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Uptake of WR-2721 Derivatives by Cells in Culture: Identification of the Transported Form of the Drug[1]

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ABSTRACT

When V79-171 cells are incubated in medium to which WR-1065 has been added the cells accumulate WR-1065 and disulfides of WR-1065 (WRSS) in a ratio of about 10:1. Analysis of the culture medium showed that it contained primarily WR-1065 but that significant levels of the symmetrical disulfide WR-33278 and of the mixed disulfide of WR-1065 with cysteine were also present. Since incubation of cells with either of the latter disulfides did not lead to uptake it was concluded that WR-1065 is the form of the drug taken up. The uptake rate on a per cell basis was shown to be independent of cell density, to be first order in the WR-1065 concentration in the incubation medium, to increase as $[H^+]^{-1.2}$ at medium pH values from pH 6.8 to 8.0, and to have a Q_{10} value (rate increase per 10°C temperature increase) of 2.9 ± 0.3 between 2 and 37°C. Rates of WR-1065 uptake measured for HeLa, HT29/SP-1d, Me-180-VCII, Ovary 2008, and WI-38 cell lines were found to be similar to that measured for V79-171 cells. The results are consistent with uptake by nonmediated, passive diffusion of the uncharged form of WR-1065 across the plasma membrane but uptake mediated by a membrane transport system could not be rigorously excluded. Based upon these results and earlier findings it is postulated that the lower drug uptake seen in tumors as compared with normal tissues in animals treated with WR-2721 results from a combination of (*a*) slower conversion of WR-2721 to WR-1065 in tumors as a consequence of the lower inherent level of alkaline phosphatase and lower pH in tumors and (*b*) a decreased uptake rate of the WR-1065 present in tumors as a consequence of their lower pH.

INTRODUCTION

The drug WR-2721[4] has been the subject of increasing attention following the work of Yuhas and coworkers (1–3) suggesting that it is capable of selectively radioprotecting normal *versus* tumor tissues in animals. Subsequent extension of these observations showed that it can also protect against lethal effects of chemotherapeutic agents (3–5) and it has been recently reported that it, or its thiol form (WR-1065), protects against carcinogenic and mutagenic affects caused by radiation (6, 7) and chemotherapeutic drugs (8, 9). The basis for the apparent selective protection of normal tissue has been elusive. Using radiolabeled WR-2721 it was established that tumors accumulated the drug much less effectively than most normal tissues (10, 11). *In vitro* studies of radiolabel uptake with tissue preparations by Yuhas and coworkers (12, 13) were interpreted to indicate that WR-2721 is actively transported into normal tissues but is only slowly absorbed by solid tumors through facilitated diffusion. However, WR-2721 has generally been found not to protect cultured cells (14–17) unless "activated" by tissue extracts added to the medium and capable of dephosphorylating the drug (16). Human kidney T-cells constitute an exception and were shown by Purdie and coworkers (18, 19) to slowly dephosphorylate WR-2721 with a corresponding appearance of radioprotection.

A major difficulty in attempting to understand the factors influencing radioprotection by WR-2721 in culture has been the lack of data on the specific forms of the drug present in the medium and in the cells during the course of incubation and subsequent irradiation. In studies with V79-171 cells in culture we were unable to detect any drug uptake or radioprotection when cells were exposed to WR-2721 in medium alone but when alkaline phosphatase was added to the medium efficient uptake leading to appreciable cellular levels of WR-1065 and its disulfide forms (WRSS) was observed (17). This uptake was preceded by conversion of WR-2721 to WR-1065 and WRSS in the medium. Alkaline phosphatase had been previously shown to dephosphorylate 2-aminoethylphosphorothioate ($H_2NCH_2CH_2SPO_3H_2$) and it had been established that this activity is widely distributed in mammalian tissues (20). This suggested that WR-1065 or a WRSS form of the drug might be the species taken up and that alkaline phosphatase might play an important role in the uptake of the drug. In the present paper we extend our studies with V79-171 cells to identify the form of the drug taken up and the kinetics of the uptake process. The generality of the findings are tested in other cultured cell lines. In the following paper (21) we examine the role of intracellular and extracellular drug in protecting against radiation damage.

MATERIALS AND METHODS

Drugs. WR-1065 was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute and was stored in a dessicator at −20°C. WR-33278 was synthesized from WR-1065 by saturating a 30-mM solution containing 0.1 μM copper sulfate with oxygen at room temperature until no thiol could be detected by titration with Ellman's reagent (22). Lyophilization yielded crystalline product containing 0.002% WR-1065 as determined by labeling with mBBr and HPLC analysis. The mixed disulfide of WR-1065 and cysteine (WRSSCys) was prepared by mixing 1 mM WR-1065 with 1 mM cysteine in 100 ml of 100 mM ammonium carbonate adjusted to pH 9.0 with ammonium hydroxide. Oxygen was bubbled through the mixture until no thiol was detectable by titration with Ellman's reagent. The sample was lyophilized, reconstituted in water, and adjusted to pH 2.0 with HCl, and lyophilized again. Analysis of the resulting solid by HPLC with electrochemical detection indicated the composition to be 77% WRSSCys, 10% cystine, and 13% WR-33278. The mixed disulfide of glutathione and WR-1065 was prepared in analogous fashion.

Uptake Studies. Cells were prepared as described in the following paper (21). All procedures were carried out at room temperature (22–24°C) except as noted and pH was measured with a Brinkmann Model 103 pH meter equipped with a Metrohm AG9100 combination electrode equilibrated at the measurement temperature. Unless otherwise indicated cells were resuspended in BME medium with Hanks' salts containing 10% fetal bovine serum and 50 μg/ml gentamicin. Stock solutions of WR-1065 (100–800 mM) were prepared by weight in 0.01%

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[4] The abbreviations and trivial names used are: WR-2721, *S*-2-(3-aminopropylamino)ethylphosphorothioic acid ($H_2NCH_2CH_2CH_2NHCH_2CH_2SPO_3H_2$); WR-1065, *N*-(2-mercaptoethyl)-1,3-diaminopropane; WR-33278, symmetrical disulfide of WR-1065; WRSSCys, mixed disulfide of WR-1065 and cysteine; WRSSG, mixed disulfide of WR-1065 and glutathione; WRSS, sum of all readily reducible disulfide forms of WR-1065; HPLC, high-performance liquid chromatography; BME, basal minimal Eagle's medium.

acetic acid and filtered (Acrodisc, 0.2-μm filters, Gelman); thiol levels determined by titration with Ellman's reagent (22) agreed to within ±10% with the values based upon weight and were used to calculate the volume of drug stock needed to produce the desired final concentrations of WR-1065. An equal volume of double strength BME was added to the incubation tubes to maintain isotonicity. The volume of 5 N NaOH necessary to produce the required final pH was predetermined by preparing a cell-free replicate incubation tube at the cell suspension pH, adding the required amount WR-1065 stock solution, and titrating with 5 N NaOH to the desired pH.

The following procedure for determination of the initial rate of drug uptake (V_0) at 20 mM WR-1065 (pH 7.4), 23°C and 4×10^6 cells per ml illustrates the methodology. A total of 3×10^7 cells was resuspended in 7 ml BME (Hanks') medium (pH 7.4), 244-μl double-strength BME was added, 12 μl of 5 N NaOH was deposited on the side of the tube, 244 μl of 615 mM WR-1065 was added, and the suspension mixed. A digital timer (GCA/Precision Scientific, ±0.01 min) was simultaneously activated to measure the incubation times. Sampling for the first time point was begun immediately. Duplicate cell aliquots containing either 1×10^6 (WR-1065 determination) or 4×10^6 (WRSS determination) cells were removed from the incubation mixture and gently layered over 300 μl of silicone oil (Versilube F50, General Electric) in 1.5-ml microfuge tubes (MC-15, Cole Scientific). The cells were pelleted through the oil in a Brinkmann Model 5414 Microfuge (15000 rpm, 3 min) to separate them from the medium (23). The sample time was recorded at the time each centrifugation was initiated. After removal of 50-μl samples for analysis of WR-1065 and WRSS in the medium, the tubes were gradually inverted during aspiration of the remaining medium and oil, and during subsequent wiping with ethanol and dry swabs to ensure removal of all of the medium. Samples were then immediately processed as described under "Drug Analysis." During the course of the experiment the incubation mixtures were vortexed sufficiently frequently (at least every 10 min at 23°C) to ensure adequate oxygenation and the pH was monitored.

Measurement of V_0 was facilitated when uptake was very rapid by using two centrifuges to permit multiple processing of samples and omitting the disulfide analyses to reduce the number of samples to be processed for a given time point. When cell metabolism is rapid or during extended incubations, the pH of the incubation medium may drift significantly. When this occurred the pH change could be minimized by carrying out the incubations at a lower cell density (1×10^6 cells per ml) and increasing the volume of cell suspension used for WR-1065 analysis to 1 ml. Incubations at 37°C were also carried out at this lower cell density to decrease the surface-to-volume ratio of the sample aliquot so that air cooling in the short interval between removal from the bath and centrifugation would not produce a significant error in the measured rate. For measurements at 37°C the microfuge tubes and oil were preequilibrated at 37°C to further minimize cooling of the sample aliquot prior to completion of the centrifugation. Uptake at 2°C was carried out in an environmentally controlled cold room through completion of the centrifugation step and collection of the medium and cell pellet samples.

All data were corrected for extracellular drug carried through the silicone oil during centrifugation by extrapolating the linear portion of the uptake curve to zero time. Such zero-time values for WR-1065 and for WRSS were pH independent and for V79-171 cells averaged 0.1 nmol/10^6 cells/mM substrate concentration in the medium.

Drug Analysis. Thiols were determined by HPLC analysis with fluorescence detection following their conversion to a fluorescent derivative by reaction with monobromobimane (mBBr, Behring Diagnostics). For determination of WR-1065 in the medium, 50 μl of medium was mixed with 50 μl of $(2X + 5)$ mM mBBr (X = medium concentration of WR-1065) in 40 mM Tris-methanesulfonate, pH 8.0 (Reagent M) and the mixture stored on dry ice pending further processing. After thawing and allowing to stand at room temperature for 5–15 min, 100 μl of 4 M sodium methanesulfonate containing 0.2 M methanesulfonic acid (Reagent SA) was added, the mixture vortexed, and precipitate removed by centrifugation in a microfuge for 4 min. For determination of WR-1065 in cells, a cell pellet (1×10^6 cells) was vortexed with 50 μl of Reagent M until cells were fully suspended and the sample placed on dry ice pending further processing. The sample was freeze-thawed and vortexed three times, 50 μl of Reagent SA added, the sample vortexed again, and the precipitate removed by centrifugation.

For determination of WRSS in medium, thiols were first blocked by reaction with *N*-ethylmaleimide. A 50-μl sample of medium was mixed with 50 μl of 12 mM *N*-ethylmaleimide in 40 mM Tris-methanesulfonate (pH 8.0) containing 6 mM EDTA (Reagent N) and the mixture stored on dry ice. After thawing and allowing to stand for 5–15 min at room temperature, the sample was extracted once with 300 μl of benzene to remove unreacted *N*-ethylmaleimide and 10 μl of 100 mM dithiothreitol was added to reduce disulfides; the mixture was vortexed and allowed to react 10 min. Next, 27 μl of 100 mM mBBr in acetonitrile was added and the mixture reacted for 3 min in the dark. After addition of 137 μl of Reagent SA the mixture was vortexed and the precipitate removed by filtration through a 0.2-μm Bas filter. A similar procedure was used for determination of WRSS in cells. A cell pellet (4×10^6 cells) was suspended in 50 μl Reagent N and frozen on dry ice. After freeze-thawing and vortexing three times, the mixture was extracted with 150 μl of benzene, mixed with 5 μl 100 mM dithiothreitol, and allowed to react 10 min at room temperature. Next, 27 μl of 100 mM mBBr in acetonitrile was added and the mixture allowed to stand 3 min in the dark. After mixing with 68 μl Reagent SA the precipitate was removed by filtration through a 0.2-μm Bas filter.

The sample supernatants for WR-1065 and WRSS analysis were stored at −70°C prior to reversed-phase HPLC analysis with fluorescence detection by Method 3 which has been fully described elsewhere (24).

For analysis of specific disulfides, samples were prepared as described above for WRSS analysis but the reactions with dithiothreitol and mBBr were omitted. HPLC analysis of specific disulfides was carried out on a 250- x 4.0-mm Microsorb C-8 column (Rainin Instrument Co., 80-325) equipped with a Brownley RP-8 (OS-GU) guard column. The column was eluted at 1 ml per min with a buffer containing 0.1 M monochloroacetic acid, 10 mM 3,5-dimethylcyclohexyl sulfate, and 5 mM *N,N*-dimethyloctylamine in 10% methanol/water titrated to pH 2.5 with NaOH. The elution buffer was maintained oxygen-free in a reservoir under an atmosphere of nitrogen. Disulfides were detected using a Bioanalytical Systems Model LC-4B electrochemical detector equipped with dual gold-mercury electrodes and operated as described by Allison and Shoup (25). Retention times (min) were: cystine, 4; WRSSCys, 7; WRSS-glutathione, 9; WR-33278, 50. Disulfide levels were estimated from peak height measurements calibrated using standard samples.

RESULTS

Unless otherwise indicated, each measurement represents the average of duplicate determinations and the range of values is indicated by the error bar (if larger than the symbol in the figures) or by the average deviation listed. In most cases the experiments have been repeated at least once and were found to be highly reproducible if conditions (especially pH) were closely controlled. Cell recovery for the centrifugation of cells through silicone oil was examined using [^{3}H]thymidine-labeled cells and determined to be 95 ± 3%.

When V79-171 cells were incubated in the presence of 4 mM WR-2721 plus alkaline phosphatase (21) or in the presence of 4 mM WR-1065 significant levels (~1 mM) of WRSS accumulated in the incubation media. Specific analysis of the disulfide components in the medium by HPLC indicated that the major component was WR-33278, with WRSSCys present at an order of magnitude lower level. No other low molecular weight disulfides were detected but protein-WR-1065 mixed disulfides are not detected by the HPLC methodology used to measure specific disulfides and may also be present. In order to determine whether the WR-33278 or WRSSCys are efficiently taken up by V79-171 cells, cells were incubated in media to which each drug form had been added and the level of WR-1065 and WRSS

associated with the cells determined after 5 and 60 min (Table 1). Low residual levels of drug were observed for incubations in WR-33278 and WRSSCys but these probably represent primarily extracellular drug pulled through the oil when the cells were separated from the medium since they increased little or not at all with incubation time. The largest increase was for incubation in WR-33278 where the cellular level of WRSS increased about threefold between 5 and 60 min. However, the WR-1065 level did not increase significantly and the 60-min level of both forms together was over 20-fold lower than found for incubations with 4 mM WR-1065 where the level of WR-33278 in the medium is 1 mM or less. Thus, uptake of WR-33278 and subsequent reduction within the cell cannot account for the observed uptake. The levels of WR-33278 and WRSSCys remained essentially constant in the medium throughout the incubations with these forms of the drug (data not shown).

The possibility that a protein-WR-1065 mixed disulfide, or some component other than WR-1065 present in the medium, is responsible for uptake was explored by preincubating medium plus 4 mM WR-2721 and alkaline phosphatase (1 U per ml) at 37°C overnight; subsequent analysis of the medium showed that it contained <50 μM WR-2721, <20 μM WR-1065, and 3.0 mM WRSS. When V79-171 cells were incubated for 60 min in this medium no significant increase (<5%) over the zero-time values of cellular WR-1065 or WRSS was observed. This indicates that none of the disulfide forms of the drug formed from WR-1065, or other stable components formed from WR-2721 plus alkaline phosphatase, are taken up by cells at significant rates. However, when cells were incubated in medium containing WR-1065 a significant increase in the cellular WRSS level and a marked increase in the cellular WR-1065 level was observed (Table 1). This indicated that WR-1065 itself is the form of the drug taken up and more detailed studies of this process were undertaken.

A time course of the uptake of drug by V79-171 cells incubated in 4 mM WR-1065 is shown in Fig. 1. The levels of WR-1065 and WRSS were monitored in the medium and a slow oxidation of WR-1065 to WRSS was evident over the 90-min incubation period. The cellular level of WR-1065 and of WRSS increased throughout this period, the level of WRSS being roughly an order of magnitude lower than that of WR-1065. Cells were also analyzed for their specific disulfide content after a 60-min incubation and the values (nmol per 10^6 cells) found to be: WRSSCys, <0.05; WRSSG, <0.05; WR-33278, 0.25 ± 0.05. Thus, all of the measured WRSS (Table 1) can be accounted for as WR-33278 (1 equivalent WR-33278 = 2 equivalents WRSS). Since WR-33278 is not taken up by the cells at a rate adequate to account for this level of cellular WR-33278 (Table 1), it must arise primarily from intracellular oxidation of WR-1065.

In the standard protocol cells were incubated at 4×10^6 cells per ml and it seemed prudent to establish whether uptake is dependent upon cell density. Using the same cell preparation incubations were carried out at 1×10^6, 4×10^6, and 10^7 cells per ml in 4 mM WR-1065 and the cellular WR-1065 levels were determined after 60 min to be 5.2, 5.5, and 5.5 nmol per 10^6 cells, respectively, with WRSS levels again being an order of magnitude lower. Thus, the rate of WR-1065 uptake per cell is not cell density dependent in the range of cell density used in these experiments.

Table 1 *Cellular levels of WR-1065 and WRSS determined in V79-171 cells after incubation for 5 or 60 min in media to which various drug forms had been added*

	Cellular level (nmol/10^6 cells)[a]			
	5 min		60 min	
Drug added	WR-1065	WRSS	WR-1065	WRSS
WRSSCys, 1 mM	0.12 ± 0.01	0.17 ± 0.04	0.22 ± 0.01	0.15
WR-33278, 1 mM	0.11 ± 0.04	0.06	0.15	0.21 ± 0.05
WR-1065, 4 mM	1.0	0.09	7.0 ± 0.1	0.41 ± 0.02

[a] Average of duplicate determinations which gave identical results where no average deviation is given.

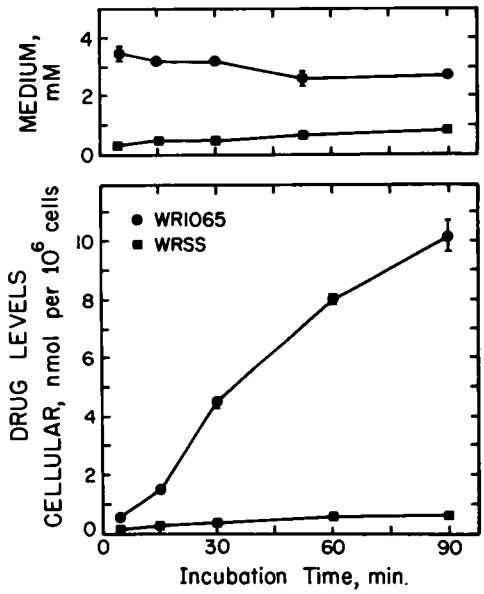


Fig. 1. Levels of WR-1065 (●) and WRSS (■) found in the medium (*top*) and associated with cells (*bottom*) as a function of incubation time when V79-171 cells were incubated in BME (Earle's) medium containing 4 mM WR-1065 at 23 ± 1°C; pH initially 7.2 but not monitored throughout the incubation.

In the course of studying the kinetics of WR-1065 uptake it became clear that the rate of uptake is a very sensitive function of temperature and pH, and that precise and reproducible rate measurements can be obtained only if these factors are closely controlled and monitored as described in "Materials and Methods." Typical time courses obtained by these methods are shown in Figs. 2 and 3; only cellular WR-1065 levels are shown and WRSS levels were generally an order of magnitude lower. The data in Fig. 2 illustrate the dependence of uptake on the concentration of WR-1065 in the medium at a single pH. Medium values for WR-1065 are the average of those measured during the initial course of the incubation. Fig. 3 illustrates the dramatic increase in uptake rate with increasing pH. Uptake is so rapid at alkaline pH (Fig. 3) that a substantial decline in rate was seen within the 13-min incubation interval as the intracellular level approached the extracellular drug level (10^6 cells ≅ 1.75 μl) but a good assessment of the initial rate of uptake, V_0, could be obtained from the early linear portion of the curve.

The dependence of the initial velocity of drug uptake (V_0) upon extracellular drug concentration and upon pH is illustrated in the log-log plot shown in Fig. 4. At each pH the slope of the line is unity, demonstrating a first-order dependence upon WR-1065 concentration. On a linear plot (not shown) these initial velocities extrapolate to zero at zero WR-1065 concentration. Thus, the uptake velocity follows a simple first-order rate law at each pH with the first-order rate constant being given by $V_0/[\text{WR-1065}]_{med}$.

The pH dependence of the first-order rates of drug uptake for the data shown in Fig. 4, as well as for data at other pH values, is presented in Fig. 5 as a logarithmic plot of $V_0/[\text{WR-}$

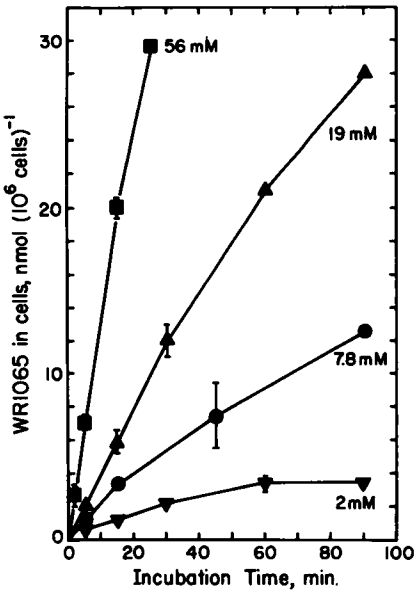


Fig. 2. Levels of WR-1065 in cells as a function of incubation time at 23 ± 1°C and pH 7.2 for cells incubated at 4×10^6 cells/ml in medium containing 56 mM (■), 19 mM (▲), 7.8 mM (●), or 2 mM (▼) WR-1065. Media WR-1065 levels, average of values measured throughout the incubation; these were generally constant to ±10%.

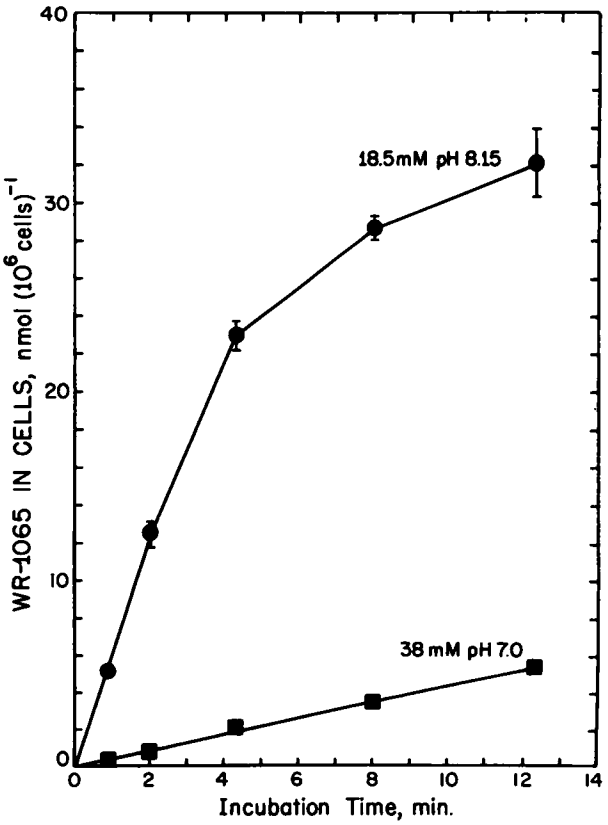


Fig. 3. Levels of WR-1065 associated with cells as a function of incubation time at 23 ± 1°C for V79-171 cells incubated at 4×10^6 cells per ml in medium containing 18.5 mM WR-1065 at pH 8.15 (●) or 38 mM WR-1065 at pH 7.0 (■).

1065]$_{med}$ *versus* the pH of the medium. The slope of the line is 1.2 showing that the rate has an inverse dependence upon hydrogen ion concentration which is slightly greater than first-order.

The temperature dependence of the uptake velocity was also examined at pH 7.2 (Table 2). At 2°C the rate was quite slow

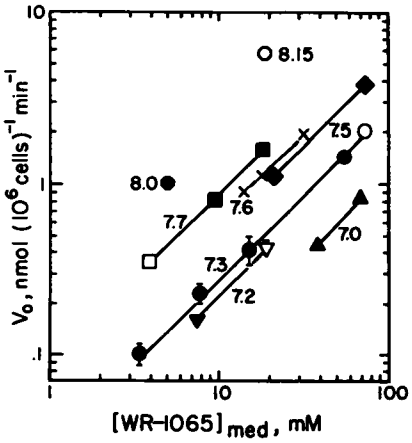


Fig. 4. Log-Log plot of the initial velocity of uptake of WR-1065 (V_0) by V79-171 cells as a function of WR-1065 concentration in the medium for experiments conducted at 23 ± 1°C and different pH values as indicated. *Solid symbols*, runs in which the pH was monitored throughout the incubation; *open symbols*, runs in which the pH was measured only at the beginning of the kinetic run.

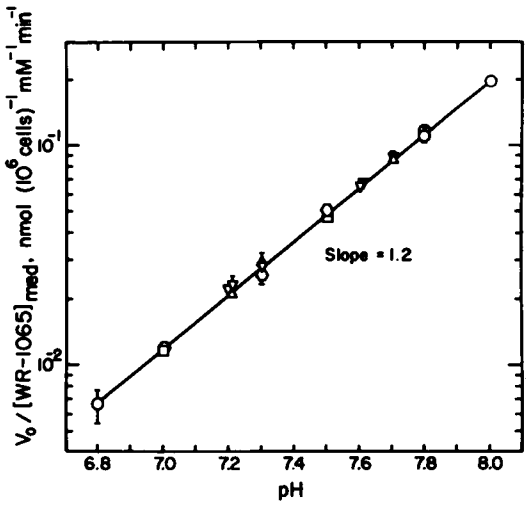


Fig. 5. Logarithmic plot of the rate constant (V_0/[WR-1065]$_{med}$) for uptake of WR-1065 by V79-171 cells at 23 ± 1°C as a function of the pH of the incubation medium. The general ranges of extracellular WR-1065 concentration into which the value for a given experiment fell are as follows: ○, 1–5 mM; △, 6–10 mM; ▽, 11–20 mM; □, 21–40 mM; ○, 41–80 mM.

Table 2 *Temperature dependence of uptake of WR-1065 by V79-171 cells at pH 7.2*

Temperature (°C)	[WR-1065]$_{med}$ (mM)	V_0/[WR-1065]$_{med}$ [nmol(10^6 cells)$^{-1}$min^{-1}mM^{-1}]
2[a]	3.2	0.0009 ± 0.0006
24[b]	3.8	0.018 ± 0.001
37[b]	3.7	0.067 ± 0.002

[a] At 4×10^6 cells/ml.
[b] At 1×10^6 cells/ml; incubations conducted in a NAPCO water bath at the indicated temperature.

and the value for V_0 is subject to a large error. Even so, an Arrhenius plot of the data (not shown) was quite linear so the temperature dependence of the rate corresponds to that expected for a single kinetic process. From the slope of the Arrhenius plot an activation energy of 78 ± 4 kJ/mol was calculated and the change in rate from 2 to 37°C corresponds to a value for Q_{10} (rate increase per 10° interval) of 2.9 ± 0.3. Thus, the uptake rate is also strongly temperature dependent and the ±1°C range of uncertainty in our incubation temperatures contributes a ±12% error to the measured rates.

The extent to which these findings with V79-171 cells can be

generalized to other cultured cells was also examined. In Table 3 the measured values of the first-order rate of uptake, $V_0/[\text{WR-1065}]_{med}$, are tabulated for a number of human cell lines. Originally derived from human tumors, HeLa, HT29, Me-180, and Ovary 2008 are cytogenetically distinct human tumor cell lines capable of tumor formation in the nude mouse (21). Homologous with the hamster derived V79-171 cells, WI-38 cells are human diploid lung fibroblasts, are nontumorigenic in nude mice, and are considered a model for "normal" cells. In the case of HeLa cells it is seen that the first-order rate shows a similar sharp increase with increasing pH of the medium to that seen with V79-171 cells. The absolute values of the rate of uptake of WR-1065 are, within a factor of two, the same as found for V79-171 cells for all five human cell lines shown in Table 3. In order to make more precise comparisons of the uptake rate it would be necessary to correct for differences in cell size.

DISCUSSION

The present results, along with those of the preliminary study (17), clearly exclude WR-2721, WR-33278, WRSSCys or other disulfides present in the incubation medium as drug forms which are readily taken up by V79-171 cells in culture. The only other form of the drug present in substantial amounts when cells are incubated in WR-1065, or in WR-2721 plus alkaline phosphatase, is WR-1065 itself, and the present studies show that WR-1065 is taken up by a process which is kinetically first order in the concentration of WR-1065 in the medium and is independent of the cell density.

Uptake of the drug might, in principle, occur by unmediated passive diffusion through the membrane or by a process facilitated by a membrane transport system (26). The two processes could be distinguished if it could be shown that uptake becomes saturated at high WR-1065 concentration since such saturation kinetics are consistent with a mediated uptake mechanism but not with an unmediated passive diffusion process. However, as long as care was taken to ensure that all measurements were at the same pH, no evidence of deviation from a strictly first-order dependence upon WR-1065 concentration could be found, even at WR-1065 concentrations as high as 80 mM (Fig. 4).

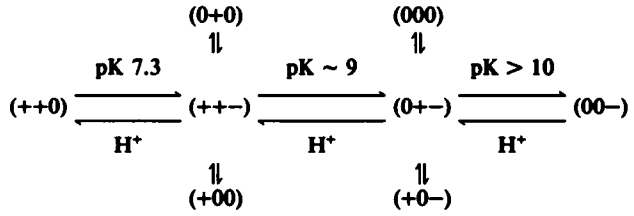
The temperature dependence might be expected to provide a basis for distinguishing a mediated uptake mechanism from one involving strictly passive diffusion. The activation energies associated with nonmediated diffusion of small molecules in biological membranes are ~60 kJ/mol, comparable to the value of 78 kJ/mol observed here for WR-1065, whereas mediated processes for uptake of natural substrates appear to have significantly lower values based upon the limited data available (26). However, if WR-1065 were taken up by a mediated process then it is evident that the protein involved did not evolve to transport WR-1065 and it would not necessarily be expected to transport WR-1065 efficiently. It follows that a mediated process might well have an activation energy for transport of WR-1065 which is significantly higher than for transport of substances which are its natural substrates. Thus, no definitive conclusion can be drawn from the observed temperature dependence concerning the operation of mediated *versus* nonmediated transport processes.

Table 3 *Uptake of WR-1065 by selected human cell lines at 23 ± 1°C*

Cell line	pH[a]	$[\text{WR-1065}]_{med}$ (mM)	$V_0/[\text{WR-1065}]_{med}$ [nmol(10^6 cells)$^{-1}$min^{-1}mM^{-1}]
HeLa	7.7[b]	9.6	0.11
	7.2	8.0	0.016
HT29/SP-1d	7.2	6.5	0.044
Me-180-VCII	7.2	7.5	0.034
Ovary 2008	7.2	7.8	0.026
WI-38	7.26[b]	4	0.022

[a] pH at start of incubation, except as noted.
[b] pH measured throughout incubation.

The rate of drug uptake shows a marked dependence upon pH in the range of 6.6 to 8.2, exhibiting a dependence upon hydrogen ion concentration to the −1.2 power. The drug undergoes a change in ionization state in this pH range but the pK values are not known with certainty. The drug certainly exists as a dication below pH 6 having the form $H_3N^+CH_2CH_2CH_2N^+H_2CH_2CH_2SH$. This is abbreviated as (++0) where the zero indicates the uncharged thiol group. A pK_a for the thiol ionization, presumably to form predominantly the (++−) form, of 7.3 has been indirectly determined (27). As shown in the equation, this form is in equilibrium with two other forms having a net charge of +1:



Further ionization of the ammonium groups leads to neutral forms of the drug and ionization of the second ammonium group yields an anionic form. Rough estimates of the ionization constants are included in the above equation. The observed pH dependence suggests that uptake occurs at least in part via a form less positively charged than the monocation since there is a steep inverse dependence upon hydrogen ion in the pH range where predominant conversion to the monocation has already occurred. This pH dependence is consistent with uptake of a form of the drug having a zero net charge. Passive, unmediated diffusion of the uncharged (000) form into the cell constitutes one mechanism of uptake which meets this requirement. If the (000) form is assumed to constitute ~10% of the total concentration of the three forms having net zero charge then it can be roughly estimated using the pK_a values of the equation that the (000) form will constitute ~0.1% of the total drug present at pH 7 and ~1% of the total at pH 8. Uptake of the (00−) form should be proportional to $[H^+]^{-2}$ between pH 6 and 8 and is therefore incompatible with the observed pH dependence.

Although the results are consistent with uptake by unmediated diffusion, a mediated uptake mechanism involving the (000), (0+−) or (+0−) forms cannot be rigorously excluded on the basis of the pH dependence. If a mediated process is involved then the system facilitating the uptake may have an inherent pH dependence which will be superimposed upon the effects of pH on the ionization state of WR-1065 making the overall pH dependence potentially even more complex.

If uptake of WR-1065 involves passive diffusion of the (000) form then the rate should be determined by the composition of the plasma membrane and should be essentially the same for all mammalian cell lines. This appears to be the case (Table 3). Uptake by a mediated process would not necessarily be expected to occur at the same rate in different cell lines but such a mechanism is not rigorously excluded by this finding. Thus, all of the evidence points to passive diffusion of the (000) form as the mechanism of WR-1065 uptake but it does not rigorously exclude a mechanism mediated by a membrane transport system.

To what extent are these findings relevant to uptake of the drug in animal tissues? In animals injected with WR-2721 we found WR-1065 in tissues at roughly 1-mM levels within 15 min after injection (28). This would correspond to an uptake rate of ~0.07 mM min^{-1} which is equivalent to a rate of about 0.12 nmol $(10^6 \text{ cell})^{-1}min^{-1}$ in V79-171 cells at 37°C or 0.027 nmol $(10^6 \text{ cell})^{-1}min^{-1}$ at 23°C. From Fig. 5 the rate constant at the approximate extracellular pH of 7.3 is 0.025 nmol $(10^6 \text{ cell})^{-1}$ $min^{-1}mM^{-1}$ from which we can estimate that a concentration of ~1 mM WR-1065 would be required to produce the observed rate. This concentration is greater than the measured values of WR-1065 in plasma which are in the micromolar range for rats following injection of WR-2721 but there are technical problems in obtaining reliable measurements and the true value could be larger (29). It is also possible that the value measured in venous samples of plasma is not representative of tissue capillary levels which could be higher due to rapid dephosphorylation by alkaline phosphatase which occurs at high levels in the endothelium of arteries and arterioles (30). In any case, the rate of uptake of WR-1065 measured here with V79 cells is clearly of the correct order to explain the observed uptake in animals.

There are a number of implications of the present findings for the selective uptake of the drug by normal tissues as compared with tumors. The "normal" and "tumor" cell lines studied here behaved similarly with respect to the uptake of WR-1065 so that selective uptake by normal tissues in animals injected with the drug WR-2721 is unlikely to result from inherent differences in their cellular response to WR-1065. The basis for selective uptake by normal tissue must therefore be sought in differences in the microenvironment at the tissue level. One important difference involves alkaline phosphatase. Alkaline phosphatase is a cell surface enzyme which occurs at high levels in capillaries (30, 31) and efficiently dephosphorylates WR-2721 (17, 32, 33). Since tumors are poorly vascularized it is expected that they would have low levels of alkaline phosphatase and this is often the case (31). However, those capillaries which do occur in tumors also appear to have lower alkaline phosphatase activity than capillaries of normal tissue (34) which may be another key factor. In addition, tumors generally have a lower pH than normal tissues (35, 36) which means that what alkaline phosphatase they do contain will be operating less efficiently than the same amount of enzyme in a normal tissue. Thus, one reason for the low uptake of drug in WR-2721-treated animals seems to be the limited ability of tumors to convert WR-2721 to WR-1065, the form of the drug taken up by both normal and tumor cells.

A second factor likely to prove important in the selective action of WR-2721 is the pH dependence of the uptake of WR-1065 by cells. The predominantly anaerobic metabolism of tumor tissues causes their pH to be lower than that of normal tissues, typically by several tenths of a pH unit or more (35, 36). The sharp decrease in the rate of uptake of WR-1065 with decreasing pH (Fig. 5) predicts a more than twofold fall in uptake rate as the pH is decreased by 0.3 units. This means that even if WR-1065 does become available to tumor tissue it will not be absorbed by the cells at a rate comparable to that of normal tissue where the extracellular pH is higher. It thus seems likely that both pH and alkaline phosphatase activity are determinants of WR-2721 uptake and that low uptake by tumors is a combined effect of both of these factors being low in tumors relative to normal tissues.

The present view of the mechanism of uptake differs markedly from that of Yuhas (12) who ascribed the selective uptake to an active transport of WR-2721 in normal tissues as contrasted with a slower uptake by tumors via passive diffusion of WR-2721 across the plasma membrane. Using 1 mm^3 tumor and liver cell cubes incubated with 100 μM [^{14}C]WR-2721 he observed a 4-fold greater uptake of radiolabel by normal than by tumor cells. He assumed that WR-2721 was the form of the drug taken up but it is possible that alkaline phosphatase associated with the tissues, and present to a greater extent in liver than in tumor, converted the drug to WR-1065. The observed rate for uptake by the liver preparation was ~1 μM min^{-1} (12) at 37°C. Using our rate constant at 37°C and pH 7.2 (Table 2) and converting to mM units we can estimate that a WR-1065 concentration as low as 25 μM could account for this rate.

Yuhas also observed that the radiolabel from [^{14}C]WR-2721 was concentrated about twofold in liver cubes and this was taken as evidence for active transport (12). However, if uptake and export of a basic substance such as WR-1065 occurs via the uncharged form then it will be concentrated in cells (37) or subcellular organelles (38) if the pH is lower there than in the extracellular medium. Thus, intracellular concentration is compatible with uptake by passive diffusion of the uncharged form of WR-1065, providing that the effective intracellular pH is lower than the extracellular pH. Such pH effects on the equilibrium values of the intracellular:extracellular WR-1065 ratio represent an additional factor to the kinetic considerations discussed above which will have an impact upon the level of drug that accumulates in a given tissue. The selective uptake by normal as compared with tumor tissue may involve equilibrium as well as kinetic factors. The relative importance of kinetic *versus* equilibrium control in determining the distribution of WR-1065 in animals treated with WR-2721 could be important in establishing the most effective time course for drug delivery. Experimental studies of the relationship between the equilibrium for WR-1065 distribution and pH may therefore prove useful.

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