 or 5.91 mM atropine sulphate, the mean ratios decreased at an increasing rate with the length of the incubation (Fig. 2). With 5.91 mM atropine sulphate, the ratio became 1 after 3 hr. The dose responses of other chemicals were tested after a 3 hr incubation.

The dose-response curves of the ratios of all 10 chemicals are shown in Fig. 3. Although the curves showed a decrease of the ratio with an increase in the concentration of each test chemical, the extent of the change differed considerably. Quinidine sulphate decreased the ratio to 1 in a narrow range of concentrations of below 0.3 mM, whereas a concentration of 125 mM nicotine was required to decrease the ratio to 1. The concentration

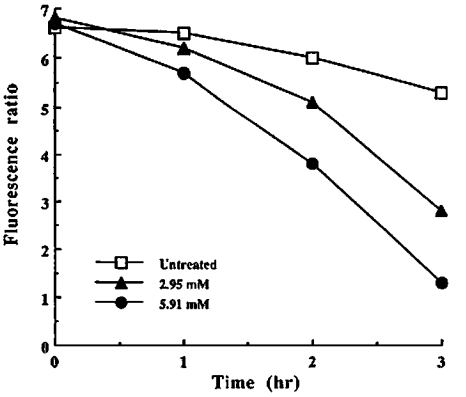

Fig. 2. Time courses of changes in fluorescence ratios induced by atropine sulphate. Mean ratios of bright and adjacent dark points (each 5×5 pixel squares) are plotted.

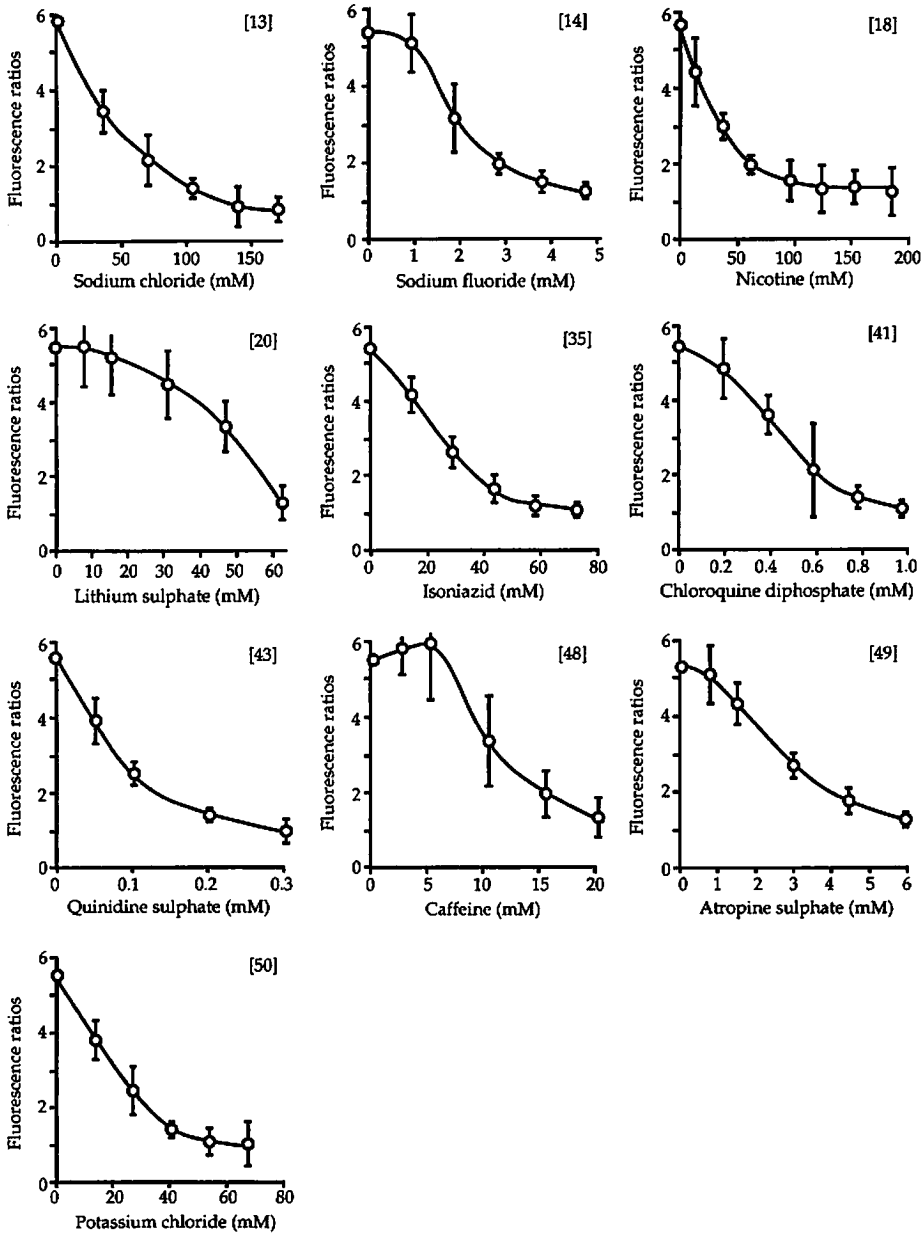

Fig. 3. Dose-response curves of the 10 MEIC chemicals.

The number in the top right of each graph is the code number of the chemical given by the Multicenter Evaluation of In vitro Cytotoxicity (MEIC), organized by the Scandinavian Society for Cell Toxicology. The values of fluorescence ratios are means for 12–15 pairs of measurements in cells treated with each chemical. Standard deviations (SD) of mean values are indicated by bars.

required for a 50% reduction of [the mean maximum ratio-1] was defined as the ED_{50}.

Table 1 lists the ED_{50} values obtained from each curve in Fig. 3 together with those determined by two other *in vitro* assays, MTT and LDH release. For the MTT assay, RERFLC-AI cells were incubated with the chemicals for 48 hr. The ED_{50} values determined by LDH release assay using the human lung carcinoma cell line SQ-5 are cited from our previous paper (15). In this study SQ-5 cells were incubated with the chemicals for 48

Table 1. ED_{50} of 10 MEIC chemicals determined by fluorescent image processing

MEIC Chemical	Image processing	MTT assay		LDH release assay	
	ED50(mM)	ED50(mM)	MTT/Fluo	ED50(mM)	LDH/Fluo
13. Sodium chloride	35	31.6	0.90	151	4.31
14. Sodium fluoride	1.9	0.98	0.51	2.10	1.11
18. Nicotine	30	6.78	0.23	21.6	0.72
20. Lithium sulphate	47	44.1	0.94	36.5	0.78
35. Isoniazid	24	17.5	0.73	49.2	2.05
41. Chloroquine diphosphate	0.42	0.18	0.44	0.41	0.98
43. Quinidine sulphate	0.07	0.12	1.73	0.20	2.86
48. Caffeine	11	2.57	0.23	5.70	0.52
49. Atropine sulphate	2.6	1.18	0.45	2.90	1.12
50. Potassium chloride	20	29.0	1.45	61.7	3.09
Average			0.76		1.75

First column shows the MEIC number of the chemicals. MTT/Fluo and LDH/Fluo are the ratios of ED_{50} values determined by MTT assay and our previous LDH release assay (15) to those determined by fluorescent image processing, respectively.

hr and the LDH activity remaining in the cells was measured to calculate those of the ED_{50}. In this assay, the largest ED_{50} was for lithium sulphate and the smallest for quinidine sulphate. These results were consistent with the largest and the smallest ED_{50} values determined by the MTT assay. However, in the LDH release assay, sodium chloride had the largest ED_{50} of 151 mM, whereas in the present assay, it was only 35 mM. Thus the ratio of the ED_{50} determined by LDH release to that determined in the present assay (LDH/Fluo) was 4.3. Relatively large discrepancies were also observed in the ED_{50} value of nicotine: 30 mM in this assay but 6.78 mM in the MTT assay resulting in the ratio, MTT/Fluo, of 0.23 (Table 1). Similar differences were observed with caffeine. However, sodium fluoride, isoniazid, chloroquine diphosphate, quinidine sulphate, atropine sulphate, and potassium chloride had relatively similar ED_{50} values in these three assays.

Correlation of results with those of MTT and LDH release assays

In Table 1, the average of MTT/Fluo was 0.76 and that of LDH/Fluo was 1.75, suggesting that sensitivity of the new method compared with that of the other two *in vitro* cytotoxicity assays is closer to the MTT assay. However, the logs of the ED_{50} values determined by this method correlated well with those determined by both of the MTT ($r=0.9492$)(Fig 4A) and the LDH release ($r=0.9482$) (Fig. 4.B) assays.

Discussion

Mitochondria have a significant membrane potential with a negative internal charge. Lipophilic compounds with a positive charge are taken up by mitochondria to a much greater extent than by other organelles, so there have been many studies on specific fluorescent probes of mitochondrial membrane potential (19–24, 26, 27–36). Since this potential is generated by proton pumps which in turn are driven by respiratory electron transport chains and because the energy stored in this gradient is primarily responsible for the conversion of $ADP+P_i$ to ATP (26), the change of the mitochondrial membrane potential caused by toxic chemicals should correlate with some aspects of toxicological mechanisms.

Smiley *et al.* found that mitochondrial membrane potentials are intracellularly heterogeneous (35), but the fluorescence intensity measured here was limited to small 5×5 pixel

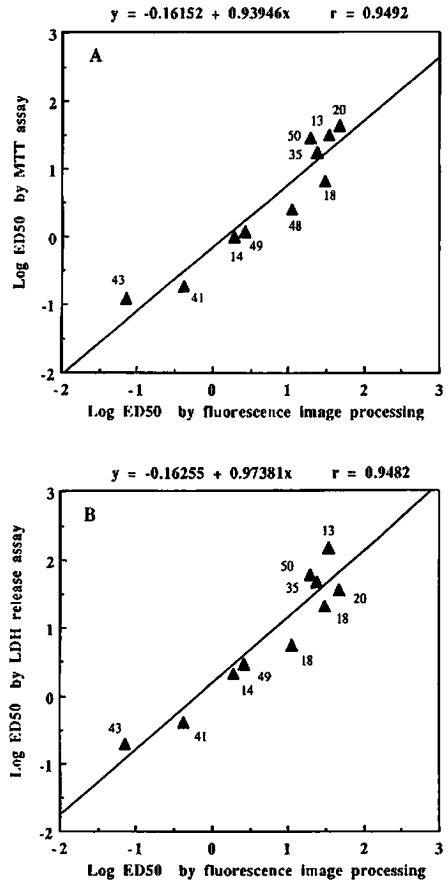

Fig. 4. Correlation of the results by fluorescence image analysis with those by MTT and LDH release assays.

(A) and (B) show the correlations of log ED_{50} values determined by image processing with those obtained by the MTT and LDH release assays, respectively. The lines, formulae and correlation coefficients of the linear regression lines are indicated. The numbers by the points are the MEIC code numbers.

squares that correspond to 21.8 μm^2. The ratios of the fluorescence intensity of these small areas to those of the adjacent cytoplasmic areas were therefore, considered to directly reflect the activities of small numbers of local mitochondria (Fig. 1).

After incubating the cells with increasing concentrations of chemicals for 3 hr, there were quantitative changes in the fluorescence intensity and the ratios (Figs. 1 and 2). Our method is faster than *in vitro* cytotoxicity assays that measure growth inhibition or cell death as indicators of toxicity (1–3, 6–17) and required several days to produced result. Image analysis of intracellular and/or cell surface ionic concentrations such as Ca^{++} require less than a second (37). In this study, we could not trace the same mitochondrial area quantitatively because repeated observation of a cell by repeated irradiation of the mitochondria with blue light excitation caused an apparent decrease of the fluorescence intensity. Therefore, we determined the ratios of the fluorescence intensity of area rich in mitochondria and adjacent cytoplasmic regions of at least 12 cells in a population.

The MTT assay depends mainly on the quantity and function of dehydrogenase in the mitochondrial matrix, so the ED_{50} values obtained by this assay were correlated closely with those obtained by the present assay (Fig. 4A). However, we found that 8 chemicals produced lower ED_{50} values by the MTT assay than by the present assay (Table 1). The average MTT/Fluo ratio was 0.76, suggesting that in general, the psent assay is less sensitive than MTT assay. One reason for this would be the duration for which cells were exposed to chemicals such a longer incubation (48 hr in the MTT assay) with a chemical would presumably result in a more severe toxic effect. Another reason would be that results in the MTT assay includes the effects of chemicals on cell proliferation. Chemicals would be more toxic to cells if they acted on both the mitochondrial membrane potential and the machinery for cell proliferation including DNA synthesis, protein synthesis, cytoskeleton metabolism, and mitochondrial replication.

The LDH release assay detects LDH activity released into the surrounding culture medium and that maintained in the cells (11, 14, 15). We cite the ED_{50} values obtained for LDH activity in cells incubated with chemicals for 48 hr, which may reflect not only cell proliferation but also the LDH content of enlarged cells. Sodium chloride and potassium chloride at lower concentrations, but higher

than those in normal medium, increase the LDH content of the cells without apparently increasing the number of cells (38). This may partly explain why the ED_{50} values of the chemicals determined by LDH release were higher than that of the present assay (Table 1) (15).

We developed an assay for Rh123 excretion inhibition using flow cytometry with primary cultured human natural killer cells (18). In this method, the fluorescence intensity of a whole cell is measured after a 3 hr exposure to a chemical. If Rh123 released from mitochondria remains in the cytoplasm for a considerable period (24), the results of this assay will be affected by the rate of Rho123 passing through the cytoplasmic membrane. In this flow cytometric assay, quinidine sulphate inhibited Rh123 excretion from the cells with an IC_{50} (equal to the ED_{50} in the present report) of 0.8 μM (18). This concentration is 1/88 of the ED_{50} determined by the present assay (Table 1). There is an apparent difference in the end points in the present assay, which essentially measures the mitochondrial membrane potential and the flow cytometric assay, which measures dye excretion from a whole cell. However, the large difference in the ED_{50} values for quinidine sulphate implys that the primary site of its toxic action is the cytoplasmic membrane rather than the mitochondrial membrane. Quinidine sulphate may be a powerful inhibitor of cell membrane P-glycoprotein (18), a multidrug resistance-1 (MDR-1) gene product that is an ATP-driven efflux pump for lipohilic drugs (39). We consider that image analysis as presented here is a powerful tool for evaluating toxic actions of chemicals on subcellular organelles *in situ* in living cells.

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