Summary in 96-Well Microplate-Based Cytotoxicity and Development of

lines [16,17]. Moreover, it has shown excellent linearity and a wide dynamic range (0 – 750,000 RLU) in RPMI-8226 cells [18]. Furthermore, a major advantage of the ATP assay over other microplate-based cytotoxicity tests is that the ATP assay can be used to assess cytotoxicity in cell suspensions and monolayers [19].

Figure 2: Photograph of cells treated by nicotine or acetaminophen. Fluorescence images showing staining with Hoechst 33342 (dark blue, nuclei) and Cell Mask (red, cytoplasm).

E ect of cell cycle-speci c inhibitor-induced cell hypertrophy on the accuracy of cytotoxicity tests

ere are a few reports concerning chemically induced cell hypertrophy and increased ATP content through cell cycle. Methotrexate (MTX) was shown to induce an increase in K562 cell size with arresting S phase [30]. Reversine, a synthetic purine, has been shown to induce cell cycle arrest in G2/M phase followed by an increased cell size of PC3 cell [31]. Moreover, DNA damage caused by anti-cancer drug elevated cellular ATP content [32-34]. Oyama et al. reported that H2O2 and doxorubicin induced G2/M cell cycle arrest with an increase of p21 expression in H9c2 cells, which was related to cellular hypertrophy [35]. Moreover, in normal cells, there are also few reports concerning change in cell size or intracellular ATP through cell cycle. Barberis et al. reported the following: RNA and proteins increase exponentially, whereas the DNA content shows a typical double amount with increasing cell size until the cells divide to generate a newborn daughter from G1 phase to M phase [36]. Marcussen et al. indicated that the cellular ATP concentration varies through the cell cycle, reaching a peak at G2/M phase and minimum at late G1/early S

Reaction product	Neutral red	MTT formazan (insoluble, dark blue)	WST-8 formazan (water-soluble, orange)	Resorufin (red)	Light	Hoechst33342: Blue fluorescence CellMaskRed fluorescence
Extraxtion step	Yes (1030 min)	Yes (1030 min)	No	No	No (Simultaneous with reaction)	No
Measuring device	Plate reader	Plate reader	Plate reader	Plate reader	Plate reader	Image analyzer
Detection reagent	Absorbance (reference)	Absorbance (reference)	Absorbance (reference)	Fluorescence (Ex/Em)	Chemi-luminescence	Fluorescence (Ex/Em)
	540 nm	570 (650) nm	450(650) nm	560/590 nm		H33342: 350/461 nmCellMask588/612 nm
Required time (one plate)	5 h	5 h	3 h	3 h	0.5 h	1.5 h
Operability	A little hassle	A little hassle	No hassle	No hassle	No hassle	No hassle
Correlation vs. Automatic cell counter	Good	Good	Good	Good	Good	Very Good

1) There were some chemicals showing abnormal values 2) Correlation is low when chemicals induce cell hypertrophy

con rmed that the result actually judged under the microscope and that of HCIA assay coincided (data not shown), but all rounded cells cannot be evaluated because most of them are detached during the washing process. As for the cell cycle, in order to verify whether HCIA assay can evaluate the cell cycle correctly, cells treated with cell cycle G1 phase inhibitor, rapamycin [46-48]; cell cycle S phase inhibitor, aphidicolin [49]; and G2/M inhibitor, Cytochalasin B [50] were co-stained with Hoechst 33342 and CellMask (Table 2). Rapamycin was con rmed to increase the ratio of G1 phase to 80% at almost all doses, whereas the G1 phase ratio of the control was 70%. Aphidicolin increased the population of G1 phase and S phase, consistent with the report of Costa et al. [51]. Cytochalasin B had a signi cantly higher population of S phase and G2/M phase, similar to the report by Gu et al.

Chemicals	Dose	Cell viability (%) calculated from counting cell number	Area of nuclei (% of cont.)	Area of cell (% of cont.)	Micronucleus appearance (ratio of cont.)	% of round cell	Cell cycle (%)		
							G1	S	G2/M
	μM	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
Control	0.00	100.0	100.0	100.0	1.0	3.5	70.0	21.4	7.8
Rapamycin	0.08	58.8	114.3	139.3	0.8	0.8	83.1	13.8	2.4
	0.16	71.4	112.5	136.6	0.8	1.2	84.8	12.7	1.9
	0.31	67.0	113.7	141.0	1.0	1.1	81.8	15.1	2.6
	0.63	61.2	118.6	144.9	0.9	1.1	83.4	13.5	2.6
	1.25	57.8	116.3	144.0	0.9	1.1	80.3	16.0	3.2
	2.50	58.4	114.9	143.2	1.0	1.3	76.6	18.4	4.4
	5.00	55.0	112.9	133.5	0.7	2.1	72.7	20.4	6.4
	10.00	35.9	92.4	91.1	2.1	10.5	61.4	21.1	16.2
Aphidicolin	0.08	57.3	115.0	142.4	1.4	1.8	76.8	18.5	3.9
	0.16	56.4	123.4	160.5	1.7	1.2	76.1	20.1	3.2
	0.31	47.1	140.2	190.4	3.0	1.2	70.3	25.3	3.5
	0.63	36.1	163.9	237.3	4.5	0.9	67.3	28.4	3.5
	1.25	31.8	194.9	293.9	3.4	0.5	76.1	20.6	2.3
	2.50	30.9	189.5	281.8	1.8	0.4	75.9	20.4	2.7
	5.00	29.4	185.6	280.7	1.6	0.7	80.0	16.8	2.1
	10.00	31.7	178.6	253.9	1.6	1.1	81.7	14.5	2.3
Cytochalasin B	0.08	70.6	100.0	110.4	1.4	1.3	75.7	19.1	4.4
	0.16	87.9	96.3	100.8	1.1	1.6	75.6	19.1	4.6
	0.31	66.8	93.1	92.2	1.5	1.6	77.5	17.0	4.8
	0.63	60.1	109.1	106.5	1.7	2.9	56.6	24.8	17.5
	1.25	36.2	139.1	148.1	2.1	1.5	34.2	30.9	33.1
	2.50	33.8	136.6	160.7	1.9	0.2	29.8	33.8	33.8
	5.00	32.8	124.1	167.2	2.8	0.3	33.8	37.1	27.9
	10.00	31.7	122.8	158.6	2.1	0.4	33.7	39.6	25.7

Table 2: Measurement of biological parameters using HCIA assay in CHL cell treated by representative.

Conclusion

In this study, we showed that cytotoxicity assays, such as NRU, MTT, WST-8, Alamar blue, and ATP, are highly accurate cytotoxicity tests, and the results generated correlate well with those of automatic cell counter when chemicals do not induce cell hypertrophy. However, we also showed that these cytotoxicity assays underestimate

cytotoxicity when chemicals induce cell hypertrophy and suggested that this is caused by an increase in intracellular ATP and NADH content accompanied by an increase in cell and mitochondrial area.

ese cytotoxicity assays utilizing biological indicators, such NRU uptake and NADH and ATP content, are therefore not suitable for assessing cytotoxicity when chemicals possessing cell hypertrophy-inducing potential-like mutagens and carcinogens are measured for cytotoxicity. erefore, we showed that the HCIA assay can directly count the number of cells by image analysis and evaluate cytotoxicity with high accuracy without being in uenced by intracellular ATP or NADH content. Furthermore, since this assay can not only measure the area of cells and nuclei as well as count cell number but also evaluate cell circularity, micronucleus appearance ratio, and cell cycle phase, we can gain further insight from the cytotoxicity results.

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