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ORIGINAL ARTICLE

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Circumvention of P-glycoprotein-mediated multidrug resistance by S16020-2: kinetics of uptake and efflux in sensitive and resistant cell lines

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Abstract *Purpose*: In contrast to Adriamycin (ADR), the novel olivacine derivative S16020-2 has demonstrated potent antitumor activity in vitro and in vivo against cell lines displaying the P-glycoprotein (Pgp)-mediated multidrug-resistance phenotype (MDR), suggesting that this compound is not transported by Pgp. The purpose of this work was to study the accumulation of S16020-2 in Pgp-overexpressing cells. *Methods*: The kinetics of accumulation and retention of radiolabeled S16020-2 and ADR in sensitive KB-3-1, P388, and S1 cells and their resistant counterparts KB-A1, P388/VCR-20, and S1/tMDR cells were investigated. *Results*: The rates of efflux of S16020-2 and ADR were similar and were higher in KB-A1 cells than in KB-3-1 cells. A modulator of MDR, S9788, inhibited the efflux of both compounds only in KB-A1 cells. These results demonstrate that S16020-2 is effectively transported by Pgp overexpressed by KB-A1 cells with an efficiency close to that of ADR. A similar conclusion was obtained with the P388/VCR-20 cell line. In addition, the initial rate of uptake and the accumulation of S16020-2 were markedly higher than those of ADR in the cell lines tested. *Conclusions*: The cytotoxic potency of S16020-2 toward tumor cells overexpressing Pgp is thus likely to be due to its rapid rate of uptake, which bypasses Pgp and thus leads to a high cellular accumulation.

Key words Accumulation · Adriamycin · Efflux · MDR · P-glycoprotein · S16020-2 uptake

Introduction

S16020-2 (NSC-659687) is a novel olivacine derivative that binds to DNA by intercalation and stimulates DNA cleavage mediated by topoisomerase II by stabilizing the covalent enzyme-DNA complex [17]. This derivative is a potent cytotoxic agent [14, 19] that has demonstrated a broad spectrum of antitumor activity against a panel of murine tumor models and human tumors xenografted into nude mice [8, 9, 15]. Due to its favorable pharmacokinetic characteristics and acceptable toxicity in different species [25], this compound was recently subjected to phase I clinical trials.

Among the pharmacological properties of S16020-2, the sensitivity of cell lines displaying the P-glycoprotein (Pgp)-mediated multidrug-resistance phenotype (MDR) was noteworthy, since previously described olivacine derivatives were markedly less potent against resistant cell lines than against their parental sensitive counterparts [2]. Although S16020-2 was as potent as Adriamycin (ADR) when tested in vitro on a panel of non-MDR tumor lines, cell lines overexpressing Pgp were more sensitive to S16020-2 than to ADR [19]. Moreover, S16020-2 retained marked antitumor activity in vivo against MDR sublines of P388 leukemia, including a highly resistant cell line transfected by human MDR1 cDNA, against which ADR was either moderately active or inactive [8]. Since the main mechanism of resistance of these cells relies on the ability of Pgp to expel the drugs from the cells, it was hypothesized that S16020-2 was not recognized by Pgp, unlike ADR, which is a good substrate.

Therefore, the kinetics of uptake and efflux of radiolabeled S16020-2 were investigated in pairs of sensitive and resistant cells to better our understanding of the exact mechanism by which this drug circumvents MDR. ADR was chosen as a reference compound because the two drugs probably share a closely related molecular mechanism of action [3, 17]. A modulator of MDR, S9788 [10, 21, 24], was used to inhibit the Pgp-mediated efflux of the two radiolabeled drugs in KB sublines. This modulator has been shown to sensitize MDR cells to cytotoxic agents by increasing their accumulation and retention [18, 23].

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Materials and methods

Chemicals and radiochemicals

ADR was purchased from Carlo-Erba (Paris, France); S16020-2 and S9788 were synthesized as previously described [5, 11]. [^{3}H]-S16020-2 (Commissariat à l'Energie Atomique, Saclay, France; specific activity 1443 GBq mmol^{-1}, radiochemical purity 97%) was first synthesized in a limited amount and was then replaced by [^{14}C]-S16020-2 (Isotopchim, Ganagobie-Peyruis, France; specific activity 2110 MBq mmol^{-1}, radiochemical purity ≥ 98%). The radiochemical purity of labeled S16020-2 was regularly controlled by high-performance liquid chromatography (HPLC). [^{14}C]-ADR (specific activity 2110 MBq mmol^{-1}, radiochemical purity 95.1%.) was obtained from Amersham (Les Ulis, France).

Cell lines and inhibition of cell proliferation

P388 and P388/VCR murine leukemias were acquired from the National Cancer Institute (NCI, Bethesda, Md., USA). The MDR subline P388/VCR-20 was established in our laboratory by culturing of P388/VCR cells in the presence of 20 n*M* vincristine (VCR) [23]. The KB-3-1 human epidermoid oral carcinoma cell line and its MDR subline KB-A1 were obtained from Dr. M. Gottesman (NCI). The human lung epidermoid carcinoma cell line S1 and the S1/tMDR subline, which is transfected by human MDR1 cDNA, were a generous gift from Dr. F. Bass (Netherlands Cancer Institute, Amsterdam, The Netherlands). The Pgp expression was estimated by flow cytometry with the monoclonal antibodies C219 and MRK16 as described elsewhere [18, 23].

Cells were cultured at 37 °C in an atmosphere containing 5% CO_2 either in DMEM (KB-3-1 and KB-A1 cells) or in RPMI1640 medium supplemented with 10% decomplemented fetal calf serum, 2 m*M* L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml, and 10 m*M* HEPES buffer, (pH 7.4). All media and supplements were obtained from Life Technologies (Cergy-Pontoise, France). The proliferation assay was performed essentially as described [19]. Briefly, celles were seeded in 96-well microplates at a density of 750 cells/well (KB-3-1, KB-A1) or 450 cells/well (S1, S1/tMDR) 48 h before the experiment. For the non-adherent cells (P388, P388/VCR-20), the drugs were diluted first in 75 μl of complete medium and 75 μl of the cell suspension at a density of 5×10^4/ml were then added. Cells were exposed to the drugs for 48 h (P388 cell lines) or 96 h (KB and S1 cell lines). At the end of this period, the plates were stained by 3-(4, 5 dimethylithiazol-2yl)-2,5-dipheyyltetrazolium bromide and the IC50 was determined. Paired experiments were performed and the mean IC50 ± SEM was calculated.

Accumulation and retention of labelled compounds

KB-3-1, KB-A1, S1 and S1/tMDR cells. Cell monolayers in 6-well plates (about 10^6 cells/well) were incubated at 37 °C in 2 ml of complete culture medium containing the labelled drug. At various times, 3 identical wells were rapidly aspirated and washed twice with 2 ml of PBS at 0 °C. Cells were then lyzed overnight with 1 ml of 1% Triton X100 and radioactivity was counted. The cells of three control wells were harvested with trypsin and numerated. The drug retention was measured in the same experiment as follows. Cells were incubated for 2 h as above, washed twice with 2 ml of complete culture medium at 0 °C and further incubated in 2 ml of drug-free culture medium at 37 °C. At the indicated times, 3 identical wells were processed as above.

P388 and P388/VCR-20 cells. For uptake analyses, suspensions of P388 and P388/VCR-20 cells, at a density of 10^6cells/ml, were incubated with 100 nM [^{14}C]ADR or [^{14}C]S16020-2 at 37 °C. At the indicated times, 3 samples of 1 ml were layered on glass microfibre filters GF/B (Whatman, Maidstone, England) previously coated with 2 ml of complete culture medium, and gently aspirated. Filters were then rapidly washed 4 times with 1 ml of PBS at 0 °C and the radioactivity was counted. For retention studies the cell suspensions, which had previously been incubated with the labeled drugs for 2 h at 37 °C, were washed with complete culture medium at 0 °C and then resuspended in drug-free complete culture medium at 37 °C. At various times, three aliquots of 1 ml were sampled and processed as described above.

For all cell lines the cell-associated radioactivity measured after 5 s of incubation at 0 °C was considered to represent nonspecific adsorption of the drugs to the cell surface and was therefore subtracted from each uptake value. Results are expressed as picomolds of drug associated with 10^6 cells. The apparent initial rate of uptake was estimated from the values measured within the first 5 min of incubation and was expressed as picomoles per minute per 10^6 cells.

Table 1 Accumulation and retention of 100 n*M* radiolabeled S16020-2 or ADR in comparison with their cytotoxicity

Cell lines	KB-3-1	KB-A1	P 388	P388/VCT-20	S1	S1/tMDR
Pgp overexpression:						
MRK16	1	11.8 ± 0.7			1	9.4 ± 0.8
C219			1	3.4 ± 0.4		
Cytotoxicity–IC_{50} (n*M*):						
ADR	10.7 ± 1.9	5403.0 ± 712.9	16.0 ± 2.4	266.3 ± 24.5	39.6 ± 4.4	124.5 ± 16.1
S16020-2	16.6 ± 1.9	124.2 ± 27.3	5.5 ± 1.8	6.7 ± 1.4	48.6 ± 16.9	25.3 ± 2.6
VCR	0.9 ± 0.3	858.0 ± 157.0	0.6 ± 0.2	49.0 ± 10.0	2.5 ± 0.2	414.0 ± 52.0
Initial rate of uptake (pmol min^{-1}/10^6 cells)[a]:						
ADR	0.5	0.6	16.1	4.9	0.5	0.5
S16020-2	12.5	14.8	17.6	13.7	8.8	8.0
Accumulation (pmol/10^6 cells)[b]:						
ADR	78.7 ± 3.6	30.2 ± 0.2	18.8 ± 2.7	16.3 ± 0.5	26.6 ± 1.2	21.4 ± 0.6
S16020-2	110.7 ± 3.9	53.9 ± 1.4	42.7 ± 0.9	29.7 ± 4.0	73.1 ± 1.0	73.5 ± 1.6
Retention (pmol/10^6 cells)[c]:						
ADR	49.2 ± 0.9	6.8 ± 0.2	12.6 ± 0.5	4.9 ± 0.2	9.8 ± 0.5	6.7 ± 0.3
S16020-2	53.1 ± 3.4	22.3 ± 0.5	36.1 ± 0.8	18.0 ± 2.4	41.5 ± 0.3	38.1 ± 0.1

[a] Apparent initial rate of uptake estimated from values measured within the first 5 min of incubation
[b] Accumulation of radiolabeled drugs after 2 h of incubation
[c] Retention after further incubation for 2 h in drug-free medium (mean ± SEM, $n = 3$)

Results

Inhibition of cell proliferation

The IC_{50} values listed in Table 1 show that although the P388/VCR-20 and S1/tMDR cell lines were significantly resistant to ADR, they were as sensitive as their parental counterparts to S16020-2. The KB-A1 cell line was highly resistant to ADR (about 500-fold resistance) but displayed only 7-fold resistance to S16020-2.

Uptake and retention of radiolabeled drugs

KB-3-1 and KB-A1 cells

The uptake of [^{3}H]-S16020-2 or [^{14}C]-ADR by KB cell lines was measured at different concentrations (50–1100 n*M*) in the culture medium. The uptake of the two drugs at 2 h increased linearly as a function of their concentration (data not shown), which indicates that their accumulation was not saturated in these conditions. Whatever the concentration, the resistant KB-A1 cells accumulated both drugs less efficiently (2-fold less on average) than did the sensitive KB-3-1 cells. In addition, for each tested concentration and for both cell lines the uptake of [^{3}H]-S16020-2 was higher than that of [^{14}C]-ADR.

The kinetics of accumulation of 100 n*M* [^{3}H]-S16020-2 or [^{14}C]-ADR by the KB cell lines is reported in Fig. 1. The accumulation of [^{14}C]-ADR was slow and increased regularly for at least 2 h without reaching a plateau, and

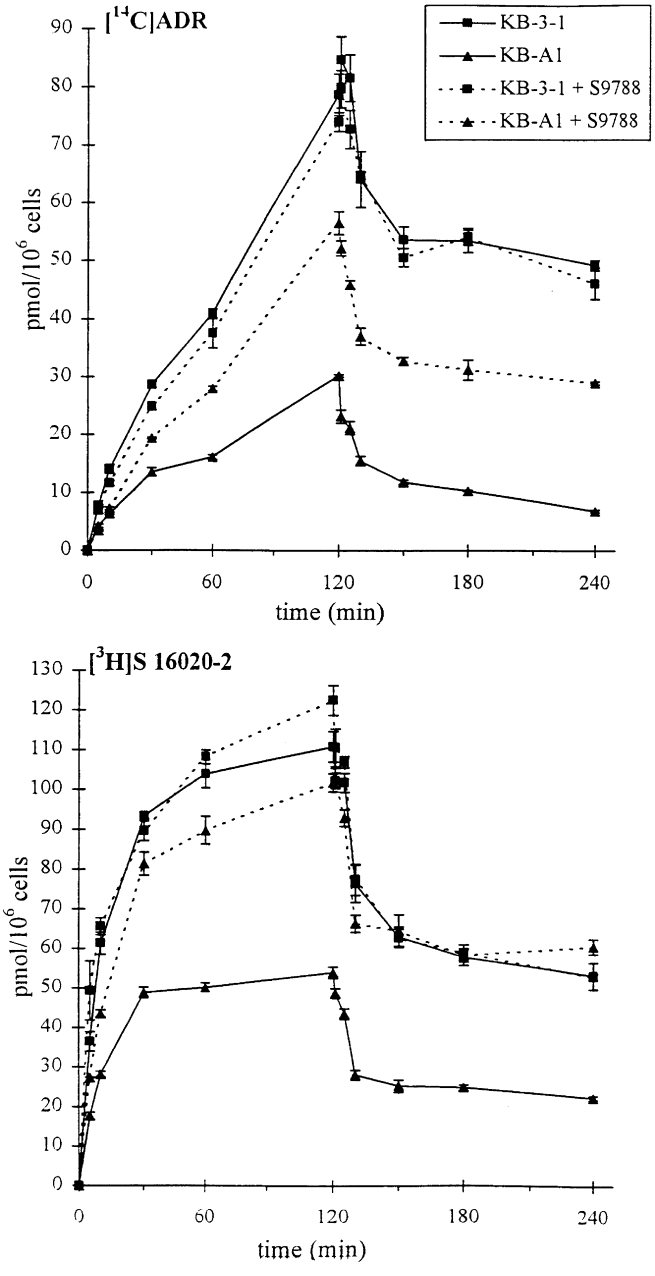

Fig. 1 Effect of S9788 on the accumulation and retention of [^{14}C]-ADR or [^{3}H]-S16020-2 by KB-3-1 and KB-A1 cells. KB-3-1 (■) or KB-A1 (▲) cells were incubated with 100 n*M* [^{3}H]-S16020-2 or [^{14}C]-ADR without (—) or with 5 μ*M* S9788(—). After 120 min of incubation, cells were washed and incubated in radiolabeled drug-free culture medium either without (—) or with 5 μ*M* S9788 (- - - -). Cell-associated radioactivity was measured at the indicated times

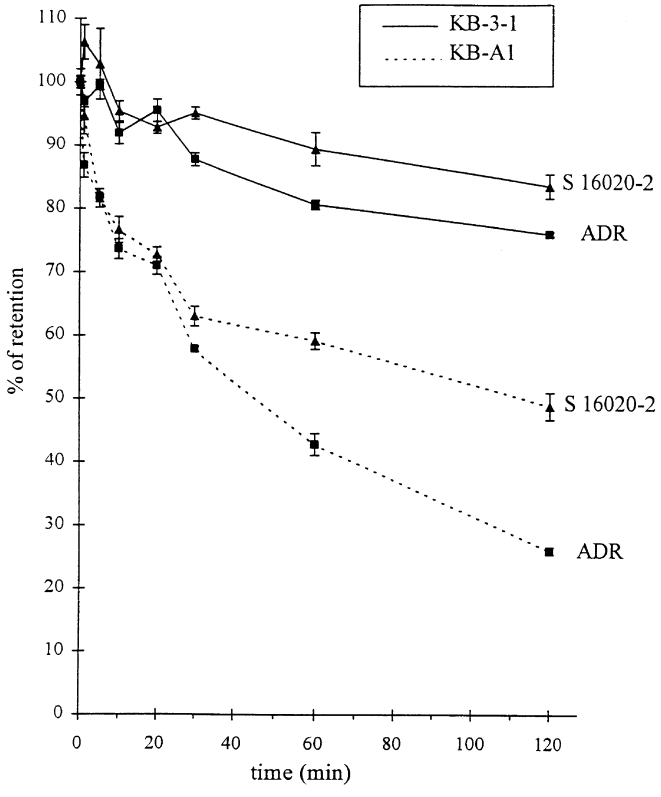

Fig. 2 Efflux of [^{3}H]-S16020-2 or [^{14}C]-ADR from KB-3-1 and KB-A1 cells. KB-3-1 cells were incubated with 200 n*M* [^{3}H]-S16020-2 (▲—▲) or 200 n*M* [^{14}C]-ADR (■–■) and KB-A1 cells, with 300 n*M* [^{3}H]-S16020-2 (▲—▲) or 400 n*M* [^{14}C]-ADR (■—■) for 2 h at 37 °C. Cells were washed and incubated in drug-free culture medium (time 0 of efflux corresponds to time 120 of uptake). The retention of the labeled drugs was measured at the indicated times, expressed as a percentage of retention, and plotted as a function of time; 100% of retention accounted for 66–106 pmol/10^6 cells

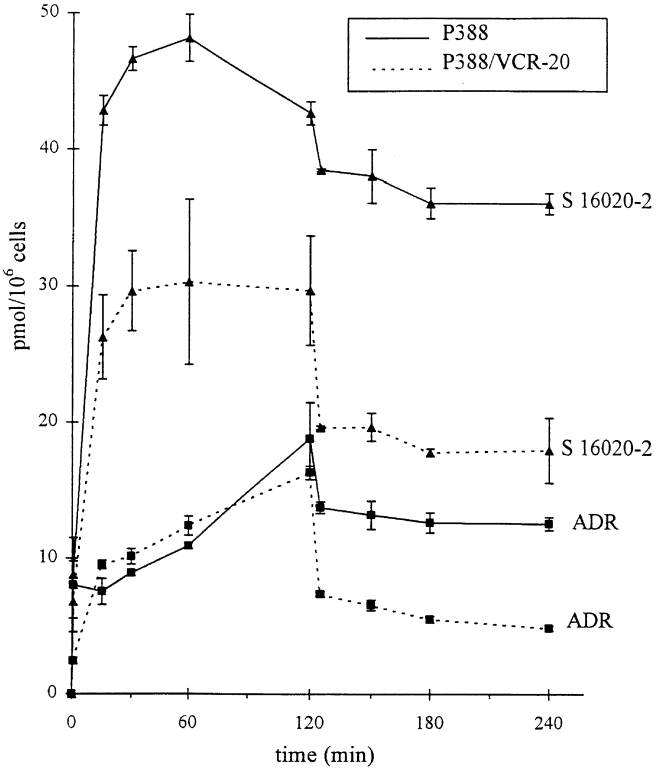

Fig. 3 Accumulation and retention of [^{14}C]-S16020-2 or [^{14}C]-ADR by P388 and P388/VCR-20 cells. P388 (—) or P388/VCR-20 (—) cells were incubated with 100 n*M* [^{14}C]-S16020-2 (▲) or [^{14}C]-ADR (■). After 120 min of incubation, cells were washed and incubated in drug-free culture medium. Cell-associated radioactivity was measured at the indicated times

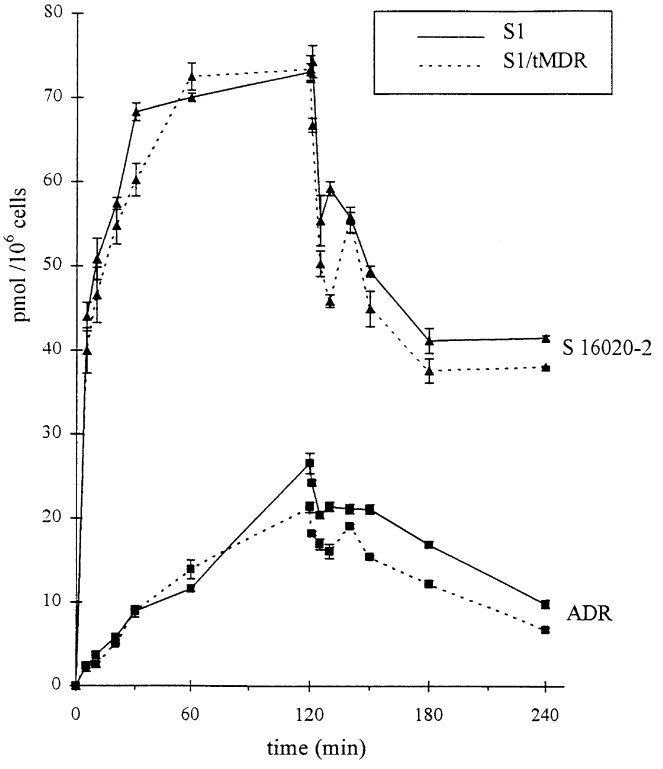

Fig. 4 Accumulation and retention of [^{3}H]-S16020-2 or [^{14}C]-ADR by S1 and S1/tMDR cells. S1 (—) or S1/tMDR (—) cells were incubated with 100 n*M* [^{3}H]-S16020-2 (▲) or [^{14}C]-ADR (■). After 120 min of incubation, cells were washed and incubated in drug-free culture medium. Cell-associated radioactivity was measured at the indicated times

both accumulation and retention were markedly lower in KB-A1 cells than in KB-3-1 cells. The accumulation of [^{3}H]-S16020-2 by the KB cell lines was rapid and reached a plateau after 60–120 min in KB-3-1 cells and after 30 min in KB-A1 cells. At the plateau the uptake of [^{3}H]-S16020-2 was 2-fold higher in KB-3-1 cells than in KB-A1 cells, and after 2 h of efflux in drug-free medium the amount of [^{3}H]-S16020-2 remaining in KB-3-1 cells was 2.4-fold higher than that measured in KB-A1 cells (Table 1). In both sensitive and resistant KB cells the apparent initial rate of uptake, the accumulation, and the retention of [^{3}H]-S16020-2 were consistently higher than those of [^{14}C]-ADR, as shown in Table 1.

The accumulation and the retention of [^{14}C]-ADR or [^{3}H]-S16020-2 by the sensitive KB-3-1 cells were not modified by S9788. The accumulation and retention of [^{14}C]-ADR were increased 1.9- and 4.3-fold, respectively, by S9788 in KB-A1 cells, albeit without reaching the levels measured in KB-3-1 cells. The modulator had a more pronounced effect on the uptake and retention of [^{3}H]-S16020-2 by KB-A1 cells. The accumulation of [^{3}H]-S16020-2 by these resistant cells was increased by S9788 to 92% of the value attained in KB-3-1 cells. In addition, the retention of [^{3}H]-S16020-2 by KB-A1 cells exposed to S9788 was similar to that of untreated KB-3-1 cells (Fig. 1).

The Pgp-mediated efflux of a compound can be indirectly estimated by comparison of the efflux measured in Pgp-overexpressing cells with the efflux measured in the parental sensitive cells. However, a direct comparison of the efflux from different cell types previously loaded with the same concentration of drugs is not informative since the accumulation can differ by a factor of up to 4, depending on which drugs and cell lines are being considered; thus, the extent of passive drug diffusion is not identical. For a more accurate comparison of the rates of Pgp-mediated efflux the KB cells were incubated with concentrations of [^{14}C]-ADR or [^{3}H]-S16020-2 yielding approximately the same accumulation after 2 h. Under these conditions the initial rates of efflux of [^{14}C]-ADR and [^{3}H]-S16020-2 were similar in the two cell lines and were higher in resistant KB-A1 cells than in sensitive KB-3-1 cells (Fig. 2), demonstrating that both drugs were transported by Pgp with the same efficiency.

P388 and P388/VCR-20 cells

The accumulation of [^{14}C]-ADR in sensitive P388 cells and resistant P388/VCR-20 cells increased rapidly during the first few minutes and then slowly up to 2 h (Fig. 3). Globally, [^{14}C]-ADR accumulation was similar in the two cell lines except that the initial rate of uptake

was 3-fold higher in P388 cells than in P388/VCR-20 cells (Table 1). The accumulation of [^{14}C]-S16020-2 by the two cell lines was also rapid for the first 15 min, and a plateau was reached by 30 min of incubation. The initial rate of uptake and the accumulation of [^{14}C]-S16020-2 were about 1.4-fold higher in P388 cells than in P388/VCR-20 cells (Table 1). After 2 h of incubation the accumulation of [^{14}C]-S16020-2 was about 2-fold higher than that of [^{14}C]-ADR in both sensitive and resistant cells.

The rate of efflux of [^{14}C]-ADR was higher in P388/VCR-20 cells than in P388 cells, and the amount of [^{14}C]-ADR remaining associated with the cells after 120 min was 2.6-fold lower in resistant cells than in sensitive cells. Similarly, the efflux of [^{14}C]-S16020-2 was more rapid in resistant cells than in sensitive cells, and after 2 h, P388/VCR-20 cells contained 2-fold less [^{14}C]-S16020-2 than did P388 cells. The retention of [^{14}C]-S16020-2 was markedly superior to that of [^{14}C]-ADR in both cell lines (Table 1).

S1 and S1/tMDR cells

Figure 4 shows the kinetics of uptake and retention of [^{14}C]-ADR or [^{3}H]-S16020-2 by S1 cells and MDR1-transfected S1/tMDR cells. The accumulation of [^{14}C]-ADR by S1 or S1/tMDR cells increased regularly and was similar in these two cell lines. The accumulation of [^{3}H]-S16020-2 was more rapid and was similar in the two cell lines, a plateau being reached at 30–60 min. The retention of [^{14}C]-ADR was slightly lower in S1/tMDR cells than in S1 cells, whereas the efflux of [^{3}H]-S16020-2 was similar in sensitive and resistant cells. The uptake and the retention of [^{3}H]-S16020-2 were markedly higher than those of [^{14}C]-ADR in both cell lines (Table 1).

Discussion

The aim of the present study was to investigate the cellular kinetics of S16020-2, a new olivacine derivative, to improve our understanding of some of its pharmacological properties, namely, its ability to circumvent MDR in vitro and in vivo and to exert a potent cytotoxic effect even after a short-term exposure of the cells [19]. To study the transport of S16020-2 by Pgp we measured the kinetics of accumulation and retention of radiolabeled S16020-2 in three resistant cell lines, differing in the mode of induction of their resistance, in comparison with their parental sensitive counterparts.

The resistance of KB-A1 cells was selected using 2 μ*M* ADR, a relatively high concentration, which implies a high degree of resistance to ADR linked to a high level of expression of Pgp and the possible contribution of other mechanisms in the overall resistance to ADR and compounds interacting with topoisomerase II. Both the accumulation and the retention of ADR were markedly lower in KB-A1 cells than in KB-3-1 cells, indicating that this drug is actively transported by the Pgp overexpressed by KB-A1 cells. The accumulation and retention of S16020-2 were 2- and 3-fold lower, respectively, in resistant cells than in sensitive KB cells, and the rate and extent of efflux of closely related intracellular concentrations of S16020-2 and ADR were similar and were higher in KB-A1 cells than in KB-3-1 cells.

To confirm that S16020-2 was actively effluxed by Pgp in KB-A1 cells we used S9788, a modulator of Pgp-mediated MDR. The accumulation of ADR or S16020-2, although not modified by S9788 in KB-3-1 cells, were markedly increased by this modulator in KB-A1 cells. Moreover, the retention of S16020-2 by KB-A1 cells exposed to S9788 was similar to its retention by KB-3-1 cells. Taken together, these results demonstrate that S16020-2 was transported by Pgp expressed by KB-A1 cells.

The resistance of the P388/VCR-20 cell line was selected using 20 n*M* VCR, which a priori ruled out mechanisms of resistance linked to the mechanism of action of ADR or S16020-2. With regard to ADR the main difference between P388 and P388/VCR-20 cells was found in the efflux, which was 2- to 3-fold higher in the resistant cells. A similar difference was observed for S16020-2, which was retained to a 2-fold greater extent by sensitive cells than by resistant cells. These results show that these two drugs were equally transported by the Pgp expressed by P388/VCR-20 cells.

The S1/tMDR cell line was obtained by transfection of the S1 cells with human MDR1 cDNA. This line was shown to be markedly resistant to actinomycin D and to vinca alkaloids such as vinblastine and vincristine and to be weakly resistant to ADR and etoposide (VP-16) [24]. In a previous study the uptake and the retention of VCR were found to be 5- and 30-fold lower in transfected cells than in parental cells, demonstrating a functional Pgp in the resistant cells [23]. Surprisingly, the retention of ADR was only slightly lower in S1/tMDR cells than in S1 cells, whereas S16020-2 was similarly accumulated and retained by both cell lines. These results demonstrate that in contrast to VCR, ADR was only weakly transported by the Pgp overexpressed by S1/tMDR cells and that S16020-2 was not a substrate for this protein. Although the molecular mechanisms underlying the substrate specificity of Pgp remain unresolved, one possibility would be that resistant lines overexpress isoforms of Pgp displaying a specific pattern of substrate specificity and, thereby, a specific pattern of cross-resistance. This hypothesis is supported by observations that the Pgp that have mutated in certain domains have an altered pattern of cross-resistance that correlates with modifications in the accumulation of the drugs [12, 26].

A rapid and high level of uptake of S16020-2 was observed in the cell lines used as compared with ADR. Drug uptake of protonable compounds is thought to occur through passive diffusion of the neutral form of the molecule. The nitrogen atom of the side chain of S16020-2 is mainly under the protonated form at phys-

iological pH, its p*K*a being about 8.3. Consequently, S16020-2 does not greatly differ from other protonable antitumor drugs. Rather than the charge of anthracyclines, their lipophilicity was proposed to be the main determinant for their passage through biological membranes [6]. Several anthracycline derivatives of varying lipophilicity were more potent than ADR in MDR cells, although they were similarly transported by Pgp in those cells [1, 20, 22]. Consequently, the lower degree of cross-resistance of MDR cells to lipophilic anthracycline derivatives appeared to be due to an increased level of uptake rather than a decreased extent of transport by Pgp. In sensitive and resistant P388 and KB cell lines we found similar properties for S16020-2, which is significantly more lipophilic than ADR as shown by a logP value of 3 versus 0.08 [16], respectively.

According to the "hydrophobic vacuum cleaner" model of Pgp function [7], the compounds are expelled before they reach their intracellular targets. One possibility is that S16020-2 would be capable of interacting rapidly with the lipid bilayer because of its optimal physicochemical properties and would therefore saturate the limited number of binding sites on the Pgp. A significant proportion of the molecules bound to the membrane would then rapidly enter the intracellular space and reach their nuclear target, the DNA-topoisomerase II complex. The moderate cross-resistance of KB-A1 cells to S16020-2 could be due to the high number of Pgp molecules expressed by this cell line or to other modifications such as altered distribution [13, 27, 28] or modification of topoisomerase II [4, 29] observed in MDR cell lines selected using ADR.

In conclusion, our results show that Pgp transports ADR and S16020-2 with about the same efficiency. The accumulation of the two drugs by resistant cells then depends mainly on the rate and extent of their uptake. The cytotoxic potency of S16020-2 on Pgp-overexpressing cells is thus likely to be due to its high uptake rate, which, in turn, can be linked to its lipophilicity. Whatever the precise mechanism involved, the activity of S16020-2 on MDR tumors observed in experimental models in vitro and in vivo might translate to the human diseases, conferring to this drug a therapeutic advantage against Pgp-overexpressing tumors.

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