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Restoration of intracellular drug accumulation in MDR cell line K562r does not mean reversal of its drug resistance[1]

HU Xun, CHEN Wan-Yuan
(Cancer Institute, Zhejiang Medical University, Hangzhou 310009, China)

ABSTRACT Our purpose was to test whether drug sensitivity and drug accumulation in MDR cell erythroleukemic K562r could be restored by incubating cells with 3 anthracycline antibiotics in combination. Drug sensitivities of cells were assessed with MTT assay, in which doxorubicin, epirubicin, daunorubicin, or the 3-drug mixture was applied with concentrations ranging from 1 to 3125 $ng \cdot ml^{-1}$. The IC_{50} of K562r cells were 1.0, 1.0, 0.1, and 0.2 $\mu g \cdot ml^{-1}$, respectively, about 22, 16, 10, and 20 times higher than those of K562 cells. After cells were exposed to doxorubicin (2—32 $\mu g \cdot ml^{-1}$) for 1 h, the drug concentrations in K562r cells were all higher than those in K562 cells. Similar results were obtained for epirubicin or daunorubicin. After 1-h incubation of cells with the 3-drug mixture (3 to 192 $\mu g \cdot ml^{-1}$), there were no considerable differences of drug accumulation between K562r and K562 cells with only 3 exceptions in 21 groups. It is concluded that restoration of intracellular drug accumulation in MDR cell line K562r was not correlated with reversal of its drug resistances.

KEY WORDS doxorubicin; epirubicin; daunorubicin; drug combinations; cultured tumor cells; drug resistance.

Chemotherapy is always hampered by drug resistance[1]. Hyperexpression of the P-glycoprotein in cancer cells, which mediated the energy-dependent drug efflux of intracellular drugs, was generally considered as a major mechanism of drug resistance[2]. Though most of the typical MDR cancer cell lines established *in vitro* exhibited a decrease of intracellular drug accumulation and an increase of intracellular drug retention, the magnitude of reduced drug uptake was obviously not enough to account for the drug resistance acquired by the drug-resistant cells[3]. The ratios of doxorubicin between nucleus and cytoplasm in drug-resistant cells were 1/2 to 1/3 folds less than those in their parent drug-sensitive cells[4]. The ratios of DNA-bound doxorubicin to free doxorubicin in drug-resistant cancer cells were one order lower than those in their drug-sensitive cells[5]. The drug sensitivity of typical MDR cell lines can be partially or completely restored in the presence of verapamil or other MDR reversal agents[6]. Verapamil behaved as a competitive agent and thus blocked the active extrusion of intracellular drugs by P-glycoprotein[7]. Based on this assumption, we assumed that concomitant incubation of MDR cells with anthracyclines in combination would resume their intracellular drug accumulation because anthracyclines are structurally similar (Fig 1) and could act as competitive inhibitors to P-glycoprotein with each other and, thus, resume the drug sensitivity. In previous study, a typical MDR cell variant K562r was established and showed cross resistance to anthracyclines, Vinca alkaloids, MMC etc[8,9]. This study was to test whether intracellular drug accumulation and

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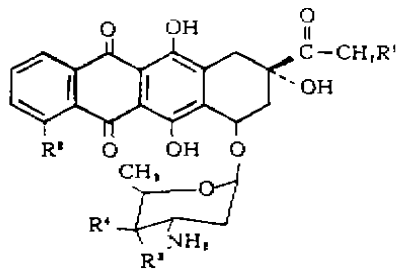
drug sensitivities of MDR cell line K562r could be recovered by incubation of cells with 3 anthracyclines in equimolar combination.



	R^1	R^2	R^3	R^4
Doxorubicin	OH	OCH_3	OH	H
Epirubicin	OH	OCH_3	H	OH
Daunorubicin	H	OCH_3	OH	H

Fig 1. Doxorubicin, epirubicin, and daunorubicin.

MATERIALS AND METHODS

Drugs and chemicals Doxorubicin (Dox) (Kyowa). Epirubicin (Epi) and daunorubicin (Dau) (Farmitalia Carro Erba). RPMI-1640 (Sigma). 3-(4,5-dimethylthlazolyl)-2, 5-diphenyl tetrazolium (MTT) (Serva) and trifluoroacetic acid (TFA, HPLC grade) Acetonitrile (ACN, HPLC grade) (Merck).

Cell line The human erythroleukemic cell line, K562, was grown as cell suspension in RPMI-1640 supplemented with 10 % foetal calf serum (Institute of Hematology, Chinese Academy of Medical Sciences), penicillin 100 IU·ml^{-1} and streptomycin 100 μg·ml^{-1}. The drug-resistant subline, K562r, was derived from its parent cell line, K562, by exposure of the logarithmically growing cells to vincristine 20 μg·ml^{-1} for 2 h and were collected by centrifugation and resuspended in fresh drug-free complete RPMI-1640 medium. This treatment with vincristine were recycled for 20 times until the resistances to Vinca alkaloids and anthracyclines were gradually acquired. The cells were diluted to 1 cell·ml^{-1} of the growth medium, and 0.2 ml of medium was distributed to each of 96 wells in a microtest plate. After 1 wk, wells containing a single colony were marked. The cloned cells were amplified and stored in liquid nitrogen until use. There were no significant differences of cell size and doubling time between K562r and K562 cells.

MTT assay[10] The cytotoxic effects of Dox, Epi, and Dau were determined by seeding logarithmically growing cells (2×10^4 cells/well) into a 96-well plate. As a single drug was applied, drug concentrations were a series of 5-fold increment started from 0.001 μg·ml^{-1} to 3.125 μg·ml^{-1}. While 3-drug mixture was applied, total drug concentrations were with 3 drugs in equimolar proportions. The cells were incubated in a humidified and CO_2-enriched incubator. After a 72-h incubation, each well was added with 50 μl of MTT (1 mg·ml^{-1}). Four hours later, 100 μl of acidified isopropanol were added into each well until the formed crystals were completely dissolved. The plate was then read at λ 570 nm with an ELISA reader. The inhibitory concentration which caused 50% reduction of the absorbance relative to untreated cells was defined as IC_{50}.

HPLC A Shimadzu LC-6A HPLC equipment was used. All separations were performed on a Lichrosorb RP-18 column (10 μm, 250 mm × 4.6 mm). Solvent A was 0.1% TFA and 10 % ACN. Solvent B was 0.1 % TFA and 90 % ACN. The gradient used for HPLC analysis started at 20 % of solvent B and programmed linearly to 50 %. Dox, Epi, and Dau were monitored at λ 234 nm and eluted out at 11.4 min, 12.5 min, and 15.4 min, respectively (Fig 2). The analytical variables of this performance was listed in Tab 1.

Tab 1. Analytical variables of HPLC on analysis of doxorubicin, epirubicin, and daunorubicin.

	Linear range (ng·ml^{-1})	γ	CV %	Analytical recovery/%
Doxorubicin	10—1 000	0.9989	6.5	95.6—104
Epirubicin	10—1 000	0.9991	5.7	96—99
Daunorubicin	10—1 000	0.9981	7.8	96—106

Anthracycline accumulation Cells were incubated with a series concentrations of Dox, Epi, and Dau alone or combined. As for loading of cells with a single drug, drug concentrations were a series of 2-fold increment started from 1 μg·ml^{-1} to 64 μg·ml^{-1}. As to

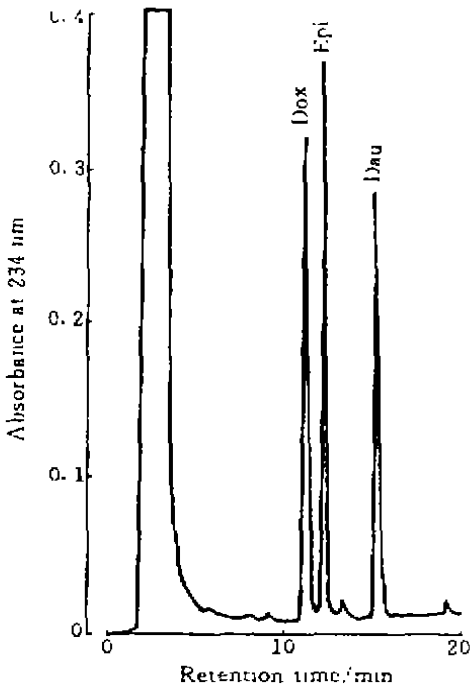


Fig 2. Chromatogram of mixture of doxorubicin, epirubicin, and daunorubicin (1.67 μg each).

the 3-drug mixture, total drug concentrations were a series of 2-fold increment started from 3 $\mu g \cdot ml^{-1}$ to 192 $\mu g \cdot ml^{-1}$ with 3 drugs in equimolar proportions. After 1-h incubation at 37 ℃, cells were centrifuged (6000×g, 10 min). Cell pellet was collected and washed with ice-cold PBS twice. Intracellular anthracyclines were extracted as described previously[11], and determined by HPLC.

Statistics Unpaired t test was used.

RESULTS

Drug accumulation When cells exposed to Dox, Epi, or Dau separately, intracellular drug accumulation was reduced remarkably in K562r cells *vs* that in K562 cells. However, when cells were incubated with the 3-drug mixture, drug uptake by K562r cells was nearly the same as that by K562, cells with only 3 exceptions in 21 groups. These results indicated that concomitant incubation of MDR cells with multiple drugs could resume their normal drug uptake (Fig 3).

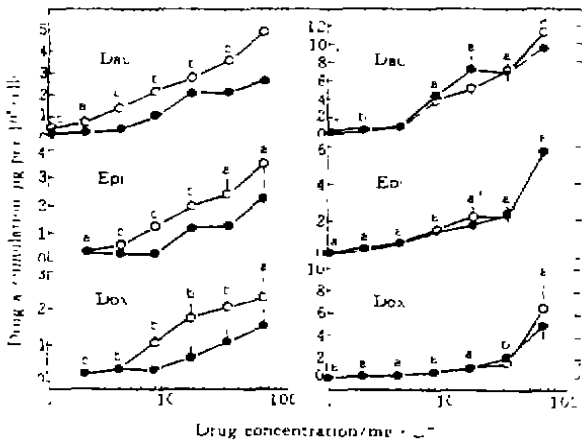


Fig 3. Intracellular drug accumulation in K562 and K562r cells. Left column, incubation with Dox, Epi, and Dau separately. Right column, incubation with Dox, Epi, and Dau concomitantly. ○ K562 cells. ●, K562r cells. $n=3$ determinations. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$, statistical significance between K562 (○) and K562r (●).

Drug resistance The RF (resistance factor, defined as IC_{50} (K562r)/IC_{50} (K562)), values of K562r to these drugs were in the order of Dox > Epi > Dau, when K562r cells were exposed to each of them separately. When the cells were exposed to the 3-drug mixture, the RF value was 20, close to the average value of either drug applied separately (Tab 2). These results indicated that the

Tab 2. Drug sensitivities of K562 and K562r cells.

	IC_{50}/mg·L^{-1} K562r	K562	RF
Doxorubicin	1.0	0.045	22
Epirubicin	1.0	0.06	16
Daunorubicin	0.1	0.01	10
Mixture	0.2	0.01	20

drug resistance of K562r remained nearly the same despite of the restoration of intracellular drug accumulation.

DISCUSSION

The cellular pharmacological characteristics of K562r was in favor of existence of multifactorial mechanisms. Though incubation of K562r cells with Dox, Epi or Dau separately resulted in a remarkably lower intracellular drug intake compared with that of K562, incubation of cells with the 3-drug mixture did not give rise to substantial differences in the drug uptake between K562 and K562r cells. This finding was in our expectation, because Dox, Epi and Dau are structurally similar and could all bind with P-glycoprotein and thus are competitive inhibitors mutually similar to that in the case of verapamil. The crucial point was that despite restoration of the drug accumulation in k562r cells, drug resistances of the cells still kept the same. These results indicated that restoration of drug accumulation in K562r cells was not related to reversal of their drug resistances. As an alteration of intra-nuclear DNA topoisomerase II simultaneously with the amplification and over-expression of mdr1 gene was detected (unpublished data), K562r cell might be a type of "multifactorial"-mediated other than "pure" P-glycoprotein-mediated MDR cell. Similar data with different approaches demonstrating that increment of P-glycoprotein production or reduction of drug accumulation was only associated with the initial occurrence of drug resistance, but not correlated with the degree of drug resistances were reported recently[12]. These findings suggested that reduction of intracellular drug accumulation due to "pure" P-glycoprotein mechanism appeared to be far inferior to explain even classical MDR.

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MDR 细胞株 K562r 细胞内药物积聚的恢复并不代表其抗药性的逆转

胡 迅，陈万源
（浙江医科大学，肿瘤研究所，杭州310009，中国）

摘要 本文探讨细胞内药物积聚与细胞抗药性的相关性. MTT 法揭示 K562r 细胞对 Dox、Epi、Dau 及此三药混合物的 IC_{50}分别比 K562 细胞提高22、16、10及20倍. K562r 细胞分别与 Dox、Epi 或 Dau (2—32 $\mu g \cdot ml^{-1}$)温育1 h，其细胞内药物含量显著低于 K562细胞. 但细胞与三药混合物温育1 h，二者细胞内药物积聚除3个例外均无显著差异，提示 K562r 细胞内药物积聚与其药敏无相关性.

关键词 阿霉素；表柔比星；柔红霉素；合并用药；肿瘤细胞培养；抗药性

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Protein c-*fos* induction in rat brain and spinal dorsal horn by sincalide and opioids *in vitro*

CHEN Su-Zhen[1], HAN Ji-Sheng
(*Neuroscience Research Center, Beijing Medical University, Beijing 100083, China*)

ABSTRACT Sincalide (CCK-8) is an antiopioid substance. In this study, we investigated the effects of sincalide and opioids on c-*fos* expression and their interaction in brain and spinal dorsal horn *in vitro*. Immunoprecipitation was used for detection of c-*fos* protein. The results indicated that 0.1 $\mu mol \cdot L^{-1}$ sincalide induced c-*fos* expression markedly in both brain (a 3.8-fold increase in c-*fos* protein level) and in spinal dorsal horn (a 3.6-fold increase). NDAP (a κ receptor agonist) 0.1 $\mu mol \cdot L^{-1}$ showed some activating effects on c-*fos* expression, the c-*fos* protein level increased 2.7 and 2.6 times respectively in brain and spinal dorsal horn. Ohmefentanyl (Ohm, a μ receptor agonist) 0.1 $\mu mol \cdot L^{-1}$ also exhibited an inducing effect on c-*fos* protein production. Sincalide and NDAP exerted some additive effects on c-*fos* protein production in spinal cord. In contrast, the effect of sincalide on c-*fos* protein production is antagonistic to that of Ohm. The results suggested that there were changing patterns of interaction on c-*fos* expression between sincalide and opioids.

KEY WORDS proto-oncogene proteins c-*fos*; sincalide; narcotics; brain; spinal cord; precipitin tests; autoradiography.

Sincalide (CCK-8) showed antiopioid activities in pain modulation[1], food intake[2], and brain dopamine turnover rate[3]. Sincalide could modify κ and μ opioid receptors[4], and reverse the inhibitory effect of opioids on synaptosomal Ca^{2+} intake[5]. Nuclear proto-oncogene c-*fos* protein function as a part of

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[1] Now in *Institute of Genetics, Fudan University, Shanghai 200433, China.*

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