Biochemical Pharmacology, Vol. 55, pp. 1777–1783, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00603-5

ELSEVIER

KW-2149 (7-*N*-[2-[γ-L-glutamylamino]ethyldithio-ethyl] mitomycin C): DNA Interactions and Drug Uptake Following Serum Activation

Simon R. McAdam,§ Richard J. Knox,† John A. Hartley* and John R. W. Masters‡*

*CRC Drug-DNA Interactions Research Group, Department of Oncology, University College London, 91 Riding House St, London W1P 8BT; †Department of Medical Oncology, Charing Cross Hospital, Fulham Palace Road, London, W6 8RF; and ‡University College London, Institute of Urology and Nephrology, 67 Riding House Street, London W1P 7PN, U.K.

ABSTRACT. 7-*N*-[2-[γ-L-glutamylamino]ethyldithio-ethyl] mitomycin C (KW-2149) is a mitomycin-C analogue currently being evaluated in clinical trials. It has been shown that KW-2149 is unusual in that it is activated by serum, resulting in an increase in potency of up to 200-fold. To investigate the mechanism by which KW-2149 is activated, the abilities of mitomycin-C, KW-2149 and its metabolites M-18 (symmetrical disulphide dimer) and M-16 (methyl sulphide form) to interact with DNA were compared, and the influence of serum and glutathione on the sequence-specificity of KW-2149-DNA interactions was determined. Following reduction by glutathione both KW-2149 and M-18 are more efficient crosslinking agents of naked DNA, with the metabolite M-18 showing superior activity. The efficiency of DNA interstrand crosslinking in cells by KW-2149 was also increased by the addition of serum. Using the potassium/SDS precipitation method it was found that KW-2149 and M-18 crosslink protein to DNA whilst mitomycin C and M-16 do not. All four compounds produced almost identical patterns of adducts. Serum and glutathione did not alter the pattern of DNA adducts, but did increase the efficiency of adduct formation. Our earlier studies had indicated that the mechanism of activation of KW-2149 by serum is related to cellular uptake, and we therefore studied the effects of certain metabolic inhibitors, temperature and competitive inhibition on drug uptake. The results suggest that uptake is passive, and this indicates that a component in serum modifies KW-2149 to a form that passively enters cells more rapidly. BIOCHEM PHARMACOL **55**;11:1777–1783, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. anticancer agent; mitomycin-C; KW-2149; serum; drug metabolism; drug uptake; DNA damage

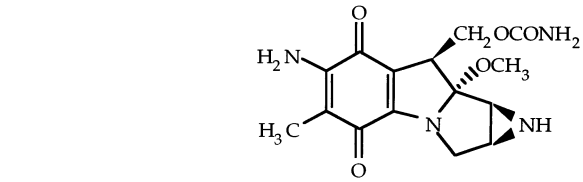
KW-2149¶ (Fig. 1) is a water-soluble analogue of mitomycin-C [1] which is currently in clinical trial [2]. It has a similar spectrum of antitumour activity to mitomycin-C (MMC) against a range of experimental murine tumours and human tumour xenografts *in vivo* but is less myelosuppressive [3] and lacks cross-resistance against mitomycin-C resistant tumours *in vitro* and *in vivo* [4–7]. The cellular target of MMC is believed to be DNA [8]. Enzymatic reduction of the quinone group by cellular enzymes leads to the formation of reactive metabolites that alkylate and crosslink DNA [9]. The cytotoxicity and antitumour activity of MMC *in vivo* is dependent on enzymatic reduction and hence resistance of tumour cells to MMC is related to deficient reductase activity [10]. The requirement for enzymatic reduction is therefore a limiting factor in the therapeutic efficiency of the drug.

It was demonstrated previously that KW-2149 is unique amongst anticancer agents as it is activated by serum. KW-2149 was approximately 200-fold more toxic in the presence of 5% human serum than in medium alone (comparing IC_{50}s, the concentrations reducing colony forming efficiency by 50%). The degree of activation depended on the batch of serum and its species of origin, and the effect of serum could be replaced partially by glutathione [11]. Serum increased the toxicity of KW-2149 through a mechanism which either stimulated the uptake of the drug or converted KW-2149 to a form which entered cells more readily [11]. The goal of this study was firstly to determine the effect of serum and glutathione on the efficiency and sequence specificity of binding of KW-2149 to DNA. Comparisons were made of the drug-DNA interactions of mitomycin-C, KW-2149 and its metabolites M-18 and M-16 [12, 13] (Fig. 1). Secondly, it was determined whether the uptake of KW-2149 into cells is controlled by an active or a passive process by comparing

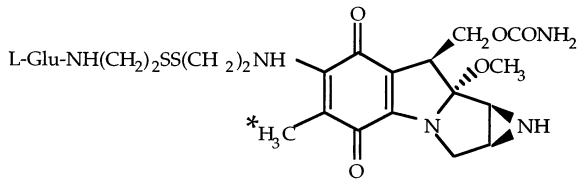
§ Corresponding author: Simon McAdam, CRC-Drug-DNA Interactions Group, Department of Oncology, University College London, 91 Riding House St, London W1P 8BT, U.K. Tel. 0044-171-436-8222; FAX 0044-171-436-2956; E-mail: s.mcadam@ucl.ac.uk.

¶ *Abbreviations:* K/SDS, potassium/SDS; KW-2149, 7-*N*-[2-[γ-L-glutamylamino]ethyldithio-ethyl]mitomycin C; M-16, 7-*N*-[2-(methylthio)-ethyl]mitomycin C; M-18, 7-*N*,7′-*N*′-dithiodiethylenedimitomycin C; MMC, mitomycin C.

Received 28 May 1997; accepted 16 October 1997.

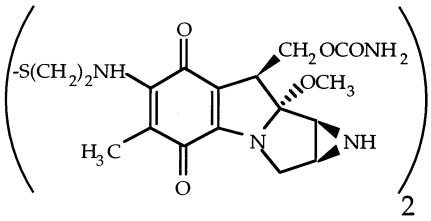


Mitomycin C

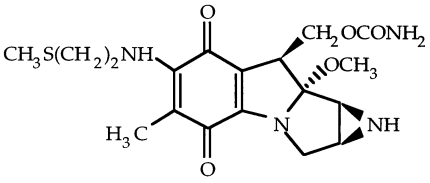


KW-2149

FIG. 1. Structures of mitomycin-C, KW-2149 and its metabolites M-16 and M-18. *Indicates the tritium-labelled position on KW-2149 used for the uptake experiments.



M-18



M-16

the effects of various metabolic inhibitors, temperature and competitive inhibition on the uptake of tritiated KW-2149.

MATERIALS AND METHODS

Chemicals

KW-2149, tritiated KW-2149, mitomycin-C and the KW-2149 metabolites M-16 and M18 were supplied by Kyowa Hakko Kogyo Ltd. The metabolic inhibitors: Ouabain, Sodium Flouride, Iodoacetate, 2-deoxy-D-glucose, cordycepin and 2,4-dinitrophenol were all purchased from Sigma Chemical Company.

Cells

The origin of the human bladder cancer cell line RT112 has been described [14]. The cells were grown routinely in RPMI 1640 medium supplemented with 10% fetal calf serum (Globepharm) and 2 mM of L-glutamine. The cells were used over a maximum culture period of 10 passages to minimise any changes resulting from long-term culture.

Agarose Gel Crosslink Assay

Crosslinking capacity was determined using an agarose gel method [15]. Briefly DNA from pBR322 was linearized by digestion with BamHI and dephosphorylated by treatment with bacterial alkaline phosphatase. The DNA was 5′-end-labelled using T4 polynucleotide kinase and [γ^{32}P]ATP (5000 Ci/mmol, Amersham). Approximately 10 ng of labelled DNA was used for each experimental point. Reactions were performed in 25 mM of triethanolamine, 1 mM of EDTA (pH 7.2) at 37° for 1 hr. Where appropriate

drugs were reduced by incubation with glutathione (GSH) or dithiothreitol (DTT). The reactions were terminated by the addition of an equal volume of stop solution (0.6 M of sodium acetate, 20 mM of EDTA, 100 μg/mL of tRNA). The samples were precipitated, denatured and electrophoresed as described previously [15]. Gels were dried at 80° onto filter paper and autoradiography performed at −70°. Quantitation was achieved by densitometry of the autoradiograph using a BIO-RAD imaging densitometer. For each lane the amount of single- and double-stranded DNA was determined and the percentage crosslinked (double-stranded) DNA calculated.

Alkaline Elution

DNA interstrand crosslinking in cells was measured by alkaline elution as described [16]. Cells in logarithmic growth were labelled for 24 h with 0.015 μCi of [^{14}C]thymidine/mL (specific activity 56 mCi/mmol; Amersham), centrifuged and washed three times in phosphate buffered saline (PBS) and resuspended in either fresh medium (RPMI 1640) supplemented with serum or in PBS. Cells were treated with drug for 1 hr at 37°. Single-strand breaks were introduced into the cellular DNA by exposure of cells on ice to 4Gy from an x-ray source. Elution was performed through 2 μM pore size polycarbonate filters at pH 12.1 in the presence of proteinase K.

Taq Polymerase Stop Assay

The sequence specificity of DNA adduct formation in plasmid DNA was determined using the taq polymerase stop method [17]. Prior to drug incubation pBR322 DNA (0.5 μg/sample) was linearized with *Bam*HI to generate a convenient stop site for the polymerase downstream from the primer. Drug reactions were performed in 25 mM of triethanolamine and 1 mM of EDTA, pH 7.2 at 37° for 1 hr and terminated by the addition of an equal volume of stop solution (0.6 M of sodium acetate, 20 mM of EDTA, 100 μg/mL of tRNA). Samples were precipitated with 3 volumes of ethanol, washed with 70% ethanol and vacuum-dried. A synthetic oligonucleotide primer of the following sequence was used: 5′-TAT GCG ACT CCT GCA TTA CG-3′. The primer was 5′-end-labelled using T4 polynucloetide kinase and purified by elution through a Biospin column (Bio-Rad).

Linear amplification of DNA was carried out in a total volume of 100 μL containing 0.5 μg of template DNA, 5 pmol of labelled primer, 200 μM each dNTP, 1 U of Taq DNA polymerase, 20 mM of $(NH_4)_2SO_4$, 75 mM of Tris-HCL, pH 9.0, 0.01% Tween, 2.5 mM of $MgCl_2$ and 0.01% gelatin. After gentle mixing the samples were amplified on a thermal cycler. After an initial denaturation at 94° for 3 min the amplification procedure was carried out as follows: 1 min denaturation at 95°, 1 min annealing at 60° and 1 min chain elongation at 72° for a total of 30 cycles. After amplification the samples were ethanol-precipitated and washed with 70% ethanol. Samples were resuspended in formamide loading dye, heated for 2 min at 95°, cooled on ice and electrophoresed at 2500–3000 V for approximately 3 hr on a 80 cm × 20 cm × 0.4 mm, 6% acrylamide denaturing sequencing gel (sequagel 6, National Diagnostics.) The gels were dried and exposed to x-ray film (X-Omat LS, Kodak).

K/SDS Precipitation Method for Detection of DNA-Protein Complexes

The amount of DNA-protein crosslink formation in cells was determined by the K/SDS precipitation method as described [18] with modifications [19]. Cells in logarithmic growth (2×10^6 cells/mL) were labelled with 0.1 μCi [^{14}C]thymidine/mL (specific activity 375 mCi/mmol; Amersham). After overnight incubation the cells were washed 3 times in PBS, trypsinised and resuspended in fresh medium to a final concentration of 10^5 cells/mL. One mL of cells was plated per well of a 24-well microtiter plate (Falcon) and incubated for 2 hr at 37°. The cells were then treated with a range of concentrations of drug for 1 hr at 37°. After the drug incubation the medium was removed from each well and cells were lysed by adding 1 mL of prewarmed (65°) lysis solution [1.25% SDS, 5 mM of EDTA (pH 8.0), salmon sperm DNA (0.4 mg/mL)]. The DNA in the lysate was sheared by pipetting 30 times with a C20 pipet tip (Gilson) and adding 250 μL of 325 mM of KCl. After vigorous vortexing the samples were cooled on ice for 10 min and centrifuged for 15 min at 4°. The pellet was washed again and resuspended in 200 μL of H_2O at 65°. Four mL of scintillation fluid (Ecoscint A, National Diagnostic) was added to the sample and the radioactivity counted.

Drug Uptake Into Cells

10^5 RT112 cells were plated per well in 96 well flat-bottomed plates (Nunc) and in T25 flasks (Nunc) in 200 μL or 5 mL respectively of complete medium (RPMI 1640 supplemented with 10% FCS and 2 mM of L-glutamine). After a 24-hr incubation the medium was replaced with 100 μL of fresh medium (in the presence or absence of FCS) containing 0.25 μCi of [^{3}H]KW-2149/mL (specific activity 11.0 Ci/mmol) and unlabelled drug or metabolic inhibitors where appropriate, for 1 to 3 hr. After incubation the medium was removed and the cells washed ×4 with ice-cold PBS. Five aliquots of 200 μL of 1N NaOH were added to each well and collected. The NaOH washes containing the cells were bulked and neutralised with 1 mL of 1N HCl, and 3 mL of distilled water was added. Ten mL of scintillation fluid (Ecoscint, National Diagnostics) was added and the radioactivity determined on an LKB scintillation counter.

RESULTS

DNA Crosslinking in Isolated DNA

The ability of KW-2149 to produce crosslinks in plasmid DNA was determined using an agarose gel assay. An

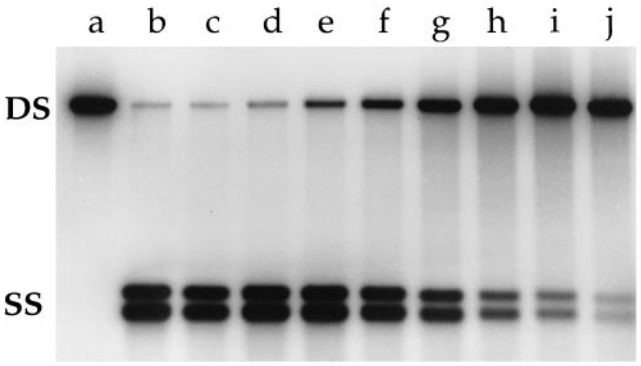


FIG. 2. DNA interstrand crosslink formation in pBR322 DNA by increasing concentrations of KW-2149 in the presence of GSH. Lane a, nondenatured control; lane b, denatured control; lane c–j contain 100 μM of GSH with KW-2149 at lane c, 0 μM, lane d, 0.1 μM; lane e, 0.5 μM; lane f, 1 μM; lane g, 5 μM; lane h, 10 μM; lane i, 50 μM; lane j, 100 μM of KW-2149. DS = double-stranded DNA, SS = single-stranded DNA.

autoradiograph from a typical gel is shown in Fig. 2. With increasing concentration of KW-2149 there is an increase in interstrand DNA crosslinking in the presence of 100 μM of GSH. In the absence of GSH, KW-2149 still forms interstrand crosslinks in DNA but with a much lower efficiency. For example, with 100 μM of KW-2149 in the absence of GSH, 16.5% of the DNA is crosslinked (data not shown), compared to >90% double-stranded DNA in the presence of GSH (Fig. 2). At high concentrations of GSH there is a reduction in the amount of DNA interstrand crosslinking, as shown in Fig. 3. At a drug concentration of 10 μM KW-2149 the optimum concentration of GSH is 500 μM. The ability of the major metabolite of KW-2149, the disulphide dimer M-18, to crosslink isolated DNA was compared to that of the parent drug (Table 1). Densitometric analysis revealed that M-18 is a more efficient crosslinker than the parent compound both in the presence and absence of GSH. The metabolite M-16 under identical conditions did not crosslink DNA in the presence or absence of GSH (data not shown).

DNA Crosslinking in Cells

The ability of KW-2149 to produce DNA interstrand crosslinks in RT112 cells was investigated using alkaline elution. Following a 1-hr exposure to drug at 37° extensive dose dependent interstrand crosslinking was observed (Fig. 4). The efficiency of crosslinking was increased by the addition of serum to 5 μM of KW-2149. Significantly more crosslinks were evident at a 10-fold lower dose of KW-2149 in the presence of serum than in its absence. There is no evidence of single-strand breaks in the unirradiated samples. The formation of DNA-protein crosslinks was measured using the K/SDS precipitation technique (Fig. 5). KW-2149 and the metabolite M-18 induce DNA-protein crosslinks in cells at similar concentrations. The metabolite

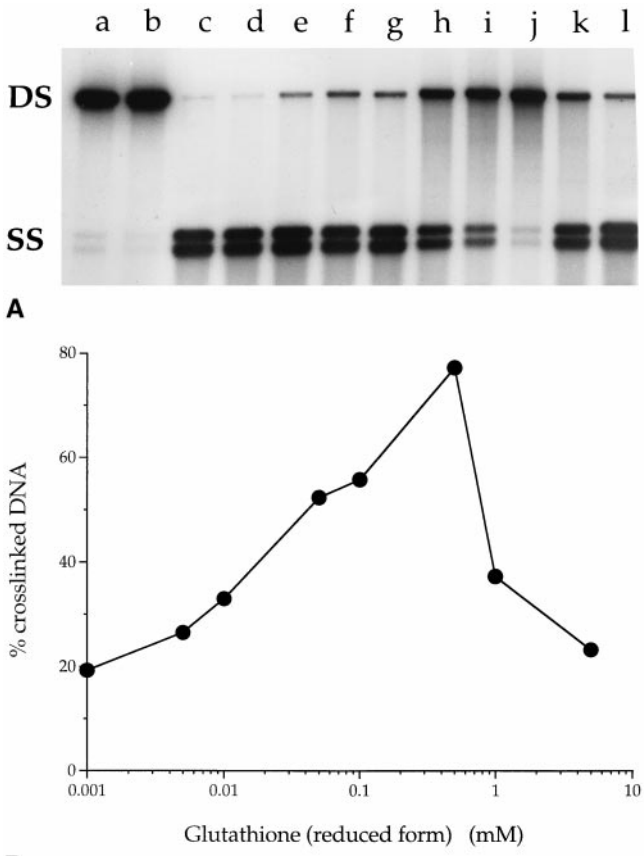


FIG. 3. (A) DNA interstrand crosslink formation in pBR322 DNA by 10 μM of KW-2149 at a range of concentrations of GSH. Lane a, nondenatured control; lane b, nondenatured control + 5 mM of GSH; lane c, denatured control; lane d, denatured control + 5 mM of GSH; lanes e–i contain 10 μM of KW-2149 with GSH at lane e, 1 μM; lane f, 5 μM; lane g, 10 μM; lane h, 50 μM; lane i, 100 μM; lane j, 500 μM; lane k, 1000 μM; lane i, 5000 μM. DS = double-stranded DNA, SS = single-stranded DNA; (B) percent cross-linked DNA with 10 μM of KW-2149 at a range of glutathione concentrations, calculated from densitometric measurements of the gel autoradiograph shown in (A).

M-16 and mitomycin-C did not produce DNA-protein crosslinks in this assay at equivalent doses.

TABLE 1. Comparison of DNA crosslink formation by KW-2149 and its metabolite M-18 under reducing conditions.

	% crosslinked DNA*†			
	KW-2149		M-18	
Dose of drug (μM)	−GSH	+GSH‡	−GSH	−GSH‡
1	ND	17	ND	17
10	ND	53	ND	100
100	16	100	50	100

*Calculated from the amount of single- and double-stranded DNA determined from densitometry of agarose crosslink gel autoradiograph (see Materials and Methods).

†Data derived from a single representative experiment.

‡1mM of GSH.

ND = not determined.

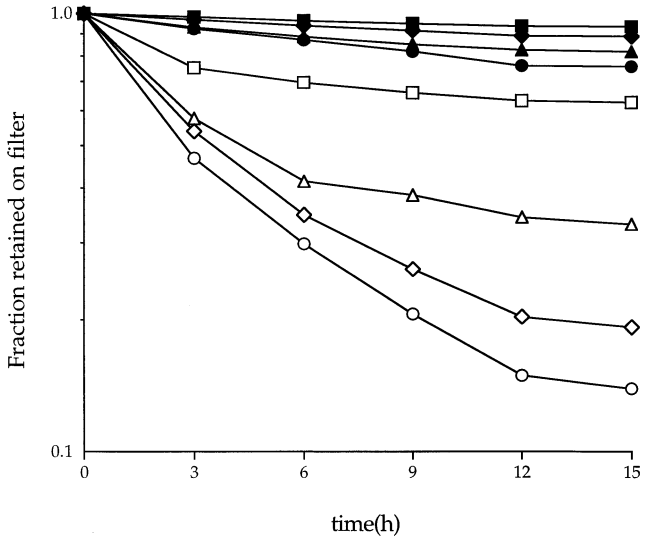


FIG. 4. DNA interstrand crosslink formation in RT112 cells. Alkaline elution profiles of DNA from untreated cells in the presence of 10% serum (●, ○) or from cells treated with KW-2149 for 1 hr at 37° at a concentration of 0.5 μM in the presence of 10% serum (▲, △); 5 μM of KW-2149 in 10% serum (■, □); 5 μM of KW-2149 without serum (◆, ◇). Cells were either irradiated (open symbols) or unirradiated (closed symbols).

DNA Sequence Specificity

DNA sequence specificity in plasmid DNA was measured using the Taq polymerase stop technique. An autoradiograph of a typical gel is shown in Fig. 6. The left and right panels are from two separate experiments. The controls containing unmodified DNA (lanes a and f), showed few sites of early termination. The pattern of blocks produced by the alkylating agent mechlorethamine, which produces adducts at guanine-N7 at 2.5 μM is shown in lane b. Blocks

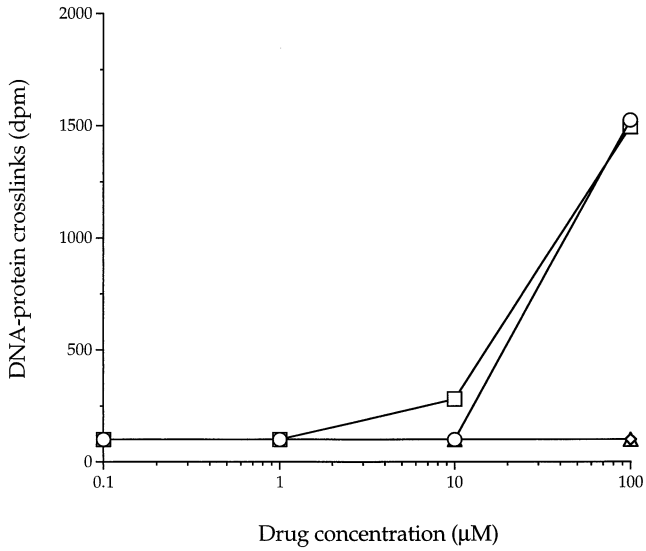


FIG. 5. DNA-protein crosslink formation in RT112 cells treated with KW-2149 (○), M-18 (□), M-16 (△) and Mitomycin-C (◇) at a drug concentration of 0.1 μM to 100 μM for 1 hr at 37°.

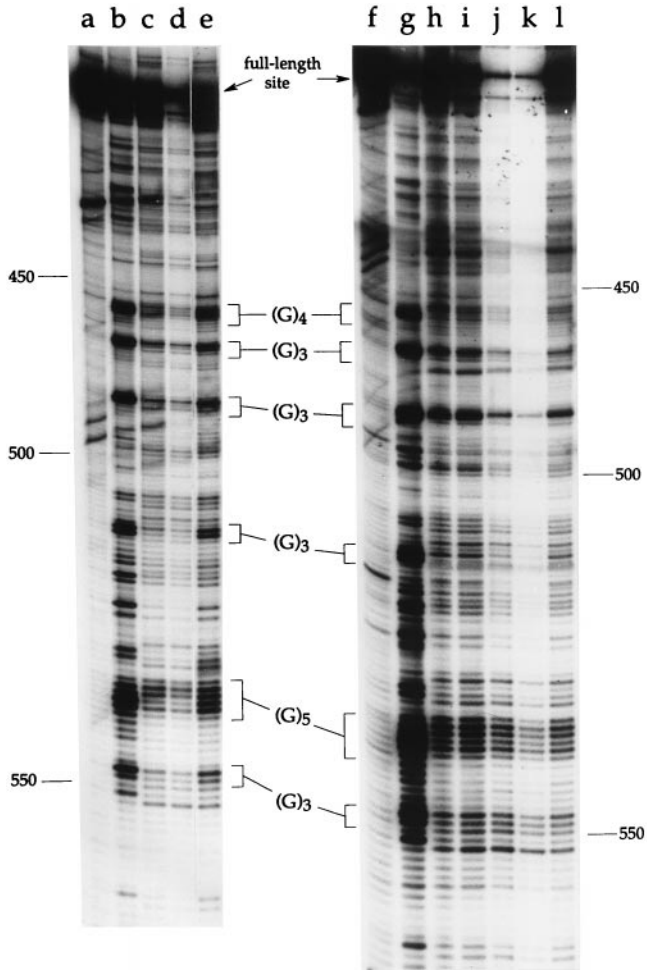


FIG. 6. Autoradiograph of a taq polymerase gel showing blocking sites on the upper strand of pBR322 DNA defined by the *Bam*HI and *Sal*I restriction sites. Lane a and f, controls; lane b and g, 2.5 μM of mechlorethamine; lane c, 1 μM of KW-2149 + 100 μM of GSH; lane d, 1 mM of KW-2149 no GSH; lane e, 100 μM of mitomycin C + 2 μg/mL of DT-diaphorase, pH 5.8; lane h, 5 μM of KW-2149 + 1 mM of GSH; lane i, 10 μM of KW-2149 + 1 mM of GSH; lane j, 5 μM of M-18 + 1 mM of GSH; lane k, 10 μM of M-18 + 1 mM of GSH; lane 1 100 μM of M-16 + 1 mM of GSH.

to the polymerase are seen preferentially at runs of contiguous guanines (e.g. the run of guanines at bases 535–539), as shown previously for this agent [17]. The sequence specificity of adduct formation for KW-2149 (lanes h and i) and the metabolites M-18 (lanes j and k) and M-16 (lane l) are almost identical, showing a similar sequence preference for runs of contiguous guanines. The binding pattern of KW-2149 is unaffected by chemical reduction with thiols, as can be seen by comparing lane c (1 μM of KW-2149 reduced with 100 μM of GSH) and lane d (1 mM of KW-2149 without reduction). Mitomycin C gave a similar pattern of adduct formation under reducing conditions (lane e). The sequence specificity of mitomycin-C has been demonstrated previously [17, 20] with a preference for runs of contiguous guanines, predominantly at 5′-GG-3′ and 5′-CG-3′ sequences. M-18 induced more DNA damage at

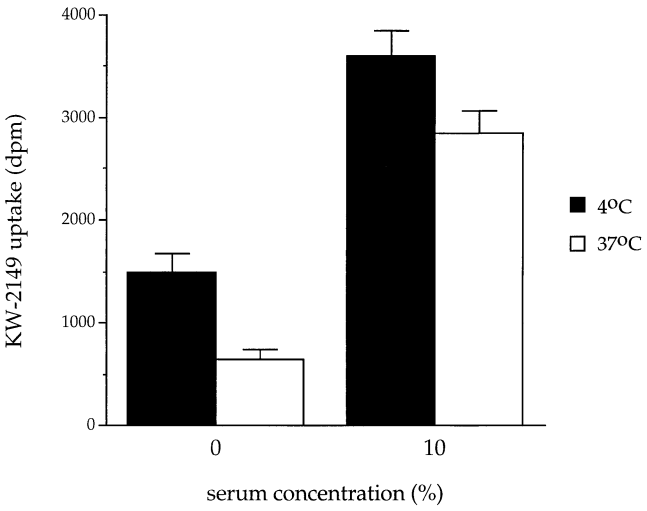


FIG. 7. Influence of temperature on the uptake of ^{3}H-KW-2149. RT112 cells were incubated with drug at 4° or 37° for 1 hr either in the presence or absence of serum (indicated).

equivalent drug concentrations to KW-2149, whilst mitomycin C and the metabolite M-16 showed a similar pattern of reactivity at a 100-fold higher concentration than KW-2149 (lane c).

Cellular Uptake Mechanisms

Several metabolic poisons were chosen (ouabain, 2,4-dinitrophenol, sodium fluoride, iodoacetate, cordycepin and 2-deoxy-D-glucose) for their ability to inhibit different metabolic pathways in cells (including Na^+, K^+-ATPase pump, electron transport/oxidative phosphorylation and glycolysis) and thus to investigate their influence on the uptake of KW-2149. After incubation with the metabolic inhibitors at concentrations known to inhibit the relevant mechanisms, uptake of tritiated KW-2149 was unaffected (data not shown). RT112 cells were incubated with tritiated KW-2149 and excess unlabelled KW-2149 or M-18 in the presence or absence of 10% serum. No inhibition of uptake of ^{3}H-KW-2149 was seen when concentrations of KW-2149 up to 10 μM were added (data not shown). Uptake of ^{3}H-KW-2149 was compared at 4° and at 37° in the presence and absence of serum (Fig. 7). Tritiated KW-2149 uptake was 2.4-fold higher in the presence of serum than in the absence of serum at 4° and 4.4-fold higher in the presence of serum than in the absence of serum at 37°. Unexpectedly, tritiated KW-2149 uptake was 1.3-fold higher in the presence and 2.3-fold higher in the absence of serum at 4° than at 37°.

DISCUSSION

KW-2149 is activated to a form with greater cytotoxicity by serum [11]. In this study, the mechanism of activation and its effects on drug-DNA interactions were studied. It appears that KW-2149, its metabolites M-18 and M-16, and mitomycin-C all form a similar pattern of adducts on DNA, although with differing degrees of efficiency and with different metabolic requirements.

Our earlier studies had shown that glutathione could partially replace serum for activation of KW-2149 [11]. This was confirmed in the present study using the crosslinking assay, indicating that the efficiency of formation of crosslinked DNA by KW-2149 was increased by glutathione. The effect of serum on KW-2149 activation could not be studied in the crosslinking assay however, because protein components in serum produce DNA degradation. A dose-response was seen for both KW-2149, as described previously [22], and glutathione in the DNA crosslinking assay. However, at high concentrations of glutathione the amount of crosslinking decreased. The major metabolite M-18 is a more efficient crosslinker than the parent compound KW-2149, reflecting its higher toxicity (on a weight basis) to cells in culture (our unpublished data). The increase in DNA crosslinking induced by glutathione was similar for both KW-2149 and M-18. Although M-18 is the major serum metabolite (11, 13, 14), the fact that M-18 also requires activation by GSH (Table 1) and serum (our unpublished data) suggests that it is not the form that enters cells more readily than KW-2149.

The DNA sequence specificity's of mitomycin-C (activated by DT-diaphorase), KW-2149 (in the presence and absence of reduced glutathione) and its metabolites M-18 and M-16 were compared and found to be almost identical. This is important as it indicates that all these compounds probably have a similar ultimate molecular mechanism of action. Nevertheless, these drugs may have different spectrums of clinical activity. The toxicity of mitomycin-C will be modified according to the levels of DT-diaphorase. Because cells that are resistant to mitomycin-C often express low levels of DT-diaphorase, these cells are not cross-resistant to KW-2149 [6, 7, 11, 23]. Thus, although the final common denominator of all these compounds in terms of DNA interaction appear to be virtually identical, the route by which the DNA interaction is achieved is different.

Using a simple but relatively insensitive assay for measuring DNA-protein crosslinks, we confirmed that KW-2149 and M-18 crosslink protein to DNA, whereas mitomycin-c and M-16 do not, as shown previously using this method [19]. Using the more sensitive technique of alkaline elution, DNA-protein crosslinks can be observed following exposure to mitomycin-C [21]. It may be concluded that KW-2149 and M-18 are more efficient DNA-protein crosslinkers than either M-16 or mitomycin-C.

Drug uptake was not significantly influenced by any of the metabolic inhibitors tested, suggesting a passive mechanism of uptake. Further evidence for passive uptake was obtained from the studies on the effect of temperature, which indicated that there was slightly higher uptake at 4° than at 37°. If the mechanism of uptake is active, we would expect a higher rate of uptake at the higher temperature. Similarly, if there was competition for an active process, the addition of unlabelled KW-2149 should have competed for

the uptake of tritiated KW-2149. The results indicate that a factor in serum converts KW-2149 to a form that can passively enter cells at a much higher rate than the parent compound. The active metabolite of KW-2149 is yet to be identified.

This project was supported by Kyowa Hakko Kogyo Co., Ltd.

References

1. Kono M, Saitoh Y, Kasai M, Sato A, Shirahata K, Morimoto M and Ashizawa T, Synthesis and antitumor activity of 7-*N*-{{2-{[γ-L-glutamylamino)ethyl]dithio}ethyl}}mitomycin C. *Chem Pharm Bull* **37:** 1128–1130, 1989.
2. Dirix L, Catimel G, Koier I, Prove A, Schrijvers D, Joossens E, De Bruijn E, Ardiet C, Evene E, Dumortier A, Clavel M and Van Oosterom A, Phase I and pharmacokinetic study of novel mitomycin C analog KW-2149. *Anti-Cancer Drugs* **6:** 53–63, 1995.
3. Ahizawa T, Okabe M, Gomi K and Hirata T, Reduced bone marrow toxicity of KW-2149, a mitomycin C derivative, in mice. *Anti-Cancer Drugs* **4:** 181–188, 1993.
4. Ohe Y, Nakagawa K, Fujiwara Y, Sasaki Y, Minato K, Bungo M, Niimi S, Horichi N, Fukuda M and Saijo N, *In vitro* evaluation of the new anticancer agents KT6149, MX-2, SM5887, Menogaril, and Liblomycin using cisplatin- or adriamycin-resistant human cancer cell lines. *Cancer Res* **49:** 4098–4102, 1989.
5. Morimoto M, Ashizawa T, Ohno H, Azuma M, Kobayas E, Okabe M, Gomi K, Kono M, Saitoh Y, Kanda Y, Arai H, Sato A, Kasai M and Tsuruo T, Antitumor activity of 7-*N*-{{2-{[2(γ-Glutamylamino)ethyl]dithio}ethyl}} mitomycin-C. *Cancer Res* **51:** 110–115, 1991.
6. Tsuruo T, Sudo Y, Asami N, Inaba M and Morimoto M, Antitumor activity of a derivative of mitomycin, 7-*N*-[2-[[2-(γ-L-glutamyl-amino)ethyl]dithio]ethyl] mitomycin C (KW-2149), against human tumors and a mitomycin-C resistant tumor *in vitro* and *in vivo*. *Cancer Chemother Pharmacol* **27:** 89–93, 1990.
7. Dirix L, Gheuens EEO, Van der Heyden S, Van Oosterom AT and De Bruijn EA, Cytotoxic activity of 7-*N*-(2-((2-(-γ-L-glutamyl-amino)-ethyl)dithio)ethyl)-mitomycin C and metabolites in cell lines with different resistance patterns. *Anti-Cancer Drugs* **5:** 343–354, 1994.
8. Tomasz M, Lipman R, Chowdary D, Pawlak J, Verdine G and Nakanshi K, Isolation and structure of a covalent cross-link adduct between Mitomycin C and DNA. *Science* **235:** 1204–1208, 1987.
9. Tomasz M, Mercado CM, Olson J and Chatterjie N, The mode of interaction of mitomycin C with deoxyribonucleic acid and other polynucleotides *in vivo*. *Biochemistry* **13:** 4878–4887, 1974.
10. Willson JKV, Long BH, Chakrabarty S, Brattain DE and Brattain MG, Effects of BMY 25282, a mitomycin C analogue, in Mitomycin C-resistant human colon cancer cells. *Cancer Res* **45:** 5281–5286, 1985.
11. Masters JRW, Knox RJ, Hartley JA, Kelland LR, Hendriks HA and Connors T, KW-2149 (7-*N*-[2-[γ-L-glutamylamino]ethyldithio-ethyl]mitomycin C): a new mitomycin C analogue activated by serum. *Biochem Pharmacol* **53:** 279–285, 1997.
12. Masters JRW, Hepburn PJ, Walker L, Highman WJ, Trejdosiewicz LK, Povey S, Parkar M, Hill BT, Riddle PR and Franks LM, Tissue culture model of transitional cell carcinoma: characterization of twenty-two human urothelial cell lines. *Cancer Res* **6:** 3630–3636, 1986.
13. Kobayashi S, Ushiki J, Takai K, Okumura S, Kono M, Kasai M, Gomi K, Morimoto M, Ueno H and Hirata T, Disposition and metabolism of KW-2149, a novel anticancer agent. *Cancer Chemother Pharmacol* **32:** 143–150, 1993.
14. Ashizawa T, Okamoto A, Okabe M, Kobayashi S, Arai H, Saito H, Kasai M and Gomi K, Characteristics of the antitumor activity of M-16 and M-18, major metabolites of a new mitomycin C derivative KW-2149, in mice. *Anti-Cancer Drugs* **6:** 763–780, 1995.
15. Hartley JA, Berardini MD and Souhami RL, An agarose gel method for the determination of DNA interstrand crosslinking applicable to the measurement of the rate of total and "second-arm" crosslink reactions. *Anal Biochem* **193:** 131–134, 1991.
16. Kohn KW, Ewing RAG, Erickson LC and Zwelling LA, Measurement of strand breaks and crosslinks by alkaline elution. In: *DNA Repair: A Laboratory Manual of Research Procedures*, Vol 1, Part B. (Eds. Friedberg E and Hanawalt P), pp. 378–401. Marcel Dekker, New York, 1981.
17. Ponti M, Forrow SM, Souhami RL, D'Incalci M and Hartley JA, Measurement of the sequence specificity of covalent DNA modification by antineoplastic agents using Taq DNA polymerase. *Nucleic Acids Res* **19:** 2929–2923, 1991.
18. Rowe TC, Chen GL, Hsiang Y-H and Liu LF, DNA damage by antitumour acridines mediated by mammalian DNA topoisomerase II. *Cancer Res* **46:** 2021–2026, 1986.
19. Fujii N, Arai H, Saito H, Kasai M and Nakano H, Induction of protein-DNA complexes in Hela S3 cells by KW-2149, a new derivative of Mitomycin C. *Cancer Res* **53:** 4466–4468, 1993.
20. Prakash AS, Beall H, Ross D and Gibson NW, Sequence-selective alkylation and cross-linking induced by Mitomycin C upon activation by DT-diaphorase. *Biochemistry* **32:** 5518–5525, 1993.
21. Dorr RT, Bowden GT, Alberts DS, and Liddel JD, Interactions of mitomycin C with mammalian DNA detected by alkaline elution. *Cancer Res* **45:** 3510–3516, 1985.
22. He Q-Y, Maruenda H and Tomasz M, Novel bioreductive activation mechanism of mitomycin C derivatives bearing a disulfide substituent in their quinone. *J Am Chem Soc* **116:** 9349–9350, 1994.
23. Lee J-H, Naito M and Tsuruo T, Nonenzymatic reductive activation of 7-*N*-{{2-{[2-(γ-L-glutamylamino)ethyl]dithio}ethyl}}-mitomycin C by thiol molecules: a novel mitomycin C derivative effective on mitomycin C-resistant tumor cells. *Cancer Res* **54:** 2398–2403, 1994.