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蒽环类药物在多药抗药细胞株 K562r 细胞内的积聚、滞留与外排[1]

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Intracellular accumulation, retention, and distribution of anthracyclines in a multidrug-resistant variant K562r[1]

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ABSTRACT A multidrug resistant variant (K562r) of the human K562 erythroleukemia cell line was obtained *in vitro* by repeated exposure of these cells to vincristine. The K562r cells were resistant to vincristine, harringtonine, mitomycin C, doxorubicin, epirubicin, and daunorubicin but retained the sensitivity to methotrexate. The resistances to vincristine and anthracyclines were reversed in the presence of verapamil. Despite being 60- or over 60-fold resistances to doxorubicin, epirubicin and daunorubicin, the net intracellular uptakes of these drugs at 2 h were reduced by only 15%, 20%, and 11%, respectively in K562r cells compared to their parental line. Verapamil did not enhance drug accumulation but inhibited drug efflux in K562r cells. Though there was no significant difference of drug content in cytoplasm between K562 and K562r cells, the drug content in the nucleus of K562r cells reduced significantly compared to that in K562 cells. The lower concentrations of anthracyclines in the nucleus of K562r cells might contribute to their acquisition of drug resistance.

KEY WORDS doxorubicin; daunorubicin; epirubicin; high pressure liquid chromatography; drug resistance; verapamil

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摘要 我们建立的抗药细胞株 K562r 表现为典型的 MDR 特性. K562r 细胞对阿霉素、表阿霉素、及柔红霉素的摄入量减少，外排量增加. 维拉帕米对 K562r 细胞的药物摄取无明显影响，但能抑制 K562r 细胞内的药物外排. 摄取药物60 min 时，1×10^6 K562r 细胞核中阿霉素、表阿霉素和柔红霉素的含量分别为809 ± 68，1238 ± 81和1664 ± 77 ng，显著低于 K562r 细胞核内的药物含量 $P<0.01$.

关键词 阿霉素；表阿霉素；柔红霉素；高压液相色谱法；抗药性；维拉帕米

肿瘤化疗失败的关键因素之一为肿瘤细胞的多药抗药性(multidrug resistance, MDR). 其特点为对结构和功能不同的多种药物尤其是蒽环类和植物碱类药物具有交叉抗药性[1]. 体外建立的抗药细胞株，若其具有 mdr1基因过表达，则往往表现为细胞内药物积聚减少[2,3]，此现象通常被认为是细胞获得抗药性的根本原因，然而细胞内药物积聚减少幅度往往较小，不足以解释何以细胞的抗药性可提高数十甚至上千倍. 本研究拟解决如下问题：1. 胞内药物积聚减少与细胞抗药性的关系；2. 抗药细胞胞内药物的分布；3. 维拉帕米对细胞药物摄取与外排的影响.

MATERIALS AND METHODS

药物与试剂 阿霉素(doxorubicin, Dox)（汕头 Turbin 制药厂）、表阿霉素 (epirubicin, Epi)、柔红霉素(daunorubicin, Dau)及维拉帕米(Verapamil, Ver)

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(Farmitalia Carro Erber). 长春新碱(vincrisitine, Vin)、三尖杉酯碱(harringtonine, Har)及甲氨蝶呤(methotrexate, Met)(杭州民生药厂). 四氮甲基唑蓝[3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-2*H*-tetrazolium-bromide, MTT]和三氟醋酸(trifluoroacetic acid, TFA)(Merck). 乙腈(acetonitril, Ace)(中国科学院上海脑研究所). RPMI-1640 (Sigma).

抗药细胞的诱导与培养 人红白血病细胞株K562常规培养于完全RPMI-1640培养液内. 取对数生长期K562细胞, 加入Vin至20 μg·ml^{-1}, 37 ℃放置2 h, 离心取细胞, 培养于完全RPMI-1640培养液中, 待细胞完全恢复正常生长后, 复用Vin处理, 直至细胞获得抗药性后, 用极限稀释法获抗药细胞克隆. 克隆细胞传代至今已半年, 抗药性稳定, 此克隆株被命名为K562r.

细胞抗药性试验[4]MTT法 对数生长期细胞接种于96孔培养板内, 每孔5×10^{4}细胞, 加入抗癌药, 药物浓度范围0.0012—100 μg·ml^{-1}, 培养48 h后, 每孔加入MTT 1 mg·ml^{-1} 5 μl, 37 ℃放置4 h后, 倾去培养液, 每孔加入100 μl酸化异丙醇, 待蓝色结晶完全溶解后, 用ELISA比色计570 nm处比色, 求出半数抑制浓度IC_{50}及药物抗性系数(resistance factor, RF) RF = IC_{50}(K562r)/IC_{50}(K562). 重复二次.

细胞的药物摄取与外排[5] 药物摄取: 1×10^{7}细胞培养于含有Dox, Epi及Dau(浓度均为9 μmol·L^{-1}的RPMI-1640完全培养液中, 在不同时间间隔各取1 ml样品, 用PBS洗2次. 细胞内Dox, Epi和Dau用HCl 0.3 mol·L^{-1}— 50% EtOH 0.1 ml在37 ℃提取1 h, 再加入10%磺基水扬酸50 μl, 9200×*g* 离心10 min, 100 μl上清上HPLC柱, 检测三种药物的含量, 实验重复2次.

药物外排: 取已摄取药物1 h的细胞, PBS洗2次, 重悬浮于RPMI-1640完全培养液中, 在不同时间间隔取1 ml细胞悬液, 离心取细胞. 细胞内3种药物的提取与检测同上. 实验重复2次.

Ver对细胞药物摄取与外排的影响 实验方法同上, 只是在药物摄取与外排的同时加入Ver至10 μmol·L^{-1}. 实验重复2次.

细胞核与细胞质中药物的分布 细胞药物摄取与上相同. 在药物摄取60 min时, 取细胞用PBS洗2次. 按Fleisher[6]报道的方法分离细胞核与细胞质, 1000×*g* 离心10 min, 分别收集沉淀与上清. 三种药物的提取与鉴定同上, 实验重复2次.

HPLC检测Dox, Epi和Dau 日本岛津LC-6A HPLC系列装置. 色谱柱填充物为Lichrosorb RP-18 (10 μm, φ4.6 mm×250 mm)溶剂A为0.1% TFA和10% ACN, 溶剂B为0.1% TFA和90% ACN, 梯度洗脱时, 溶剂B从20%至50%, 20 min完成, 洗脱速率1 ml·min^{-1}, 上样量100 μl, 检测波长为234 nm, Dox, Epi及Dau分别在11.43, 12.47和15.39 min时被洗脱(Fig 1).

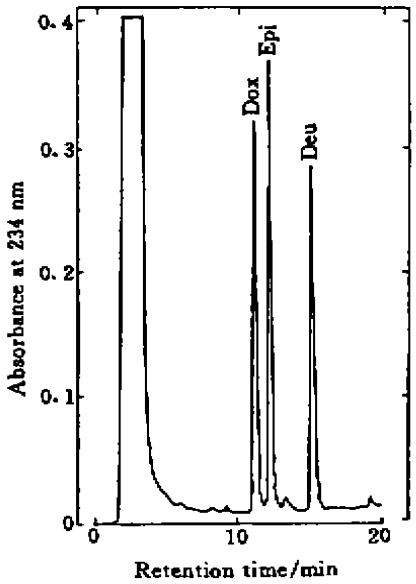

Fig 1. Chromatogram of mixture of anthracycline standards dissolved in HCl 0.3 mol·L^{-1}-50% ethanol-4% sulfosalicylic acid. Doxorubicin, epirubicin, and daunorubicin of 1.67 μg each.

RESULTS

K562r的抗药性 K562r表现为典型的MDR特性, 即对蒽环类和植物碱类药表现有交叉抗药性, 而对抗代谢药Met无交叉抗药性, 并且细胞对Vin, Dox, Epi和Dau的抗性可被Ver 10 μmol·L^{-1}逆转. (Tab 1).

细胞内药物积聚与外排 在药物摄取120 min时, K562细胞内Dox:Epi:Dau量之比为1.00 : 2.16 : 6.30 此比例在K562r细胞内为1.00:2.01:6.72 (Fig 2).

Tab 1. Drug sensitivities of K562 and K562r cells (n=18).

	$IC_{50}/\mu g \cdot ml^{-1}$ K562r	K562	RF
Dox	0.24	<0.004	>60
Dox+Ver	<0.004	<0.004	
Epi	0.24	0.004	60
Epi+Ver	0.016	0.004	4
Dau	0.24	0.004	60
Dau+Ver	<0.004	<0.004	
Vin	4	<0.0012	>3333
Vin+Ver	<0.006	<0.0012	5
MMC	18	4	4.5
Met	20	20	1

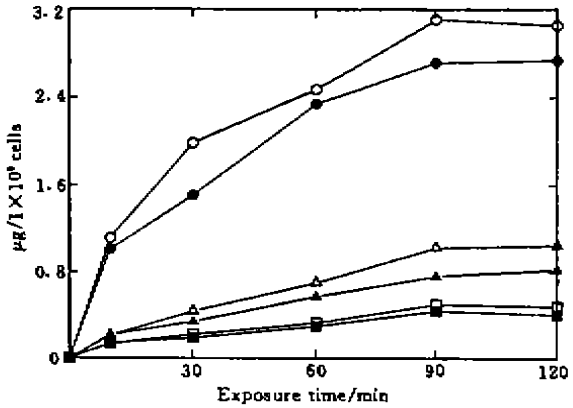

Fig 2. Accumulation of daunorubicin (○, ●, $P<0.05$), doxorubicin (□, ■, $P>0.05$), and epirubicin (▲, △, $P<0.05$) in K562 (○, □, △) and K562r (●, ■, ▲) cells.

比较K562r和K562细胞内药物积聚，发现前者Dox，Epi及Dau含量约比后者少16%，22%，与11%. 药物外排试验揭示K562r细胞内可排出的Dox，Epi及Dau大约为细胞内总量的52%，48%，55%，而K562细胞内此三种药物可排出的量约为35%，32%，42% (Fig 3).

Ver对细胞药物摄取与外排的影响 Ver对K562和K562r药物摄取均无影响(Fig 4). 但Ver能抑制K562r细胞药物外排(Fig 5).

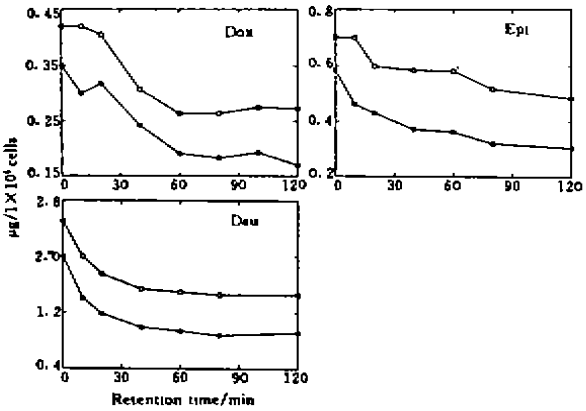

Fig 3. Retention of Dox ($P<0.01$), Dau ($P<0.01$) and Epi ($P<0.01$) in K562 (○) and K562r (●) cells.

细胞核、质内药物的分布 K562r和K562细胞质Dox、Epi和Dau的含量无显著差异，但前者细胞核内三种药物的含量均显著低于后者. (Tab 2).

Tab 2. Distribution of doxorubicin (Dox), epirubicin (Epi), and daunorubicin (Dau) in cytoplasm and nucleus of K562 and K562r cells (n=3).
$^aP>0.05$, $^cP<0.01$.

		ng/10^5 cells±s Nucleus	Cytoplasm	$\frac{N}{C}$ ratios
Dox	K562	1265±23	149±28	8.5
	K562r	809±68^c	176±31^a	4.6
Epi	K562	2340±148	297±27	8.0
	K562r	1238±81^c	240±49^a	5.2
Dau	K562	2514±37	432±33	5.8
	K562r	1664±77^c	262±68^a	4.6

DISCUSSION

K562r细胞对三种蒽环类药物的摄取减少，排出增加，但仍不足以解释其抗药性的增加. 蒽环类药物在K562r细胞核内积聚的减少可能是其获得抗药性的更重要的因素，此结果与文献结果[7,8]相符.

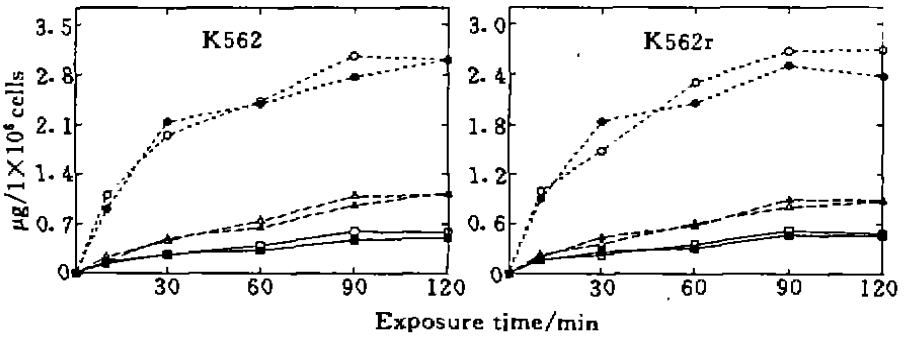

Fig 4. Effects of Ver on accumulation of Dox (□, ■), Dau (○, ●), and Epi (△, ▲) in K562 and K562r cells. (■, ●, ▲) with Ver. (□, ○, △) without Ver.

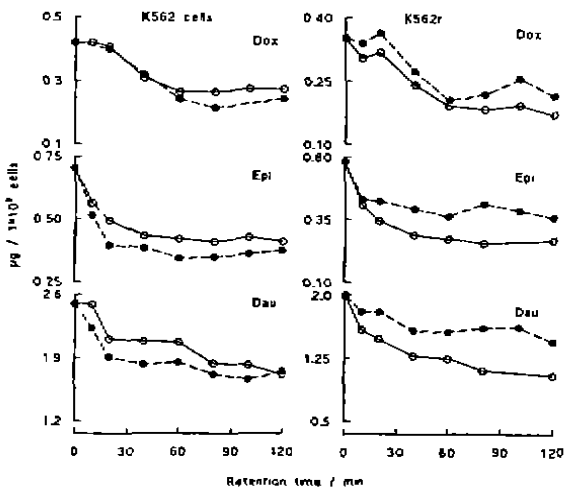

Fig 5. Effects of Ver on retention of Dox, Dau, and Epi in K562 and K562r cells. (○) without Ver. (●) with Ver.

Ver 逆转 MDR 细胞抗药性基于其能促进细胞内药物的积聚[9,10]. 本实验结果指出，Ver 可逆转 K562r 的抗药性，亦可抑制细胞内药物的外排，但不能促进其对药物的摄取，此结果与文献结果[11,12]不完全一致. 原因可能为(1) 文献报道的均为 MDR 细胞对单药的摄取，而本实验为 K562r 细胞对三种药物的同时摄取，MDR 细胞对单药和多药摄取可能存在差异；(2) 虽然 Ver 可逆转 MDR 细胞的抗药性，也可阻止 MDR 细胞内的药物外排，在此情形下，Ver 的浓度远大于总药浓度，而细胞对药物摄取时，溶液中 Ver 与总药物浓度的摩尔浓度比为1:3，提示 Ver 浓度低于总药浓度时 MDR 细胞对药物的摄取及敏感性不会受到明显的影响. 因此，有效剂量的 Ver 用于化疗也将因其心脏毒性而受到限制.

本 HPLC 法与其它检测细胞内蒽环类药物的方法[13,14]相比，具有以下优点：(1) 可清晰地检测同一样品中各种蒽环类药物，(2) 亦可用于分离检测其它药物如 Vin, Met, 5-Fu 等药物. 此法为研究细胞对多种药物的摄取与外排提供了有效的分析手段.

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苦参碱对大鼠输精管的作用与激活钙通道的关系

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Relation of effects of matrine on rat *vasa deferens* to activation of calcium channels

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ABSTRACT Effective mechanisms of matrine (Mat) in contraction were observed in isolated rat *vasa deferens*. Mat caused a strong concentration-dependent contraction of *vasa deferens*, and this contraction was competitively inhibited by prazosin (Pra, 10 μmol·L^{-1}) and nifedipine (Nif, 50 nmol·L^{-1}), with depression of maximal responses. Their pA_2 value was 5.1 and 9.29, respectively. The contraction was also inhibited by verapamil (Ver, 1 μmol·L^{-1}) with depression of maximal responses; but this antagonism was noncompetitive. Its pD_2' value was 6.07. Mat promoted $CaCl_2$-induced contraction of *vas deferens*. The effect of Mat was enhanced in proportion to increase in concentrations of $CaCl_2$. Mat markedly strengthened KCl-induced contraction of *vas deferens*. The results suggest that one of the mechanisms of the contractive effects of Mat within a certain range of concentrations was related to the activation of the calcium channel.

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