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Optimal conditions of chemotherapeutic sensitivity in K562 cell line using tetrazolium dye assay

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ABSTRACT Ten antineoplastic agents were used on K562 cell lines with the tetrazolium salts (MTT) assay to investigate optimal conditions of the chemotherepeutic sensitivity. The doubling time of K562 cell was 20.4 h in 96-well microtitre plates. The optical density in MTT assay of the no-drug controls was 0.77 ($n=45$). A 70% reduction (IC_{70}) in absorbance compared with control values using an ELISA reader at 570 nm was referred to as drug sensitive and < 70% (IC_{70}) was referred to as resistant. The inhibitory effect of the theoretical maximal plasma drug concentration (C_{max}) in plasma was compared with that of 10% C_{max} drug concentration. At 10% C_{max} after a 2-d drug exposure to daunorubicin and homoharringtonine, or after 5 d to epirubicin, 5-fluorouracil, cytarabine hydrochloride, and doxorubicin, significant cytotoxicities were shown. There were little time-dependent effects after 2-5 d drug exposure for 4 out of the 10 tested drugs, and mild or marked time- and dose-dependent effect for others.

KEY WORDS antitumor drug screening assays; antineoplastic agents; cultured tumor cells; tetrazolium salts

A number of *in vitro* tests have been tried to predict the responses of human tumors to chemotherapeutic agents, such as clonogenic assay, dye exclusion assay, and radioactive precursor incorporation. However, practical limitations have restricted their applications in routine clinical practices[1-3].

A tetrazolium dye (MTT = 3-4, 5-dimethyl-thiazol-2, 5-diphenyl tetrazolium bromide) assay[4] was based on a reductant reaction of MTT to formazan caused by living cells. The reduced formazan can then be measured with a microplate spectrophotometer. This method has been successfully applied to a new anticancer drug screening program in various incubation cell lines[5-8]. In order to search the optimal conditions, we studied the appropriate test dose for 10 drugs, the exposure times, and the endpoints for laboratory assays.

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MATERIALS AND METHODS

The K562 cell line was provided by Department of Immunology, Kiel University, Germany, and cultured under conventional conditions: 37℃, 5% CO_2 + 95% air, 100% relative humidity, in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, penicillin 100 IU·ml^{-1} and streptomycin 100 μg·ml^{-1}. 5-Fluorouracil (5-FU, Hai-pu Pharmaceutical Factory, Shanghai, China), Fluoro-deoxyuridine (F-Deo, Hai-men Pharmaceutical Factory, Zhejiang, China), cytarabine hydrochloride (Cyt, Experimental Pharmaceutical Factory of Beijing Medical University, China), vincristine (Vin, Hangzhou Min-sheng Pharmaceutical Factory, China), homoharringtonine (Hom, Hangzhou Min-sheng Pharmaceutical Factory, China), methotrexate (Met, Ebewe Australia), mitomycin C (Mit, Kyowa Hakko Kogyo Co, Tokyo, Japan), doxorubicin (Dox, Farmitalia, Italy), epirubicin (Epi, Farmitalia), and daunorubicin (Dau, Farmitalia). Stock solutions of drugs were prepared in Hank's solution. Drug concentrations included a 4-fold difference in magnitude. Five or six points were selected, which included the maximal concentration attained in the blood, assuming the total daily dose to be distributed only in the plasma[9]. We purchased MTT from

Fluka Chimie AG and RPMI 1640 from Kyowa Hakko Kogyo Co. Japan. The other reagents were all of AR.

MTT assay The original MTT assay[4] was modified. K562 cell line were seeded in 96 well microtitre plates in 135 μl / well with 8×10^4—1.2×10^5 cells. Fifteen μl of various drug stock solutions were added to each of 5 replicate wells, and 8 replicate wells without drug served as control. The plates were incubated for 1, 2, or 5 d. After the incubation, 50 μl fo Hank's solution with MTT 1 mg · ml^{-1} were added to each well and the plate reincubated for further 4 h. The plate was then inverted on blotting papers to remove the medium. The formazan crystals formed were dissolved in 100 μl of acid–isopropanol (HCl 0.04 mol / L in isopropanol) and shaked for 10 min. The plates were read on a DG 3022 ELISA reader at 570 nm. A 70% reduction (IC_{70}) in absorbance compared with control values was defined as the drug sensitive and $< IC_{70}$ was interpreted as resistance.

RESULTS

The K562 cell doubling time was 20.4 h in 96 well microtitre plates. In all of 45 panels of the control wells the mean optical density was 0.77± *s* 0.16. The different concentrations for each drug showed 3 phases: a flat slope phase; a slope phase; and a plateau phase (Fig 1). According to the differences of the inhibitory range, 3 general forms exist: (a) After a 2– or 5–d exposure for Dau and Hom a positive concentration correlation was shown in the range of 10–90% inhibition; (b) For Met the positive correlation was shown in the range of 10–60% inhibition. Although the concentration of Met was increased 100–folds to 0.1 mg · ml^{-1}, there was no further increase in inhibition; (c) The other drugs showed that with increased concentration and time the inhibition also generally increased (Tab 1). Among 2 or 5 d with Hom, Met, F–Deo, and Dau, there were no significant differences as the time increased. For other

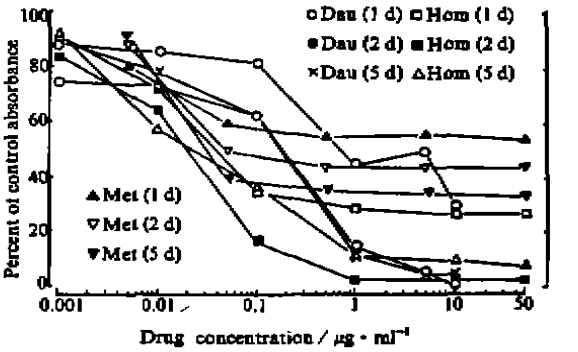

Fig 1. Drug concentrations and its inhibitery effects to K562 cell line with daunorubicin, homoharringtonine, and methetrexate after 1–, 2–, or 5–d drug exposure.

drugs added for a longer period and of higher concentrations, there were mild or marked time– and concentration–dependent relationships. After a 1–d drug exposure, no drug showed sensitive and after a 2–d exposure to Dau, Hom, or after a 5–d to Epi, 5–FU, Cyt, and Dox at 10% C_{max}, the IC_{70} sensitivity level was attained (Tab 1).

The dilution folds form the C_{max} to the concentration at which the drug effect was equivalent to IC_{30} were also presented (Tab 1). Met, Dau, Hom, and Dox showed wide ranges of effects.

DISCUSSION

This paper reports the application of MTT assay to study on the inhibitory effect of 10 chemotherapeutic drugs. In the comparative studies of 1–, 2–, or 5–d drug exposure, positive correlation between the drug effect and concentrations, as well as between drug effects and the length of the exposure time was existed within a certain range. Exception was found in little time–dependent effect during the 2–5 day continuous exposure for Hom, Met, F–Deo and Dau. The results indicate that a 2–d exposure and 10% C_{max} are fit for these 4 drugs and for rest of other 6 drugs the present method of the drug exposure time or concentrations need to be adjusted, including a relevent way to express the results

Tab 1. Inhibitory effects of 10 antineoplastic drugs on K562 cell line.

Drug	Time / d	Exposure C_{max} / $\mu g \cdot ml^{-1}$	Exposure Inhibiting rate / %	At one-tenth of the maximal concentration (C_{max}) Inhibiting rate / %	At one-tenth of the maximal concentration (C_{max}) At IC_{70} / $\mu g \cdot ml^{-1}$	At one-tenth of the maximal concentration (C_{max}) At IC_{30} / $\mu g \cdot ml^{-1}$	At one-tenth of the maximal concentration (C_{max}) Dilution folds till IC_{30}
Homoharringtonine	1	1.6	72	66	0.5	0.011	7
	2		99	87	0.05	0.005	8
	5		90	68	0.18	0.004	8
Methotrexate	1	20	47	44	(50)*	0.015	10
	2		57	57	(50)*	0.014	10
	5		60	60	(50)*	0.012	10
Fluoro deoxyuridine	1	200	48	34	(5000)*	9	4
	2		72	47	66	10	4
	5		73	47	80	9.5	4
Daunorubicin	1	12	78	51	9.8	0.2	6
	2		100	87	4.5	0.018	9
	5		96	88	4.7	0.03	8
Cytarabine hydrochloride	1	40	27	27	(500)*	(500)	
	2		60	48	(500)*	0.1	7
	5		92	77	2.2	0.1	7
Mitomycin C	1	3.2	32	24	(50)*	0.7	2
	2		64	34	4.5	0.22	4
	5		83	47	1.4	0.10	5
Vincristine	1	0.8	36	31	(10)*	0.07	4
	2		51	34	(10)*	0.06	4
	5		88	59	0.18	0.018	6
5-Fluorouracil	1	400	37	25	(500)*	100	2
	2		57	29	(500)*	50	3
	5		96	74	33	5.4	6
Doxorubicin	1	64	55	28	(20)*	1.1	6
	2		69	42	(20)*	0.19	8
	5		96	70	0.63	0.09	9
Epirubicin	1	16	46	29	(10)*	1.80	3
	2		64	61	(10)*	0.37	5
	5		98	93	0.54	0.07	7

* The highest concentration was less than the inhibiting concentration.

need to be designed for each individual drug.

As yet a well-accepted standard MTT method, using in clinical antitumor drug prediction, has not been confirmed. According to the anti-drug screening program IC_{50} was the bonderline of the sensitivity, in this study IC_{70} was adopted as the threshold of sensitivity after a 2-d drug exposure and at 10% C_{max}, this is consistent with that suggested by Bired *et al*[7]. Under this level Hom, Dau were up to standared.

In this experiment, the double dilutionfolds from C_{max} to the concentration at which the drug effect was equivalant to IC_{30} may be predictive of the effective active time of the individual drug *in vivo*, although their effective rates are different.

Since hematological malignancies have fairly effective drugs and the response is easier to be evaluated, we have started to study the clinical drug sensitivity predictions of acute leukemia patients, and intend to make some special designs to meet the characteristics of the drugs and the clinical samples. It is likely that patients will benefit from the further foundmental research and detailed understanding of the drug sensitivity test and from applying it to clinical practice.

137-140 (11)

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四氮唑盐法测定 K562 细胞化疗敏感的最适条件

R979.1

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摘要 MTT 法用于 10 种抗癌药物对 K562 细胞的体外微培养药敏试验，其中 Hom，Met，F-Deo 和 Dau 自 2 d 起对 K562 细胞的抑制作用稳定，其它药物随着作用的延长抑制作用相应增强。在按临床用药所达体内药物理论最高浓度的 10%药浓度时，K562 细胞与 Dau，Hom 接触 2 d，与 Epi，5-FU，Cyt，Dox 则需接触 5 d 获 70%以上抑瘤。

关键词 抗肿瘤药物筛选试验；抗肿瘤药物；培养的肿瘤细胞；四氮唑盐

K562细胞