PERSISTENT CYTOTOXICITY AFTER EXPOSURE TO ANTINEOPLASTIC
AGENTS: THE SUCCINATE DEHYDROGENASE INHIBITION TEST
RE-EXAMINED

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Abstract

The succinate dehydrogenase inhibition (SDI) test and human tumor clonogenic assay (HTCA) have some methodological differences. However, the effects that these differences have on cytotoxicity estimates of antineoplastic agents have not been rigorously examined. To re-examine the SDI test, the AUC (area under the concentration-time curve) values producing 50% inhibition of cisplatin (CDDP), carboplatin (CBDCA) and mitomycin C (MMC) in the conventional SDI test, without a drug-free incubation period after drug-exposure; the modified SDI test, with a drug-free incubation period; and the HTCA using a panel of PC-7, PC-9, PC-14 and K562 cells were compared with that of respective AUC in clinical pharmacokinetics. DNA histograms determined by flow cytometry using propidium iodide were estimated for cells during the drug-free incubation. CDDP, CBDCA and MMC were all confirmed to have AUC dependent cytotoxicity. The $AUC_{50}/AUC_{clin.}$ ratio for MMC as determined by the conventional SDI were significantly higher in all four cell lines than those for CDDP and CBDCA. While, in the modified SDI, the $AUC_{50}/AUC_{clin.}$ values were decreased and in agreement with those obtained by the HTCA. In the DNA histogram after the drug-free incubation, MMC showed the most cytotoxic change, which was a severe decrease in G₁ and G₂ phase and increased fragmented particles indicating apoptosis. Drugs with strong cytotoxicity that persists after exposure, such as MMC, are underestimated with respect to their cytotoxicity when the conventional SDI test without a drug-free incubation is used.

Introduction

The *in vitro* sensitivity tests for antineoplastic agents are classified into clonogenic assays, such as the human tumor clonogenic assay (HTCA) (Hamburger and Salmon, 1987) and nonclonogenic assays, such as the succinate dehydrogenase inhibition (SDI) test (Mosmann, 1983). The HTCA was first developed by Salmon and Hamburger, and

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provides an estimate of colony-forming inhibition by antineoplastic agents (Hamburger and Salmon, 1987). The conventional SDI test was subsequently developed by Mossmann (1983) and is now in wide use. In the conventional SDI test cell death is judged by the decrease of mitochondrial succinate dehydrogenase activity. Both assays are considered methodologically and conceptually established since they have been in use for over 15 years.

However, there are several methodological differences between the HTCA and the SDI test that may affect the estimates of cytotoxicity of certain drugs. For example, the HTCA involves a 14-day drug-free incubation period following drug exposure, while the conventional SDI test does not have a drug-free incubation period (Hamburger and Salmon 1987; Mossmann (1983). The duration of exposure to antineoplastic agents vary greatly, for example in the HTCA protocol cells are exposed to drugs for periods as brief as one hour, while the conventional SDI test employs drug exposure times as long as 72 hours (Hamburger and Salmon 1987; Mossmann (1983). Despite these methodological differences both assays evaluate the cytotoxicity of an antineoplastic agent by the concentration producing 50% inhibition (IC_{50}). The effects that these differences have on estimates of cytotoxicity have not been rigorously examined before.

Evaluation of cytotoxic effects of antineoplastic agents *in vitro* is an old but important theme. The objective of this study was to re-examine the SDI test with respect to the following points: 1) duration of exposure to antineoplastic agents; 2) the drug-free incubation period after drug-exposure, and 3) the dose of antineoplastic agents. We will also discuss the concept of cytotoxicity persisting after drug-exposure, comparable to post-antibiotic effect (PAE) in bacteriology (Eagle, 1949; Eagle, Fleishman *et al.*, 1950).

Material and Methods

Cell lines and chemicals

A panel containing three cell lines of human non-small cell lung carcinoma, PC-7, PC-9 and PC-14, and one human myelogenous leukemia cell line, K562, was used (Hayata and Tsuji, 1975; Lozzio and Lozzio, 1975). Cells were adjusted to a final concentration of 1×10^5 cells/ml in RPMI 1640 + 10% FCS when chemosensitivity test mentioned below was done on each of them. Cisplatin (CDDP), carboplatin (CBDCA)

and mitomycin C (MMC) were dissolved 0.9% NaCl solution, and sterilized by passage through a 0.22 μ m filter just prior to use.

In vitro chemosensitivity tests

SDI test: For the conventional SDI test without a drug-free incubation period after the drug-exposure (Mosmann, 1983), a 135 μ L aliquot cell suspension of each cell line was exposed to 15 μ L of CDDP, CBDCA or MMC, at logarithmically spaced concentrations, for 72 h at 37° C in wells of a 96-well microplate (FALCON 3072). Then, 15 μ L of 3-(4, 5-dimethyl- thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) reagent (5 mg/ml in PBS) and, 4 hours later, 100 μ L of SDS (10% n-dodecylsulfate sodium salt in 0.01N HCl) were added to each well. After 24 hours, the absorbance at 560 nm was determined with a microplate reader, Model 3550 MICROPLATE READER (BIORAD, Tokyo).

The modified SDI test had a drug-free incubation period after the drug-exposure (Kobayashi, Kudoh *et al.*, 1995). Briefly, a 0.9 ml aliquot cell suspension was exposed to 0.1 ml of CDDP, CBDCA or MMC for 1~72 h at 37° C. After washing the cells twice, they were resuspended in 1 ml of the medium. Aliquots of each cell suspension (150 μ L) were placed in a 96-well microplate and subjected to a drug-free incubation period of 72 hours at 37° C. MTT and SDS were added and the absorbance of each well was measured as described above.

HTCA

A 2.7 ml cell suspension was added to 0.3 ml of each drug and incubated for 2 hours. The cells were washed twice, resuspended in 2.7 ml of the medium, to which 0.3 ml of 3% noble agar had been added. To obtain a double-layer, soft agar medium, a feeder layer consisting of 1 ml of McCoy's 5A with 0.6% noble agar, 10% FCS and 5% horse serum albumin was overlaid with 1 ml of the cell suspension in 35-mm culture dishes. After incubation at 37° C in an atmosphere of 5% CO₂ for 14 days, colonies were counted with a colony counter, Colony Analyzer CA-7 (Oriental Instruments Ltd., Tokyo).

AUC producing 50 % inhibition

The area under the concentration-time curve values that produce 50 % inhibition (AUC₅₀) were estimated for the SDI test, the modified SDI and the HTCA. To calculate AUC₅₀, the concentrations of each drug in RPMI 1640 + 10% FCS were measured at

appropriate time points (0, 1, 2, 3, 6, 12, 24, 48 and 72 h). For CDDP and CBDCA, the concentration of non-protein bound platinum in the exposure medium was estimated. Specifically, after the drug solutions were subjected to ultrafiltration by centrifugation with an Amicon Centrifree 4104 (Amicon Corporation, U.S.A.) at $1980 \times g$ for 20 min, the concentration of non-protein bound platinum in the medium was measured by atomic absorption spectrophotometry. For MMC, the concentration of total MMC in the medium was measured by HPLC (high performance liquid chromatography). The AUC_{50} was calculated according to the trapezoidal rule.

Flow cytometry

The cytotoxic effects of the drug-free incubation period after drug-exposure was investigated by a flow cytometry using propidium iodide (PI) (Sigma Chemical Co., St. Louis, MO) after 9.5 ml cell suspensions had been exposed to 0.5 ml of CDDP, CBDCA or MMC for 72 hours at IC_{25} , the concentration producing 25% inhibition, by the conventional SDI test. The cells were washed twice to remove the drugs and resuspended in the fresh medium. The cells were incubated in the drug-free medium for 0, 24, 48, or 72 h. Samples were washed twice in phosphate-buffered saline (PBS) containing 0.5% Tween 20 by centrifugation and fixed with 70% cold ethanol for 24 h. The cells were washed twice and incubated in 1 ml of PBS containing 150 units of ribonuclease A (Sigma Chemical Co.) per milliliter of PBS for 20 min at $37^{\circ}C$. After centrifugation, the cells were suspended in 1 ml of the PBS containing Tween 20 and $20 \mu g/ml$ PI for 15 min. Flow cytometry was done with an EPICS C apparatus (Coulter Electronics, Inc., Hialeah, FL). Blue light (485 nm) from a single Inova 90 argon ion laser was used to make PI fluoresce, and the DNA content (red fluorescence from PI, 580 nm) was measured.

Statistical analysis

To estimate differences in AUC_{50} among the three methods, and the AUC_{50} per the AUC in clinical pharmacokinetics ($AUC_{50} / AUC_{clinc.}$) among the three drugs, one way factorial ANOVA and Fisher's probability least significant difference (PLSD) were used. Results are presented as means $\pm$ standard error unless otherwise noted, and a $p < 0.05$ was considered statistically significant. All analyses were performed using StatView-J4.02 and SuperANOVA.

Results

AUC producing 50 % inhibition in chemosensitivity tests

The log-log relationships between IC_{50} and exposure time for CDDP, CBDCA and MMC, as determined by the modified SDI test produced straight lines with slopes of approximately -1, indicating that the AUC required for 50% growth inhibition (AUC_{50}) was constant (Figure 1). We confirmed that CDDP, CBDCA and MMC were AUC-dependent drugs, and that AUC of their active forms, i.e., the non-protein bound platinum form (free Pt) for CDDP and CBDCA and total MMC, which binds protein to a negligible extent, were valid as doses to estimate their cytotoxic effects.

Table I present the AUC_{50} values determined for CDDP, CBDCA and MMC by the conventional SDI test, the modified SDI test and the HTCA. The AUC_{50} was highest for each drug using the conventional SDI test, followed by the modified SDI test, while the values determined by the HTCA were the lowest, the differences were statistically significant ($p < 0.01$). All three tests showed that K562 cells were most sensitive to each drug, while PC-7 cells were relatively insensitive. These data suggest that raw AUC_{50} values could be compared among cell lines for each test.

However, it is difficult to understand the results by simply comparing raw values of AUC_{50} among drugs because clinically relevant doses vary for each drug. It is necessary to calculate the AUC_{50} per the AUC in clinical pharmacokinetics ($AUC_{50} / AUC_{clin.}$). Reported AUC_{clin} values, based on non-protein bound platinum, for 30-min drip infusions of 80 mg/m^2 CDDP or 450 mg/m^2 CBDCA were $3.47 \mu\text{g/h per ml}$ or $57.4 \mu\text{g/h per ml}$, respectively (Sasaki, Tamura *et al.*, 1989). The $AUC_{clin.}$ of MMC was reported to be $1.03 \mu\text{g/h per ml}$ when 20 mg/m^2 MMC was given as an i.v. push injection (Den Hartigh, McVie *et al.*, 1983). From these data, the ratios of $AUC_{50}/AUC_{clin.}$ were calculated and the comparison of the ratios among the drugs in each test was shown in Figure 2. With the conventional SDI test, the $AUC_{50}/AUC_{clin.}$ ratios for MMC were significantly higher in all four cell lines in comparison to the other two drugs, indicating that MMC is less cytotoxic than CDDP and CBDCA. On the other hand, the modified SDI and the HTCA tests did not indicate any differences in cytotoxicity among the three drugs in PC-9, PC-14 and K562 cell lines. In PC-7 cells, MMC was judged to be the most cytotoxic agent by both the modified SDI and HTCA. In terms of the platinum compounds of CDDP and

Figure 1. The log-log relationships between IC₅₀ and exposure time for CDDP (a), CBDCA (b) and MMC (c) as determined by the modified SDI test: In the modified SDI test, 15 μ L of CDDP, CBDCA or MMC was exposed to a 135 μ L aliquot cell suspension (1×10^5 cells/ml PC-14 cells) for 1, 2, 4, 8, 24, 48 or 72 hours. The drug-free incubation after the drug-exposure was performed for 72 hours, and MTT and SDS were added and the absorbance was measured according to material and methods. The means of experiments performed in triplicate and the best fitting lines with a slope of -1 are shown.

Figure 2. The AUC_{50} per the AUC in clinical pharmacokinetics (AUC_{50} / AUC_{clin}): The ratios of AUC_{50} to AUC_{clin} in the conventional SDI test, the modified SDI test and HTCA were calculated (see the legend in Table I).

Figure 3. DNA histograms before and after the drug-free incubation for 72 h: After exposing CDDP, CBDCA or MMC for 72 h, DNA histograms before and after the drug-free incubation for 72 h were determined by flow cytometry using propidium iodide (PI). The DNA histograms of PC-14 before [(a)control, (b)CDDP, (c)CBDCA, (d)MMC] and after a drug-free incubation [(e)control, (f)CDDP, (g)CBDCA, (h)MMC] are shown.

TABLE I

cell line	cytotoxicity test	AUC $\mu\text{g/h per ml}$		
		CDDP	CBDCA	MMC
PC-7	SDI ₁	196 $\pm$ 9	4571 $\pm$ 269	106 $\pm$ 4
	SDI ₂	127 $\pm$ 8	2172 $\pm$ 217	20.9 $\pm$ 2.2
	HTCA	7.93 $\pm$ 1.48	173 $\pm$ 16	1.33 $\pm$ 0.12
PC-9	SDI ₁	44.2 $\pm$ 2.5	1135 $\pm$ 23	45.3 $\pm$ 3.2
	SDI ₂	16.1 $\pm$ 1.6	345 $\pm$ 43	4.80 $\pm$ 0.97
	HTCA	2.75 $\pm$ 0.47	36.7 $\pm$ 5.3	1.40 $\pm$ 0.37
PC-14	SDI ₁	63.9 $\pm$ 5.6	1706 $\pm$ 88	60.1 $\pm$ 5.4
	SDI ₂	34.6 $\pm$ 3.8	442 $\pm$ 30	7.46 $\pm$ 0.99
	HTCA	2.63 $\pm$ 0.48	45.0 $\pm$ 11.1	0.822 $\pm$ 0.140
K562	SDI ₁	24.8 $\pm$ 1.6	442 $\pm$ 56	16.8 $\pm$ 0.8
	SDI ₂	11.6 $\pm$ 0.7	169 $\pm$ 9	3.86 $\pm$ 0.52
	HTCA	0.420 $\pm$ 0.116	10.4 $\pm$ 1.0	0.210 $\pm$ 0.054

The AUC values required to produce inhibition equivalent to 50% of the control (AUC₅₀): The conventional SDI test without drug-free incubation after drug-exposure, the modified SDI test by drug-exposure for 72 hours with drug-free incubation and HTCA were performed to estimate AUC₅₀ for CDDP, CBDCA, and MMC (see material and methods). Experiments of both SDI tests were performed 8 times, while those of the HTCA were repeated 6 times. The values were presented as mean $\pm$ standard error.

CBDCA, the conventional SDI test using PC-7, PC-9 and PC-14 indicated CBDCA was less cytotoxic than CDDP. While, both the modified SDI and HTCA did not show any difference in the cytotoxicity between CDDP and CBDCA (Figure 2).

Effect of the drug-free incubation after drug-exposure

Figure 3 presents changes in DNA histograms of PC-14 cells, as determined by flow cytometry with PI, with a drug-free incubation period following exposure to 0.20 μ g/ml CDDP, 3.0 μ g/ml CBDCA and 0.10 μ g/ml MMC. These concentrations produced a 25% inhibition as determined by the conventional SDI test. Immediately after CDDP exposure, an accumulation of G₂M phase cells and a decrease in G₁ phase cells was observed. After a drug-free incubation for 72 h, this phenomenon was less marked and G₁ cells were increased. In contrast, an accumulation of cells in the G₂M phase and a more severe decrease in G₁ phase cells were seen immediately after MMC exposure. After a drug-free incubation for 72 h, no increase in G₁ cells and the increase of fragmented particles (< G₁ phase) indicating apoptosis were observed. This showed no restart of the cell cycle and cell death. In cell cultures exposed to CBDCA, the peak number of G₁ cells was less than that in the CDDP-exposed cultures after a drug-free incubation for 72 h.

Discussion

We found that the antineoplastic agents, CBDCA, CDDP and MMC, had cytotoxicity that persisted after drug exposure, and there were differences in the level of cytotoxicity among the drugs (Table I, Figure 2). Namely, MMC had the strongest cytotoxic effect during the drug-free incubation. The effects of platinum compounds were less marked. Of the two platinum compounds, the cytotoxicity of CBDCA was greater than that of CDDP. The DNA histograms in this estimation gave information to explain the mechanism of the cytotoxicity that persists after drug-exposure (Figure 3). Our data suggests that the conventional SDI test without a drug-free incubation period cannot estimate all dying cells. These results show the importance of determining cytotoxicity that persists after drug-exposure, which varies among anticancer drugs.

The Post-antibiotic Effect (PAE) is a bacteriological concept proposed by Eagle in 1949 (Eagle, 1949; Eagle, Fleishman *et al.*, 1950) and confirmed by other researchers (Beggs and Jeilise, 1969; Bundtzen, Gerber *et al.*, 1981; Craig and Gudmundsson, 1986;

Vogelman, Gudmundsson *et al.*, 1988). Growth was observed to be absent in cultures of gram positive bacteria for a few hours after exposure to penicillin G. PAE is the suppression of bacterial growth, which persists for some duration after limited exposure to antimicrobial agents. Differences in the magnitude of PAE among antimicrobial agents also have been recognized. We propose that the similar concept should be considered for antineoplastic agents.

Anticancer drugs have traditionally been classified as either concentration-dependent or time-dependent drugs. The studies of Ozawa, Sugiyama *et al.* (1988) clarified that the former are AUC dependent drugs. In other words, the cytotoxicity of a drug belonging to the former group is regulated not by concentration but by the AUC of the active form. From Figure 1, CDDP, CBDCA and MMC were shown to be AUC-dependent drugs, and it indicated that the AUC of their active form was valid as a drug dose. This graph also indicates that the modified SDI test can recognize the AUC-dependent cytotoxicity of the drugs, and that, in case of estimating AUC-dependent drugs, any drug exposure duration will do.

Figure 2 illustrates that the values of AUC_{50} obtained with the conventional SDI were found to be at least 7-fold higher than $AUC_{clin.}$, and were thus remarkably higher than $AUC_{clin.}$ values. The $AUC_{50}/AUC_{clin.}$ ratios for MMC by the conventional SDI test were significantly higher in all the four cell lines than those of the other two drugs. As mentioned above, drugs with strong cytotoxicity that persists after its exposure, such as MMC, may be underestimated when using the conventional SDI test without a drug-free incubation period. Figure 2 show, with the drug-free incubation, the values of AUC_{50} were decreased, and the modified SDI test yielded results indicating that MMC was as cytotoxic to PC-9, PC-14 and K562 cells as CDDP and CBDCA and had an even stronger effect on PC-7. These results were in agreement with those obtained by HTCA.

In conclusion, the conventional SDI test without a drug-free incubation is not adequate for fundamental studies to estimate the cytotoxicity of antineoplastic agents because it does not allow the determination of all of the cytotoxic effects of the drugs. The SDI test needs a drug-free incubation period to estimate the cytotoxicity that persists after the drug is removed.

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