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RAPID COMMUNICATION

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7-*N*-(2-([2-(Gamma-L-glutamylamino)-ethyl]-dithio)-ethyl)-mitomycin C (KW-2149) is more active than mitomycin C on chemonaive and drug-resistant urothelial carcinoma cells

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Abstract This in vitro study aimed to investigate the cytotoxic activity of 7-*N*-(2-([2-(gamma-L-glutamylamino)ethyl]dithio)ethyl)-mitomycin C (KW-2149) versus mitomycin C (MMC) against cell lines from human transitional cell carcinoma (TCC). Direct cytotoxicity of the two drugs was measured employing a colorimetric cytotoxicity assay on chemonaive and chemoresistant cancer cell populations. The results revealed that all cell lines ($n = 19$) were significantly more inhibited by treatment (2 h, 96 h) with KW-2149 than by MMC ($P < 0.03$–0.001). pH 6.0 decreased the stronger activity of KW-2149 ($P < 0.013$–0.004). Creatinine ≥10 mmol/l and nitrosourea ≥100 mg/l also inhibited the activity of KW-2149 significantly. Tumor cells with relative drug-resistance against MMC (RT112-MMC: 55-fold) exerted minor cross-resistance to KW-2149 (fourfold). In conclusion, the present in vitro data suggest KW-2149 to be a superior drug for intravesical therapy of patients with primary or recurrent superficial bladder carcinoma. Since pH and concentrations of creatinine and nitrosourea influence the activity of KW-2149, patients are supposed to profit from neutralizing the urinary pH and enhanced diureses.

Key words KW-2149 · Mitomycins · Transitional cell carcinoma · Intravesical treatment · Urine

Introduction

Mitomycin C (MMC) is broadly accepted as an active drug for intravesical therapy of patients with superficial transitional cell carcinoma of the bladder (pTa, pT1). After topical treatment of bladder carcinoma with MMC, patients experience complete tumor remission in approximately 50% and partial remission in approximately 30% [3, 17, 29]. Tumor progression and recurrent bladder carcinoma are suggested to result from intrinsic or acquired drug-resistant tumor cell populations against chemotherapeutic drugs, thus new compounds are warranted for treatment.

KW-2149 represents a novel, semisynthetic, MMC derivative [14]. In contrast to MMC, the biochemical activation of the compound is independent from bioreduction, for example by cytochrome P450 reductase or DT-diaphorase. It is considered to require thiol molecules, like glutathione and cysteine [15]. Besides its superior cytotoxic activity against malignancies of different origin in vitro [6, 13, 19, 22] and in vivo [19, 31], KW-2149 showed significant activity against MMC-resistant neoplasia [2, 6, 13, 15, 21, 27, 31].

The present preclinical study aimed to investigate the cytotoxicity of KW-2149 compared with MMC on cell culture cell lines derived from chemonaive and chemoresistant human transitional cell carcinoma (TCC). Moreover, it was of interest whether physiological components of human urine, including antibiotics, which are often applied as concomitant drugs, influence drug efficacy.

Materials and methods

Cell culture

Cell lines from human TCC (Table 1) were maintained under sterile cell culture conditions (6% CO_2, 37°C). Dulbecco's modified eagle medium (DMEM; GIBCO BRL, Paisley, UK), was supplemented with 15% (v/v) heat-inactivated fetal calf serum (FCS), RPMI 1640 medium with 10% FCS. Media were completed with 100 IU/ml penicillin and 100 µg/ml streptomycin (GIBCO). Subculture passages were detached by enzymatic treatment of tumor cells with trypsin-EDTA solution (0.05%/0.02%; GIBCO). Suspended vital cells were counted by methylene blue exclusion.

Origin, cell culture conditions and resistance patterns of each cell line have been described elsewhere (see Table 1). Cell cultures routinely tested negative for mycoplasma contamination.

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Table 1 The IC50-concentrations (μg/l) of KW-2149 and MMC obtained by treatment of chemonaive and chemoresistant bladder carcinoma cell lines with the anticancer drugs for 2 h or 96 h, respectively. Statistical analysis revealed that all investigated cell lines were significantly more inhibited by treatment with KW-2149 than by treatment with MMC (2 h). *Ref* reference, *G*, grade of malignancy; *ns* not significant, *n.r.* not reached

Cell line	Grade	Ref	2 h treatment			96 h treatment		
			KW-2149	MMC	$P <$	KW-2149	MMC	$P <$
Chemonaive:								
RT 4	G I	[25]	200	2200	0.03	20	20	ns
RT 112	G II	[16]	80	720	0.03	20	20	ns
647 V	G II	[9]	20	1100	0.01	20	60	0.05
HT 1376	G III	[24]	600	2600	0.03	200	300	ns
J 82	G III	[23]	60	360	0.03	20	20	ns
T 24	G III	[4]	20	300	0.03	20	50	ns
639 V	G III	[9]	20	1250	0.01	20	70	0.05
TCCSUP	G IV	[20]	60	270	0.01	20	20	ns
253 J	G IV	[10]	20	420	0.03	20	20	ns
SW 1738	G x	[7]	20	200	0.03	20	20	ns
Chemoresistant:								
RT 112-MMC		[6]	600	n.r.	–	80	1100	0.01
RT 112-CP		[32]	380	2100	0.03	100	150	ns
HT 1376-CDDP		[26]	800	n.r.	–	600	1000	0.01
HT 1376-VP16		[26]	600	n.r.	–	300	950	0.01
HT 1376-MTX		[26]	600	n.r.	–	270	950	0.01
TCCSUP-CDDP		[26]	60	230	0.01	60	60	ns
TCCSUP-VP16		[26]	250	1380	0.01	150	200	ns
TCCSUP-MTX		[26]	80	250	0.01	60	60	ns
TCCSUP-VBL		[26]	120	580	0.01	80	80	ns

Chemicals

Mitomycin C (Lot 41004-03) and 7-*N*-(2-([2-(gamma-L-glutamylamino)ethyl]dithio)ethyl)-mitomycin C (Lot 9305) were provided by the manufacturer (Kyowa Hakko Kyogyo, Düsseldorf, Germany). Anticancer agents were diluted with medium to final drug concentrations (10–3000 μg/l, 250 000 μg/l, 400 000 μg/l). Vials were always freshly prepared.

Determination of drug activity in vitro

The activity of KW-2149 and MMC was measured by use of modified sulforhodamine B (SRB-) assay [18, 28]. Vital tumor cells were allowed to settle in 96-well microtiter plates for 4 h (Becton Dickinson Labware, N.J.), before medium was replaced by drug-containing medium. After 2 h incubation, the medium was replaced by medium without anticancer drugs (94 h). Treatment of 2 h was compared with treatment of 96 h. Controls received no cytostatic agents. Results from eight wells per experiment were repeated at least twice.

The influence of pH (6.0, 7.2, 8.0), albumine (0.5 g/l, 2.5 g/l), creatinine (5 mM, 10 mM, 25 mM), glucose (2 g/l, 4.5 g/l, 10 g/l), hemoglobin (0.1 g/l, 0.5 g/l, 1.0 g/l), nitrosourea (50 mM, 100 mM, 150 mM), and different antibiotics (cefotiam: 50, 100, 150 μg/ml, sulfamethoxazol: 20, 40, 80 μg/ml, ciprofloxacin: 1.2, 2.4, 3.6 μg/ml, piperacillin: 50, 150, 300 μg/ml) was analyzed on RT112, RT112-MMC and RT112-CP cells with regard to the activity of KW-2149 and MMC. Therefore, adherent tumor cells (4 h) were exposed to media, that were adjusted to one of these parameters (2 h with, 94 h without, cytostatic drugs). Controls were adjusted to those parameters for 96 h without exposition to anticancer drugs. Results from four wells with identically treated tumor cell populations were repeated at least twice.

Finally, cells were fixed with 10% (v/v) trichloroacetic acid (60 minutes, 4°C). Plates were rinsed with deionized water and dried at room temperature (24 h). Cells were stained with 0.4% (w/v) sulforhodamine B solution (Aldrich Chemicals, Steinheim, Germany) for 10 min. Unbound stain was removed by 1% (v/v) acetic acid. Bound stain was solubilized with TRIS-buffer. Optical densities were measured at a single wavelength of 515 nm on an spectrophotometric plate-reader (EAR 400 AT; SLT Labinstruments, Crailsheim, Germany). Growth inhibition was expressed as optical density (OD) of treated cells/OD of untreated cells × 100 (%). The drug concentrations that led to a 30% or 50% inhibition of tumor growth compared with untreated controls were designated IC30 or IC50, respectively. IC30 and IC50 concentrations were derived by dose-response-curves.

Statistics

To compare different IC50 or IC30 concentrations, the optical densities of the surrounding drug concentrations (i.e., the next concentration above and below the estimated IC50 or IC30) were statistically compared using the Wilcoxon test with continuity correction of 0.5. Statistics were performed on SAS system (Statistical Analysis System, SAS Institute, Cary, N.C.) A *P*-value < 0.05 was designated as statistically significant. In case those neighbouring concentrations were statistically different, IC concentrations were also likely to differ statistically significant.

Results

KW-2149 significantly inhibited growth of chemonaive and chemoresistant urothial cancer cells more than MMC. The activity of both compounds, KW-2149 and MMC, showed a dependency on drug concentration and period of incubation (96 h $\gg$2 h) (Fig. 1, Table 1).

As expected, pleiotropic cross-resistances were found for the drug-resistant cell lines. Their relative cross-resistance for treatment with KW-2149 of MMC, i.e., the

IC concentration of the drug-resistant cells compared with the corresponding parental cells, was predominantly below resistance factor 10 (Table 1). The cell line RT112-MMC was 55 times more resistant to treatment with MMC, and exhibited a four-fold cross-resistance to KW-2149.

The activity of KW-2149 and MMC on RT112 (Fig. 2), RT112-MMC and RT112-CP was significantly influenced by pH (Table 2), and the activity of KW-2149 was significantly more influenced by pH than the activity of MMC ($P < 0.01$). However, the activity of KW-2149 at pH 6.0 still remained superior ($P < 0.0458$–0.0009) or was at least equal to MMC.

Creatinine concentration ≥10 mmol/l, and nitrosourea ≥100 mmol/l decreased the activity of KW-2149 on all three cell lines, but did not decrease activity of MMC (Table 2). Despite the decremental activity of KW-2149 in the presence of creatinine and nitrosourea, the com-

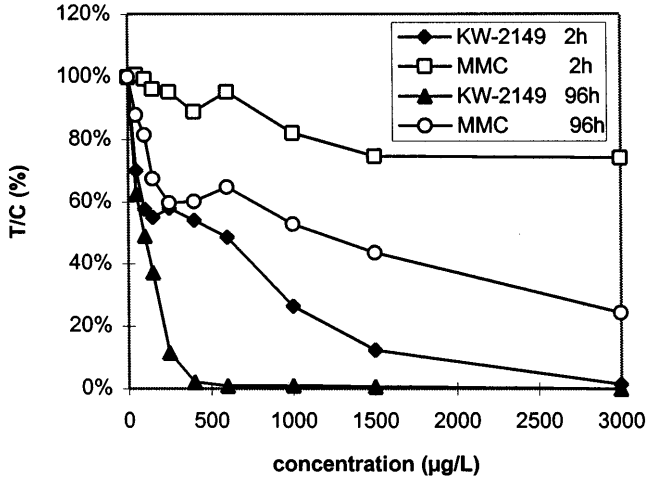

Fig. 1 Dose-response curves of RT112-MMC cells obtained by treatment with increasing concentrations of KW-2149 or MMC. Growth inhibition of tumor cells is expressed as optical density (OD) of treated cells (T) divided by the OD of untreated cells (C) ×100 (%). Growth inhibition of tumor cells is dependent on the drug concentration and increased by the duration of treatment (96 h versus 2 h)

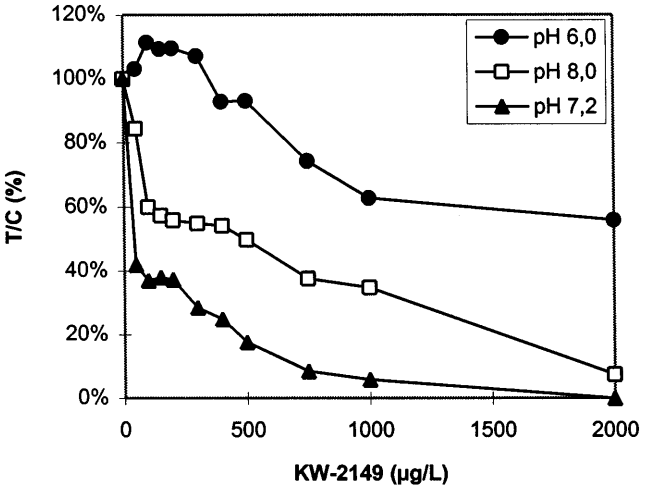

Fig. 2 pH 6.0 and pH 8.0 significantly ($P < 0.01$) decrease the activity of KW-2149 (2-h treatment) on RT112 cells, compared with pH 7.2. For key see Fig. 1

Table 2 The influence of urinary components (UC) on the activity of anticancer drugs against RT112, RT112-MMC and RT112-CP cells. Significance was calculated on the basis of growth inhibition of tumor cells by each anticancer drug. The growth inhibition, which was obtained under standard cell culture conditions with KW-2149 and MMC was compared with the growth inhibition of equal concentrations of the drugs under the influence of UC; + indicates an increased growth inhibition of the anticancer drugs by addition of UC; − indicates a decreased growth inhibition; *ns* not significant, *nd* not done

		Activity of KW-2149			Activity of MMC		
		RT112	RT112 -MMC	RT112-CP	RT112	RT112-MMC	RT112-CP
pH	6	− ($P < 0.004$)	− ($P < 0.013$)	− ($P < 0.024$)	− ($P < 0.018$)	− ($P < 0.013$)	ns
	8	− ($P < 0.013$)	− ($P < 0.013$)	− ($P < 0.018$)	− ($P < 0.018$)	− ($P < 0.032$)	− ($P < 0.024$)
Creatinine	5	ns	ns	ns	ns	ns	ns
(mM)	10	ns	− ($P < 0.010$)	− ($P < 0.004$)	ns	ns	ns
	25	− ($P < 0.023$)	− ($P < 0.018$)	− ($P < 0.010$)	ns	ns	ns
Nitrosourea	50	ns	ns	ns	ns	ns	ns
(mM)	100	− ($P < 0.013$)	− ($P < 0.018$)	ns	ns	ns	ns
	150	− ($P < 0.040$)	− ($P < 0.013$)	− ($P < 0.018$)	ns	ns	ns
Cefotiam (µg/ml)	50	ns	− ($P < 0.016$)	ns	ns	ns	ns
	100	− ($P < 0.035$)	− ($P < 0.013$)	− ($P < 0.040$)	ns	− ($P < 0.041$)	ns
	150	− ($P < 0.013$)	− ($P < 0.033$)	− ($P < 0.044$)	ns	ns	ns
Ciprofloxacin	1.2	ns	+ ($P < 0.027$)	ns	ns	ns	ns
(µg/ml)	2.4	+ ($P < 0.040$)	+ ($P < 0.013$)	+ ($P < 0.031$)	+ ($P < 0.024$)	ns	ns
	3.6	ns	+ ($P < 0.018$)	ns	ns	ns	ns
Piperacillin	50	ns	− ($P < 0.014$)	ns	ns	ns	ns
(µg/ml)	150	ns	− ($P < 0.041$)	ns	ns	ns	ns
	300	ns	− ($P < 0.031$)	ns	ns	ns	ns
TMP-SMZ	20	nd	+ ($P < 0.018$)	+ ($P < 0.024$)	ns	ns	nd
(µg/ml)	40	nd	ns	+ ($P < 0.010$)	ns	ns	nd
	80	nd	+ ($P < 0.041$)	+ ($P < 0.013$)	+ ($P < 0.024$)	ns	nd

pound still significantly remained more cytotoxic than equal concentrations of MMC ($P < 0.045$–0.001).

Albumin, hemoglobin and glucose did not significantly influence the activity of either drugs on the investigated cell lines (data not shown). The addition of different antibiotics to the drug-containing medium predominantly influenced the activity of KW-2149. Cefotiam and piperacillin decreased, ciprofloxacin and TMP-SMZ enhanced the activity of KW-2149 (Table 2).

Discussion

Previous reports provided clear evidence that the novel mitomycin C analogue KW-2149 is an active anticancer drug on different murine and human neoplasms [1, 19, 31], including some few bladder carcinoma cell lines (T24, HT-1197) and xenografts from T24 cells [19].

The present preclinical experiments confirmed, for a panel of 19 chemonaive or chemoresistant cell lines, that a treatment of 2 h duration with KW-2149 inhibited tumor cells at concentrations 3.3–55 times lower than a treatment with MMC. Such superior activity of KW-2149 (10 to 100-fold [1, 13, 19]) has been reported for other human neoplastic cells in vitro [1, 6, 12, 13, 19, 22] and in animal models [19, 21]. To explain this increased potency it has been suggested that KW-2149, unlike MMC, binds covalently with DNA and protein, leading to increased protein–DNA-complexes [11]. Moreover, it has been demonstrated, that KW-2149 forms DNA–DNA and DNA–protein cross-links 20 times more effectively than MMC [5].

In our experiments we found no major cross-resistance of KW-2149 with the mitomycin C-resistant bladder carcinoma cell line RT112-MMC. Similar observations have been reported for other MMC-insensitive cell lines, which had deficiencies in DT-diaphorase/NAD(P)H dehydrogenase (quinone) and cytochrome P450/b5 reductase activity [2, 15, 27]. Since resistance to MMC is predominantly associated with decreased levels of those enzymes, our group has previously confirmed for RT112-MMC cells that resistance to MMC was due to loss of heterogeneity (LOH) at the NQOR gene locus. This LOH results in a complete loss of NAD(P)H-chinone-oxidoreductase activity [8]. In addition, Lee and coworkers [15] described, that glutathione (GSH) and cysteine increased cytotoxicity of KW-2149 in HT-29 cells, indicating that the DNA adduct of KW-2149 is, unlike MMC, activated in the presence of thiol molecules. Consistent with this, KW-2149 is most likely to remain active in MMC-resistant cells because activation of the drug is independent of bioreductive pathways. However, MMC-resistant cell lines were described [6, 13] that remained partially resistant to KW-2149. In our experiments, the observed minor cross-resistance of RT112-MMC cells against KW-2149 (four-fold) is in agreement with the fact that RT112-MMC cells are also characterized by a decreased activity of gluthatione-transferase enzyme activity [8]. Finally, as for RT112-CP and HT1376-CDDP cells, cross-resistance of cisplatinum-resistant ovarian cancer cells for KW-2149 and MMC has also been demonstrated [6].

Furthermore, the present experiments demonstrate that activity of the drugs is influenced by different natural components of human urine, especially pH. From a pharmacochemical point of view, more than 90% of KW-2149 (1 mg/ml) remains stable in human urine at pH 6.0 since acidification of human urine to pH 4.0 reduces stability to approximately 50% after 2 h (data from Kyowa Hakko Kogyo, Japan). Similarly, more than 90% of MMC remains stable after 2 h in physiological saline at pH 6.0 (data from Medac, Hamburg, Germany). In conclusion, KW-2149 and MMC are equally stable in aqueous solutions with a pH ranging from 6.0 to 8.0 during 2 h exposure, thus the stronger dependency of KW-2149 on alterations of pH, which were found in the present experiments, cannot simply be explained by instability of the molecule. Nevertheless, our data revealed that changes of pH are capable of diminishing the superior activity of KW-2149 against urothelial carcinoma. Moreover, creatinine and nitrosourea can inhibit the activity of KW-2149 at concentrations that can be found in human urine. Finally, the activity of KW-2149 was much more influenced by antibiotics than the activity of MMC (especially on drug-resistant RT112-MMC cells). It is of interest that those antibiotics that inhibit transpeptidase (cefotiam and piperacillin) reduced the activity of KW-2149. On the other hand the antibiotics that counteract DNA (TMP-SMZ and ciprofloxacin), enhanced the activity of KW-2149. For ciprofloxacin, a inhibitor of topoisomerase II, such an increase in activity is likely due to that fact that mitomycins themselves are capable of inhibiting topoisomerase II. The differences between KW-2149 and MMC are explained by the fact that DNA-damage by KW-2149 is due to single-strand scission, indicating a mode of action different to MMC [12].

In conclusion the present preclinical experiments demonstrate that KW-2149 is a mitomycin derivative superior to MMC in the treatment of TCC in vitro. Since application of KW-2149 in dogs has already been reported for pharmacological purposes, indicating no systemic toxicity of the drug after intravesical instillation [30], KW-2149 is strongly recommended as first-line intravesical chemotherapy for patients with superficial bladder carcinoma, and as second-line therapy for recurrent bladder carcinoma after previous topical therapy with mitomycin C. In line with the fact that the stronger cytostatic activity of KW-2149 decreases in the case of acidic pH, neutralizing the pH of the patient's urine is recommended. In addition, in future clinical trials, diureses of patients should be stimulated before therapy in order to reduce the negative influence of high concentrations of creatinine and nitrosourea.

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