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HYDROXYLATION AT C-3' OF DOXORUBICIN ALTERS THE SELECTED PHENOTYPE OF CELLULAR DRUG RESISTANCE

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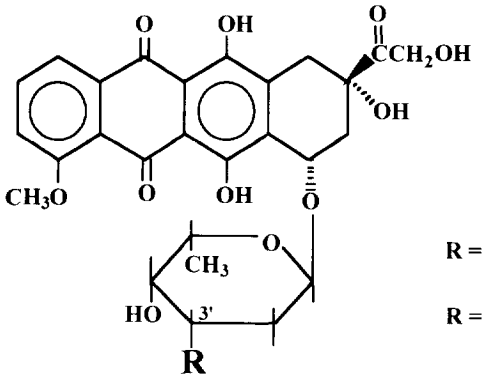
Abstract: Hydroxylation at C-3' of doxorubicin (DOX) yields the uncharged congener hydroxyrubicin, which circumvents P-glycoprotein-mediated drug resistance without loss of topoisomerase II inhibitory activity. Hydroxyrubicin-resistant cells exhibit a phenotype that is uniquely different from DOX resistance by expressing non-functional P-glycoprotein and hypersensitivity to anti-mitotic drugs.

The anthracycline antibiotic doxorubicin (DOX) is currently used in the treatment of a broad spectrum of solid tumors, lymphomas, and leukemias.[1] However, DOX activity is often impeded by the outgrowth of drug-resistant tumor cells. Cultured mammalian tumor cells can be selected for DOX resistance mediated either by overexpression of the energy-dependent transmembrane drug transporters, P-glycoprotein (P-gp) and the multidrug resistance-associated protein (MRP), or by reduced activity of topoisomerase II (topo II).[2-4] Structure-activity studies of compounds transported by P-gp suggest common structural elements among drugs of otherwise dissimilar structure and cytotoxic mechanism. The common structural features are at least one planar aromatic ring and a net positive charge at physiological pH.[5-8] We have also hypothesized that the amino group in the sugar portion of DOX, or in more general terms, presence of a basic center might be an important structural feature recognized by the P-gp multidrug transporter.[9-12] This hypothesis governed our design and synthesis of novel P-gp-circumventing drugs with improved therapeutic efficacy *in vitro* and *in vivo* against drug-resistant tumors.[10-16] In our initial efforts the basic center group of DOX at C-3' of the daunosamine moiety was replaced with a hydroxyl group to give hydroxyrubicin (WP159) (Figure 1).[9] The stereochemical orientation at C-3' during this process was not disrupted and the configurational identity of hydroxyrubicin with DOX was preserved.[13] This work has subsequently led to the selection of a new deaminated anthracycline, annamycin, for phase I clinical studies currently in progress at The University of Texas M.D. Anderson Cancer Center.[10, 12-16]

Figure 1: Anthracycline structures.



R = NH_2 **Doxorubicin (DOX)**

R = OH **Hydroxyrubicin (WP159)**

Studies comparing the efficacy of DOX and hydroxyrubicin in drug-resistant tumor cells have shown that hydroxyrubicin partially or completely circumvents P-gp-mediated drug resistance due to its decreased transport by P-gp when compared with DOX.[9,17-18] In addition, despite the loss of the basic center at C-3', hydroxyrubicin exhibits no significant difference in the degree of topo II inhibition relative to DOX.[17] In this study, we compared the drug resistance phenotypes that emerged following the selection of J774.2 murine macrophage-like cells in cytotoxic levels of either DOX or hydroxyrubicin in order to determine the effect of the removal of the basic center in the resistance selection process.

DOX-resistant (DOX/R) and hydroxyrubicin-resistant (WP159/R) cells were selected by continuous, progressive drug exposure up to 800 nM under previously described conditions.[19] The fold of resistance [R] of these cells to various lipophilic drugs was determined by the MTT cytotoxicity assay following continuous drug exposure for 72 hrs.[20] All drugs tested were known substrates for P-gp, but were identified functionally as either topo II inhibitors or tubulin/microtubule binding agents (anti-mitotic drugs) (Table 1). DOX/R cells exhibited 34.7-fold resistance to DOX and lower levels of cross-resistance to WP159, etoposide (VP-16), vinblastine (VBL), colchicine, and taxol. WP159/R cells were 10.8-fold resistant to hydroxyrubicin and, likewise, exhibited cross-resistance to other topo II inhibitors. However, at similar levels of resistance to hydroxyrubicin, WP159/R cells were 4- to 20-fold more sensitive than DOX/R cells to vinblastine, taxol, and colchicine (Table 1) and, in fact, were 2-fold more sensitive than wild-type J774.2 cells.

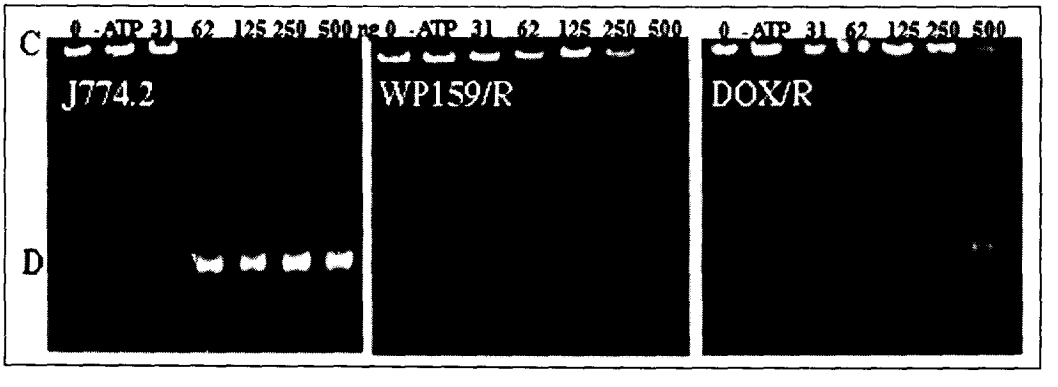
Table 1. Drug cytotoxicity in DOX/R and WP159/R cells.

	IC_{50} (nM) [R]*			IC_{50} Ratio: DOX/R to WP159/R
	J774.2	DOX/R	WP159/R	
Topo II inhibitors				
DOX	79.4 ± 4.9 [1]	2,752.5 ± 14.4 [34.7]	368.4 ± 36.5 [4.6]	7.5
WP159	107.7 ± 6.6 [1]	1,389.6 ± 78.3 [12.9]	1,159.9 ± 54.0 [10.8]	1.2
m-AMSA	900.6 ± 103.8 [1]	1,485.2 ± 68.7 [1.7]	1,353.9 ± 118.7 [1.5]	1.0
VP-16	576.7 ± 17.7 [1]	3,667.2 ± 102.1 [6.4]	2,133.3 ± 33.3 [3.7]	1.7
Anti-mitotic drugs				
vinblastine	2.1 ± 0.2 [1]	15.9 ± 0.1 [7.7]	0.8 ± 0.1 [0.4]	19.9
colchicine	93.1 ± 10.9 [1]	228.3 ± 20.5 [2.5]	51.5 ± 0.9 [0.6]	4.4
taxol	65.6 ± 3.9 [1]	332.3 ± 16.5 [5.1]	30.9 ± 4.3 [0.5]	10.8
podophyllotoxin	16.4 ± 0.7 [1]	13.4 ± 1.6 [0.8]	11.3 ± 1.5 [0.7]	1.2

*R = N-fold resistance: IC_{50} resistant cells/IC_{50} J774.2 cells

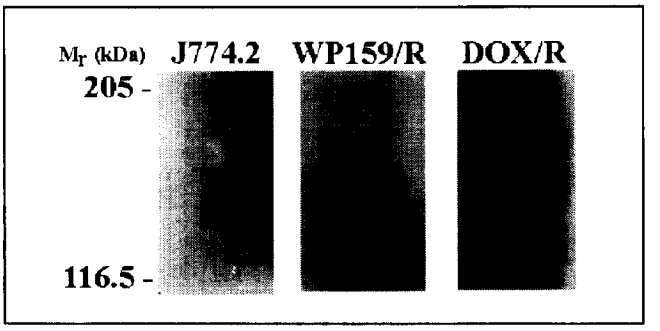
Resistance to topo II inhibitors by both DOX/R and WP159/R cells was due to decreased topo II activity. The ability of endogenous topo II from nuclear lysates of DOX/R and WP159/R cells to unlink catenated, circular kinetoplast DNA (kDNA)[21] was assessed relative to J774.2 (Figure 2).[22] Approximately 60 ng of J774.2 nuclear lysate was required to completely decatenate kDNA, while greater than 500 ng of nuclear lysate from both DOX/R and WP159/R cells were required. These results were consistent with the previous observation that hydroxyrubicin and DOX exhibited comparable inhibition of topo II in human KB carcinoma cells.[17]

Figure 2. Analysis of topo II activity in crude nuclear lysates: Values indicate ng amounts of total nuclear lysate assayed per lane. (-ATP) indicates assay of 500 ng lysate without exogenous ATP added. C - catenated kDNA; D - decatenated kDNA.



In DOX/R cells, resistance to topo II inhibitors and anti-mitotic drugs was also associated with P-gp overexpression, as demonstrated by immunoblot analysis of plasma membrane proteins with the C219 P-gp monoclonal antibody (Signet, Inc.) using previously described procedures[22] (Figure 3). WP159/R cells also overexpressed P-gp, relative to J774.2 cells, but approximately 3-fold less than DOX/R cells. In addition, the P-gp isoform in WP159/R cells exhibited a M_r of approximately 125 kDa, compared with 175 kDa in DOX/R cells. This difference in apparent size suggests cell line-specific variations in P-gp isoform expression, mutation in the core protein and/or differences in N-linked glycosylation.[23, 24]

Figure 3. Immunoblot detection of P-gp. Markers: 205 kDa, myosin; 116.5 kDa, ß-galactosidase.



P-gp overexpression in DOX/R cells correlated with reduced intracellular drug accumulation. Table 2 shows the amount (pmoles) of intracellular drug per 1 x 10^6 cells after exposure to either 1.0 or 2.0 µM drug for 2 hrs.[19] Compared with J774.2 cells, DOX/R cells accumulated 22-65% less DOX, VBL, or hydroxyrubicin. In contrast, WP159/R cells showed no change in DOX, hydroxyrubicin, and VBL accumulation, despite the overexpression of P-gp.

Table 2. Net intracellular drug accumulation.

pmoles drug per 10^6 cells ± s.e.

	Doxorubicin		Vinblastine		Hydroxyrubicin	
Cell line	**1.0 µM**	**2.0 µM**	**1.0 µM**	**2.0 µM**	**1.0 µM**	**2.0 µM**
J774.2	66.8 ± 11.9	153.4 ± 37.9	90.9 ± 13.9	163.3± 20.1	42.2 ± 12.5	64.6 ± 4.3
DOX/R	52.8 ± 0.2	87.0 ± 12.1	31.3 ± 9.0	69.7± 2.3	20.0 ± 3.8	37.2 ± 10.6
WP159/R	69.6 ± 3.6	130.5 ± 4.3	84.8 ± 9.5	140.3± 10.0	36.9 ± 4.9	77.3 ± 13.6

These results suggest that WP159/R cells overexpressed non-functional P-gp, since WP159/R cells apparently could not transport either DOX or VBL. Furthermore, initial studies have indicated that P-gp overexpression in WP159/R cells was not associated with P-gp mRNA overexpression, as has been reported previously.[23] Hydroxyrubicin, therefore, did not select for resistance conferred by P-gp. Lack of P-gp

activity would be consistent with the ability of hydroxyrubicin to circumvent, partially or completely, P-gp-mediated resistance in cell lines selected for resistance to either DOX or VBL.[9,17,25] Surprisingly, DOX/R cells exhibited significant cross-resistance to hydroxyrubicin, which could, to some extent, be attributed to reduced topo II activity. However, reduced hydroxyrubicin accumulation in DOX/R cells and the ability of the chemosensitizing agent verapamil (8 μM) to reduce hydroxyrubicin resistance by 70% suggests that P-gp in some cell lines may recognize and transport hydroxyrubicin.

The selection of anti-mitotic hypersensitivity by hydroxyrubicin, in contrast to DOX, is an important characteristic of hydroxyrubicin resistance. Initial analyses of several cloned WP159/R lines and of clones independently selected with hydroxyrubicin also suggest that anti-mitotic hypersensitivity is a consistent characteristic of hydroxyrubicin-resistant cells. Progressively increasing anti-mitotic hypersensitivity observed during selection of increasingly hydroxyrubicin-resistant cells further suggests that anti-mitotic hypersensitivity is selected with hydroxyrubicin and is dose-related. These results indicate that removal from DOX of the basic 3'-amine and eradication of the positive charge result in the selection of a unique phenotype of multidrug resistance. How anti-mitotic hypersensitivity is selected by hydroxyrubicin and whether the absence of the positively charged 3'-amine or presence of the hydroxyl group is responsible for selection is currently under investigation.

Acknowledgments

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