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KW-2149 (7-N-[2-[Y-L-glutamylamino]ethyldithioethyl]mitomycin C)

A NEW MITOMYCIN C ANALOGUE ACTIVATED BY SERUM

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ABSTRACT. KW-2149 (7-N-[2-[Y-L-glutamylamino]ethyldithioethyl]mitomycin C) is a new mitomycin-C analogue in clinical trial. This study demonstrates that KW-2149, unlike mitomycin C, is activated to a cytotoxic species by extracellular metabolism in serum. The metabolising activity differs between batches of serum and species of origin. Human serum had high activity (which resulted in a 150-fold enhancement of cytotoxicity), whereas mouse serum had low activity. In the presence of serum, the rate of uptake of ^{3}H-KW-2149 into cells increased by 8-fold and drug binding to DNA by 32-fold. The metabolising activity of serum can partially be replaced by glutathione. No anticancer drug has previously been described whose toxicity is mediated by metabolism in serum. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:279–285, 1997.

KEY WORDS. new anticancer agent; mitomycin C; intravesical chemotherapy; bladder cancer; serum factor; drug metabolism

KW-2149# is a water-soluble analogue of mitomycin C [1] which is currently in clinical trial [2]. It has a spectrum of antitumor activity similar to mitomycin C against a range of experimental murine tumors and human tumor xenografts *in vivo* but is less myelosuppressive [3] and lacks cross resistance against mitomycin C-resistant tumors *in vitro* and *in vivo* [4–7]. *In vitro* studies have demonstrated that KW-2149 is 10–100 times more cytotoxic than mitomycin C on a molar basis to 23 human cancer cell lines, including two bladder cancer cell lines [5]. However, in contrast to this study [5], we found that KW-2149 was less cytotoxic than mitomycin C to bladder cancer cells. Because of this disparity, further experiments were undertaken to compare assay techniques, cell lines and serum. We discovered that the cytotoxicity of KW-2149 is dependent on the presence of a serum factor and that there are differences in metabolising activity between species of origin and batches of serum, thus explaining the disparity between our data and those of previous reports.

MATERIALS AND METHODS

Cytotoxicity Measurements

The origins of the human bladder cell lines used in this study (see Table 1) have been described previously [8]. All the lines were grown under identical culture conditions in RPMI 1640 medium containing FCS and 2 mM L-glutamine. Each cell line was used over a maximum of 10 passages. To measure cytotoxicity, 500–3000 single cells (depending on the plating efficiency of the cell line) were plated in 5-cm plastic Petri dishes in 5 mL RPMI 1640 medium containing 5% FCS and incubated overnight to allow the cells to attach. The same batch of serum was used throughout (Imperial Laboratories, Andover, UK; batch 0890133, heat-inactivated at 56°C for 30 min). Mitomycin C and KW-2149 were supplied by Kyowa Hakko Kogyo (Tokyo, Japan) in glass vials containing 2 and 10 mg of drug as powder, respectively, and stored at 4°C in the dark. Immediately before use, the drugs were dissolved in 10 mL distilled water and further diluted in RPMI 1640 medium. Following a 1-hr exposure, the drug-containing medium was removed. After two washes with 5 mL of medium, the cells were incubated for another 10–21 days, depending on

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Abbreviations: dpm, disintegrations per minute; FCS, fetal calf serum; HPLC, high pressure liquid chromatography; IC_{50}, concentration reducing colony-forming ability by 50%; KW-2149, 7-N-[2-[Y-L-glutamylamino] ethyldithioethyl]mitomycin C; PBS, phosphate buffered saline; UV, ultraviolet.

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TABLE 1. Origins of the cell lines used in this study

Cell line	Origin	Previous therapy
PS-1	Bladder primary	None
VM-CUB-1	Bladder primary	NR
RT4	Bladder recurrence	Gold grains 2 years earlier
MGH-U1	Bladder recurrence	None
HU456	Bladder recurrence	None
HU961T	Bladder recurrence	None
COLO232	Bladder primary	4000 rads 2 months preoperatively
KK47	Bladder primary	NR
HT1376	Bladder primary	None
TCCSUP	Bladder primary	None
253J	Retroperitoneal lymph node met.	NR
HT1197	Bladder recurrence	None
SCaBER	Bladder primary	None
RT112	Bladder primary	None
RT112CP	Bladder primary	Cisplatin *in vitro*
RT112MMC	Bladder primary	Mitomycin C *in vitro*
HCV29	"Normal" bladder from patient with bladder cancer	NR
HU609	"Normal" ureter from patient with renal cell carcinoma	NR

NR = not recorded

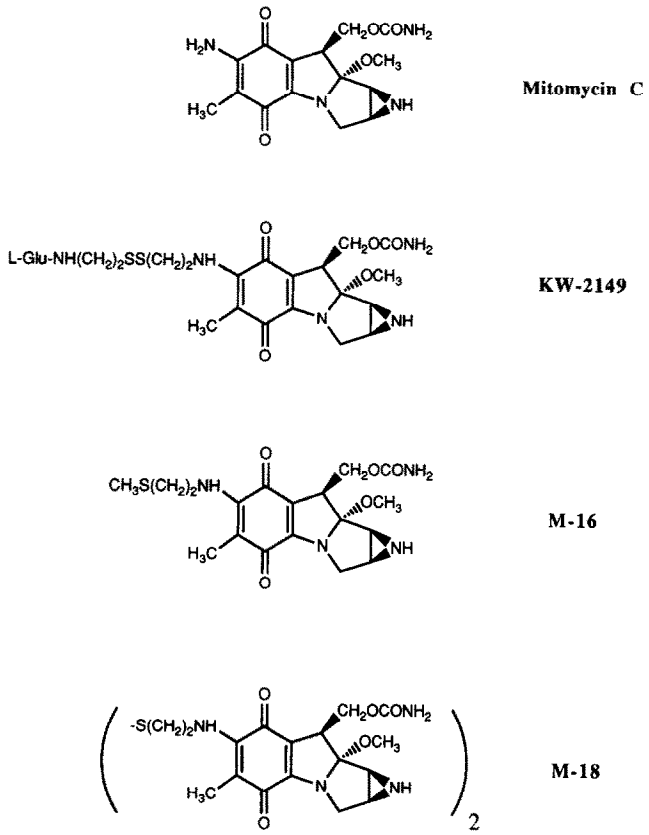

FIG. 1. Chemical structures of mitomycin C, the derivative KW-2149 and its metabolites M-16 and M-18.

the growth rate of the cells, to allow colonies to develop. The mean number of colonies at each drug concentration (average of three) was expressed as a percentage of that of the untreated controls (average of five). Using the OXSTAT program, data on the linear parts of the dose–response curves were analysed by linear regression analysis and IC_{50}s calculated. The data are derived from two or more replicate experiments.

Subsequent studies used the human bladder cancer cell line RT112 growing in RPMI 1640 medium supplemented with 2 mM L-glutamine and a range (5–50%) of the same batch of Imperial FCS (as above), 5–50% FCS (Globepharm, Esher, UK, batch 2249, heat-inactivated as above) or 10% dialysed FCS (Gibco, Paisley, UK; batch number 38N0428, heat-inactivated as above), again using 1-hr exposure to drugs. Mouse serum was obtained from Advanced Protein Products (Brockmoor, UK; batch 5545, heat-inactivated as above). Human blood samples were obtained from eight healthy volunteers (6 men aged 31–50 years and 2 women aged 31 and 33 years) and allowed to clot, and the serum was separated by centrifugation, heat-inactivated as above and stored at −20°C until use. Glutathione (GSH, Sigma, Poole, UK; product number G-6013) was dissolved in distilled water immediately before use. Mitomycin C, KW-2149 and its metabolites M-16 and M-18 [9] were kindly supplied by Kyowa Hakko Kogyo (see Fig. 1).

Drug Uptake

The 100,000 RT112 cells were plated per well in 96-well flat-bottomed plates (Nunc) in 200 mL of complete medium (RPMI 1640 supplemented with 10% Globepharm FCS and 2 mM L-glutamine) in triplicate for each experimental condition. After 24 hr incubation, the medium was replaced with 50 mL fresh medium containing 1 mC/ml ^{3}H-KW-2149 (Kyowa Hakko Kogyo; specific activity 44 Ci/mmol). After 30, 60 or 120 min exposure, the medium was removed and the cells washed four times with 200 μL complete medium. Five aliquots of 200 μL of 1 N NaOH were added to each well and collected. The NaOH washes containing the cells were bulked and neutralised with 1 mL of 1 N HCl, and 3 mL of distilled water and 10 mL of EcoScint (National Diagnostics, Atlanta, GA, USA) were added. The radioactivity was measured from triplicate wells, the background subtracted (wells without cells) and experiments repeated for each condition between two and six times.

Metabolism of KW-2149 in Serum

KW-2149 (100 μM) was incubated at 37°C in different concentrations of FCS in either RPMI 1640 medium or PBS. An aliquot (10 μL) was taken every 6.5 min and injected onto an HPLC column (Anasil Ana80 5ODS2 150 × 4.6 mm; Anachem, Luton, UK) and eluted isocratically

with 55% methanol in 100 mM ammonium acetate (pH 4) at 2.0 mL/min. The eluate was continuously monitored at 370 and 260 nm. The concentration of KW-2149 was calculated by comparing the peak area with that of a standard. In addition, the sample was eluted with a gradient (0–99% methanol in 100 mM ammonium acetate, pH 4, over 30 min) at 1.5 mL/min. The metabolism of KW-2149 also was monitored spectrophotometrically. KW-2149 (100 μM) was incubated at 37°C in PBS alone or with 25% FCS (Globepharm). The spectrum was recorded every 15 min between 250 and 600 nm.

^{3}H-KW-2149 and ^{3}H-Mitomycin C Binding to Cell and Naked DNA

DNA was prepared using a phenol extraction method [10]. Cells (2×10^7) were treated for 1 hr with ^{3}H-KW-2149 or ^{3}H-mitomycin C in RPMI 1640 alone or containing 10% FCS (Globepharm). The cells were then washed (×3) with 25 mL of ice-cold PBS and detached by trypsinisation (5 mL trypsin/versene at 37°C for 5 min) and then collected into 10 mL of PBS and pelleted. The cell pellet was lysed with a *p*-aminosalicylic acid solution (5 mL) and extracted with an equal volume of phenol solution. DNA was precipitated from the aqueous phase with ethoxyethanol, washed extensively with 70% ethanol and dissolved in 1 mL of water. This solution was treated with protease and RNAase (both at a final concentration of 10 μg/mL) for 15 min at 37°C. Sodium acetate was added to 1% (w/v) and the DNA reprecipitated with absolute ethanol. The DNA was washed extensively with 70% ethanol and then redissolved in 1 mL of water. A 50 μL aliquot was removed for determination of the concentration of DNA by UV spectroscopy. For measurement of the radioactive concentrations, 750 μL of the DNA solution were added to a scintillation vial, 1 mL of Soluene 350 (Packard) tissue solubiliser was also added and incubated overnight at 37°C. Scintillation fluid [20 mL; Uniscint BD (high salt), National Diagnostics, NY, USA] was added to each sample and the ^{3}H activity determined by liquid scintillation counting.

Calf thymus DNA (1 mL, 1 mg/mL, Type I, Sigma) was incubated with ^{3}H-KW-2149 (concentration adjusted to 100 μM with unlabelled KW-2149) in PBS alone or with 50% FCS (Globepharm) at 37°C for 2 hr. The DNA was precipitated with ethanol and extensively washed (×5) with 70% ethanol and redissolved in 1 mL of water.

RESULTS

Comparison of The Cytotoxicities of KW-2149 and Mitomycin C

In the batch of serum from Imperial Laboratories (low metabolising activity), mitomycin C was more cytotoxic than KW-2149 to bladder cancer cell lines (Table 2). The IC_{50}s were 106.2–354.6 ng/mL (318–1062 nM) for mitomycin C and 442–1708 ng/mL (738–2853 nM) for KW-2149. On a weight basis, the IC_{50}s for KW-2149 were 1.8 to 5.2-fold greater than those for mitomycin C. Two lines derived from normal urothelium, HU609 and HCV29, were within the ranges of the cancer cell lines in their sensitivities to both drugs. A cisplatin-resistant subline of RT112 was 1.4- and 1.9-fold more resistant than the parent line to mitomycin C and KW-2149, respectively, when comparing IC_{50}s. A mitomycin C-resistant subline of RT112 was 7.2-fold more resistant to mitomycin C than was the parent line when comparing IC_{50}s, but there was no cross resistance to KW-2149 (Table 2).

Influence of Serum on the Cytotoxicity of KW-2149 to RT112 Cells

Changing the batch of FCS made a dramatic impact on the cytotoxicity of KW-2149 to RT112 cells. In 5% serum

TABLE 2. Mean IC_{50} concentrations of mitomycin C and KW-2149

Cell line	Mitomycin C mean IC_{50} (ng/mL ± SE)	KW-2149 mean IC_{50} (ng/mL ± SE)	Ratio of IC_{50}s K/M
PS1	106 ± 7.5	502 ± 47	4.7
VM-CUB-I	218 ± 17.8	954 ± 73	4.4
RT4	241 ± 23.9	992 ± 355	4.1
MGH-U1	207 ± 9.7	442 ± 232	2.1
COLO232	153 ± 3.3	760 ± 167	5.0
KK47	277 ± 40.9	1095 ± 344	4.0
HT1376	283 ± 20.9	842 ± 44	3.0
TCCSUP	312 ± 19.3	560 ± 63	1.8
253J	326 ± 54.3	1708 ± 270	5.2
HT1197	231 ± 63.2	637 ± 409	2.8
SCaBER	355 ± 10.8	1210 ± 276	3.4
RT112	221 ± 33.8	952 ± 220	4.3
RT112CP	302 ± 22.7	1851 ± 63	6.1
RT112MMC	1600 ± 48.3	724 ± 298	0.5
HCV29	220 ± 21.2	803 ± 323	3.7
HU609	128 ± 36.4	585 ± 286	4.6

(Globepharm, high metabolising activity), the IC_{50} was reduced from 952 ng/mL to 27.4 ng/mL (Fig. 2A and Table 3). Cytotoxicity increased with increasing serum concentration, such that the IC_{50} was further reduced to 11.1 ng/mL in 50% serum. The cytotoxicity of KW-2149 appeared to be relatively stable because a 2-hr delay between dissolving the drug (in water or RPMI 1640 supplemented with 10% FCS) and addition to the cells did not reduce its cytotoxicity (data not shown). In the absence of serum, the IC_{50} increased to nearly 4000 ng/mL^{-1} (Fig. 2A).

In human serum, the cytotoxicity of KW-2149 was high. In 5% serum from the six men, the IC_{50}s were 11.3–31.2 ng/mL; in 5% serum from the two women, the IC_{50}s were 11.3 and 22.6 ng/ml. In contrast, in 5% mouse serum the cytotoxicity was low, with an IC_{50} of 657 ng/ml.

The serum requirement for KW-2149 cytotoxicity was replaced partially by the addition of 10 μM glutathione (maximum noncytotoxic concentration) to RPMI 1640 medium (Fig. 2B). However, the addition of glutathione did not influence the cytotoxicity of KW-2149 in medium containing 10% FCS (Fig. 2B).

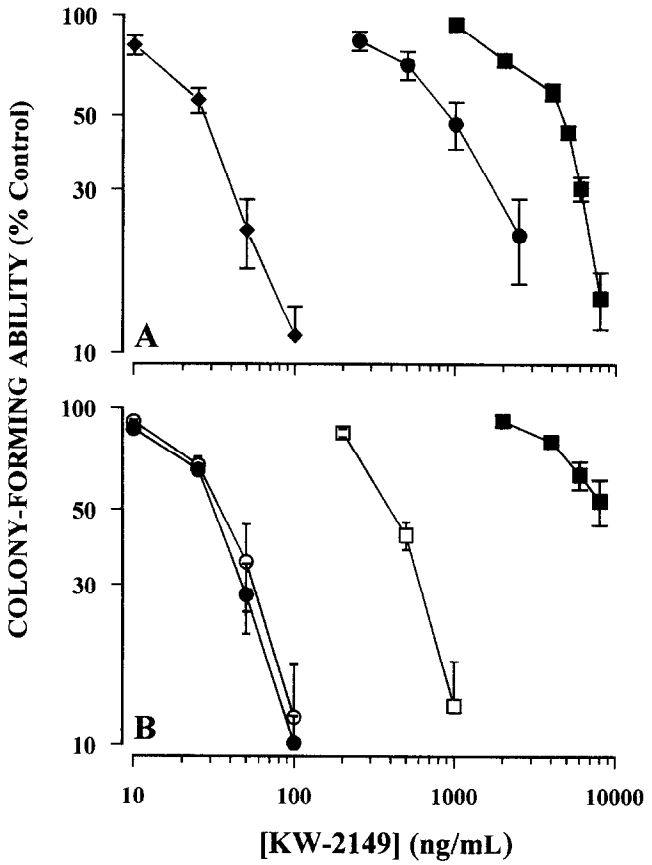

FIG. 2. The cytotoxicity of KW-2149 towards RT112 bladder cancer cells. (A) Cells were treated with a range of concentrations of KW-2149 in either RPMI 1640 medium alone (closed square) or medium supplemented with 5% FCS from either Imperial Laboratories (closed circle) or Globepharm (closed diamond). (B) Cells were treated in either RPMI 1640 medium alone (closed square) or in medium supplemented with 10% FCS (Globepharm) (closed circle) in the absence (closed symbols) or presence (open symbols) of 10 μM glutathione. All drug treatments were for 1 hr.

TABLE 3. Concentrations of KW-2149 required to reduce the colony forming ability of RT112 cells by 50% in RPMI 1640 medium supplemented with various sera

Serum	IC_{50} (ng ml^{-1})
RPMI 1640 alone (no serum)	3992 ± 457
Globepharm FCS	
5%	27.4 ± 5.4
10%	21.7 ± 2.0
20%	14.5 ± 2.2
50%	11.1 ± 3.0
Imperial FCS	
5%	952 ± 220
10%	159 ± 27.3
20%	69.5 ± 17.9
50%	48.9 ± 19.1
Dialysed FCS (10%)	42.1 ± 8.6
Mouse serum (5%)	657 ± 196
Human serum (5%)	20.7 ± 2.9

The cytotoxicity of mitomycin C was not affected by the absence of serum during drug exposure or by the addition of different types of sera. Similar cytotoxicities were obtained whether the drug was dissolved in water, PBS or RPMI 1640 alone or supplemented with serum (data not shown).

Influence of Serum on Cellular Uptake of ^{3}H-KW-2149

Drug uptake, like cytotoxicity, depended on the presence and type of serum (see Table 4). In the absence of serum, the initial rate of uptake was 2.7 ± 1 dpm min^{-1} over a 2-hr exposure period, and this increased almost 3-fold (7.2 ± 0.8 dpm min^{-1}) in the presence of 10% Imperial FCS (low metabolising activity) and 8-fold in 10% Globepharm FCS (high metabolising activity; 20.7 ± 3.8 dpm min^{-1}). In 10% dialysed serum in RPMI 1640 medium, the rate of uptake was intermediate between those of the Imperial and Globepharm sera (9.8 ± 1.1 dpm min^{-1}). In PBS, the rate of uptake was about half that in RPMI 1640 alone

TABLE 4. Rate of uptake of tritiated KW-2149 into RT112 cells under a variety of culture conditions during a 2-hr exposure period, expressed as dpm min^{-1}

Culture conditions	Rate of uptake (dpm min^{-1} ± SD)
RPMI 1640 alone	2.7 ± 1.0
RPMI 1640 + 10% Globepharm FCS	20.7 ± 3.8
RPMI 1640 + 10% Imperial FCS	7.2 ± 0.8
PBS alone	1.3 ± 0.4
PBS + 10% Globepharm FCS	10.7 ± 1.7
PBS + 10% dialysed serum	7.8 ± 1.3
RPMI 1640 + 10% dialysed serum	9.8 ± 1.1
RPMI 1640 + 10 μM glutathione	5.3 ± 0.8
RPMI 1640 + 10 μM glutathione + 10% Globepharm FCS	14.1 ± 2.1

(1.3 ± 0.4 dpm min^{-1}). Although the addition of 10% dialysed or 10% Globepharm serum to PBS dramatically increased uptake (7.8 ± 1.3 dpm min^{-1} and 10.7 ± 1.7 dpm min^{-1}), the rates of uptake were lower than those in RPMI 1640. The addition of 10 μM glutathione had no significant effect on uptake in RPMI 1640 supplemented with 10% Globepharm FCS (14.1 ± 2.1 dpm min^{-1}). In contrast, in the absence of serum, the addition of 10 μM glutathione nearly doubled the rate of drug uptake to 5.3 ± 0.8 dpm min^{-1}.

Metabolism of KW-2149 in Serum

In the absence of cells, KW-2149 was metabolised in serum-containing medium but not in PBS or RPMI 1640 medium alone (Fig. 3A). In contrast to PBS, the addition of RPMI 1640 medium to FCS increased the rate of metabolism of KW-2149. Dialysed FCS alone did not metabolise KW-2149, but metabolic activity was restored by the addition of RPMI 1640 medium (Fig. 4B). The metabolism of KW-2149 by FCS was also apparent spectrally. In the absence of FCS, the spectrum of KW-2149 remained constant for 150 min at 37°C (Fig. 4A) but changed in a time-dependent manner when FCS was present. There was a loss of absorbance of the 370-nm peak of KW-2149, with a new peak forming at 325 nm and a broad peak also forming in the visible spectrum above 430 nm (Fig. 3B). A product peak was observed in the HPLC traces. This product was compared by HPLC to the metabolites previously identified and designated M-16 and M-18 [9] and was consistent with M-18.

DNA Binding and Adduct Formation

^{3}H-KW-2149 binds highly specifically to DNA in cells. In the absence of serum, the specific binding to DNA was 176.6 ± 46.5 dpm/mg, which increased 31.8-fold to $5369 \pm$

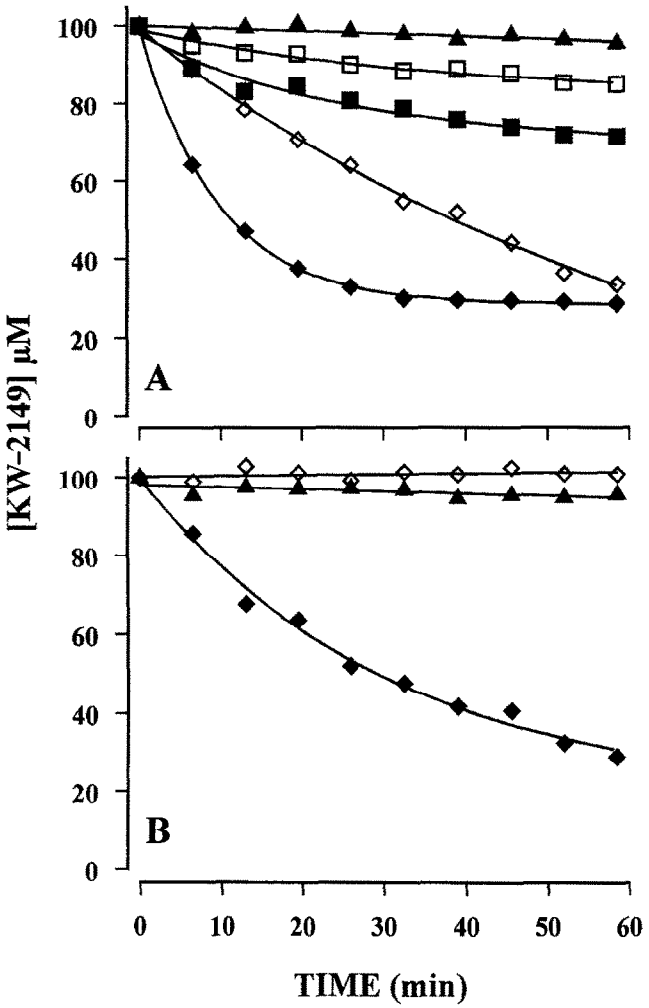

FIG. 3. The metabolism of KW-2149 in the absence of cells. (A) By undialysed FCS in the presence (closed symbols) or absence (open symbols) of RPMI 1640 medium. Solid square, 10% FCS; solid diamond 50% FCS; solid triangle, RPMI 1640 medium alone. (B) By 50% dialysed FCS (symbols as for A). The concentration of KW-2149 was monitored by HPLC, and all incubations were at 37°C.

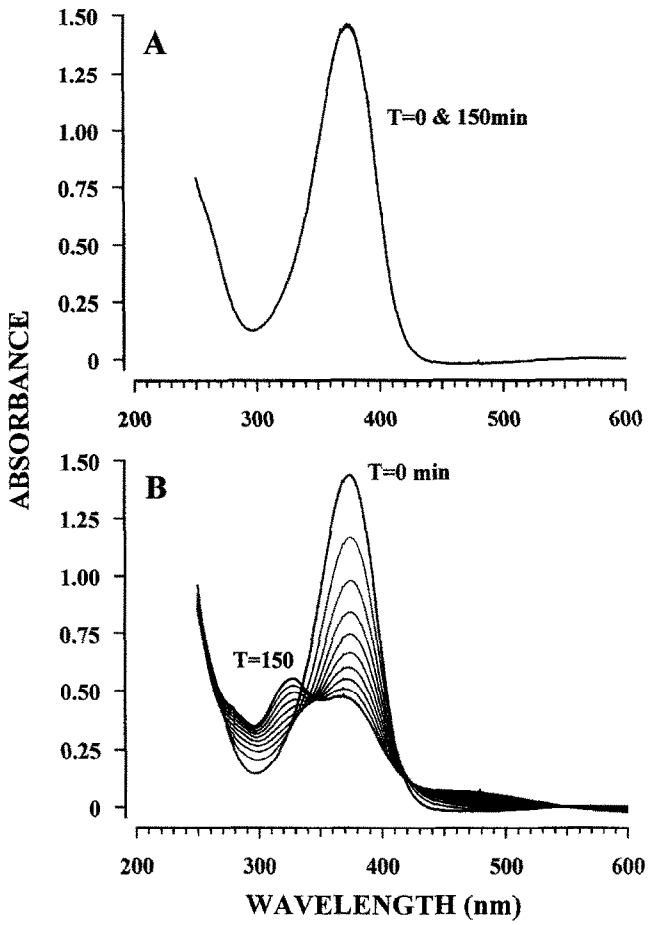

FIG. 4. The effect of serum metabolism on the UV-visible absorption spectrum of KW-2149. The initial concentration of KW-2149 was 100 μM, and spectra were recorded every 15 min over 150 min. (A) No serum. (B) 25% undialysed FCS. All incubations were at 37°C.

393.9 in the presence of 10% FCS. Similarly, an increase in DNA binding of approximately 50-fold was observed when naked DNA was reacted with KW-2149 in the presence of Globepharm FCS (high metabolising activity) compared with that in its absence (41905 vs. 836.5 dpm/mg DNA).

^{3}H-mitomycin C also bound to cellular DNA. In the presence and absence of 10% FCS, specific binding to DNA was 1139.4 ± 192.8 dpm/mg and 1125.4 ± 121.8 dpm/mg, respectively.

DISCUSSION

This study demonstrates that KW-2149 is activated extracellularly by serum, resulting in a compound with a much higher rate of cellular uptake and a greatly increased DNA binding when compared with the parent drug. These effects were not seen with mitomycin C.

Initial studies compared the cytotoxicities of mitomycin C and KW-2149. The rank order of sensitivities of the cell lines was similar for the two drugs. There was a complete lack of cross resistance to KW-2149 in a mitomycin C-resistant human bladder cancer cell line, confirming earlier findings in other cell types [5, 11]. This RT112 subline has low DT-diaphorase levels (N. W. Gibson personal communication), which may explain its resistance because DT-diaphorase can activate mitomycin C by reducing the quinone ring. In contrast, KW-2149 is a poor substrate for DT-diaphorase, and this enzyme is probably not involved in its activation.

The initial data obtained using a batch of FCS from Imperial Laboratories indicated that KW-2149 was less potent than mitomycin C *in vitro*. This finding was in contrast to previous *in vitro* data indicating that KW-2149 is up to 100-fold more toxic than mitomycin C [4–7]. The explanation for the disparity became apparent when the cytotoxicity of KW-2149 was measured in the absence of serum and in the presence of a different source of FCS. Serum enhanced the cytotoxicity of KW-2149 by approximately 150-fold as compared with medium alone.

Because of the species and batch-specific variations in the ability of serum to metabolise KW-2149, we measured the cytotoxicity of KW-2149 in human serum. There was modest variation in the capacity to metabolise KW-2149, no major sex difference, and the activating capacity was uniformly high, similar to that of the more active batch of FCS.

The mechanism underlying the increase in the cytotoxicity of KW-2149 in the presence of serum was apparent when the uptake of tritiated KW-2149 into cells and DNA binding were measured under different conditions. Serum contains a component(s) that modifies KW-2149 to a form that can enter cells more rapidly than the parent compound.

Serum can also metabolise KW-2149 in the absence of cells and a DNA-binding species is formed. The drug undergoes a significant spectral change, consistent with the conversion of the 7-aminomitosane chromophore (370 nm) to 7-aminomitosene (325 nm) by reduction [13]. Earlier studies have suggested that KW-2149 undergoes a nonenzymatic activation within cells [12], and a direct chemical reductive activation of KW-2149 has been proposed [13]. The disulphide group in KW-2149 can mediate the nonenzymatic reduction of the quinone after reaction with a thiol such as glutathione. Incubation of KW-2149 with glutathione produces a spectral change similar to that described above and activated KW-2149 to a DNA cross-linking species [13].

We also have evidence that thiols can be involved in the activation of KW-2149, but extracellularly in the serum. The addition of 10 μM glutathione to RPMI 1640 medium enhanced the uptake and cytotoxicity of KW-2149 in the absence, but not in the presence, of serum. Enhancement of KW-2149 cytotoxicity by glutathione and cysteine has been reported [12]. However, depletion of cellular glutathione by buthionine sulphoximine did not reduce the cytotoxicity of KW-2149 [12], which is consistent with our data indicating that the critical metabolic step is extracellular. However, levels of glutathione in serum are too low to account for the observed activation by serum.

In the absence of cells, KW-2149 is metabolised by serum to form a DNA reactive cytotoxic species, and this process appears to be enzymatic. This conclusion was reached because, in the absence of RPMI 1640, dialysed serum (unlike normal serum) does not metabolise KW-2149, but this activity is restored by addition of RPMI 1640. This result is consistent with the action of an enzyme that can metabolise KW-2149 but requires a low-molecular-weight cofactor or cosubstrate for activity. This factor is present in undialysed serum and RPMI 1640 medium.

The identity of the metabolising activity in serum is not yet established. Preliminary experiments indicate that the enzymes gamma-glutamyl transferase and glutathione S-transferase have no effect on the metabolism of KW-2149. Further experiments, including measurement of the activity of different size fractions of serum, are underway. However, although dialysed serum was inactive in metabolising KW-2149 in the absence of medium and cells (unless supplemented by medium), there was significant cellular uptake and cytotoxicity of the drug mediated by this serum. This finding could indicate that further activation of KW-2149 may occur in the presence of cells.

In summary, the cytotoxicity and DNA reactivity of KW-2149 are dramatically enhanced by serum activation. These effects are not seen with mitomycin C. The active metabolite of KW-2149 has yet to be identified. Further studies are also required to identify the activating and cofactor components in serum, to determine whether there is further intracellular metabolism and to analyse sequence-specific binding to DNA within cells. To our knowledge, this is the first description of an anticancer drug whose cytotoxicity is mediated by its metabolism by serum.

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