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Intracellular accumulation and DNA damage persistence as determinants of human squamous cell carcinoma hypersensitivity to the novel camptothecin ST1968

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ABSTRACT

ST1968, a novel hydrophilic camptothecin analogue of the 7-oxyiminomethyl series, is characterised by the formation of stable DNA–topoisomerase I cleavable complex and by a promising profile of antitumour activity. The present study was designed to extend preclinical evaluation of the novel camptothecin in human squamous cell carcinoma (SCC) models. ST1968 exhibited an impressive activity with a high cure rate in SCC models. ST1968 produced 100% of complete response without evidence of regrowth in tumours characterised by susceptibility to drug-induced apoptosis (FaDu, A431 and A2780). In contrast to irinotecan, ST1968 still showed an excellent, persisting activity in models less susceptible to apoptosis induction (KB, Caski and SiHa), in which drug treatment elicited a persistent DNA damage response, as documented by phosphorylation of p53, RPA-2 and histone H2AX, resulting in delayed apoptosis and senescence. This behaviour was associated with a marked cellular/tumour drug accumulation. In conclusion, ST1968 exhibited an outstanding antitumour activity superior to that of irinotecan against SCC. A high intracellular accumulation, resulting in fast apoptosis or DNA damage persistence, appeared to be a critical determinant of SCC sensitivity to ST1968.

1. Introduction

Camptothecins (CPTs) are amongst the most effective agents available for cancer treatment.[1] CPTs are the only clinically used drugs targeting topoisomerase I, which is the sole cellular target recognised for these agents. The antitumour activity of camptothecins is ascribed to their ability to stabilise the covalent DNA–topoisomerase I complex. The outstanding preclinical activity of these agents has stimulated efforts to optimise their therapeutic potential through identification and development of analogues with improved pharmacological and therapeutic profile.[1–3]

We have reported that the introduction of appropriate lipophilic substituents at the 7-position confers potential advantages in terms of cellular pharmacokinetics, stabilisation of the cleavable complex and lactone stability.[4] The ability to stabilise the cleavable complex is a favourable feature of several derivatives of the 7-oxyiminomethyl series. However, also hydrophilic derivatives of this series retain ability to stabilise the topoisomerase I-mediated cleavable complex.[5]

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In particular, the nature of the substituent at the 7-position and the presence of a free amino group in the side chain appear to be critical determinants of drug activity. Thus, in spite of a reduced cytotoxic potency, selected hydrophilic analogues of the 7-oxyiminomethyl series retain a potent activity as topoisomerase I inhibitors and a remarkable *in vivo* antitumour efficacy.[5]

The present study is a part of a preclinical evaluation aimed to define the pharmacological profile of ST1968, which is the most water-soluble analogue containing an aliphatic amine in the side chain.[5] This analogue was selected for clinical development on the basis of its preclinical pharmacological profile showing an improved efficacy and therapeutic index (manuscript in preparation). Since during preclinical evaluation, we observed that some squamous cell carcinoma models exhibited hypersensitivity to the camptothecins[5], this study was designed to extend the evaluation of preclinical activity of ST1968 to a large panel of human squamous cell carcinoma models. The results showed an impressive antitumour activity of ST1968, substantially superior to that of irinotecan.

2. Materials and methods

2.1. Drugs

ST1968 was dissolved in sodium lactate buffer (50 mM) adjusted to pH 4.0 with addition of hydrochloric acid. Irinotecan (CPT11) was dissolved in sterile, distilled water. After magnetic stirring, both drugs were administered intravenous (i.v.) very slowly in a volume of 10 ml/kg of body weight.

2.2. Antibodies

The used primary antibodies were against: p53 (Dako, Glostrup, Denmark); phospho p53 (ser^{15}), cleaved caspase 3 (Asp^{175}) (Cell Signalling Technology, Beverly, MA); RPA-2 and p21 (Neomarker, Union City, CS, USA); PARP-1 (Oncogene Science, Uniondale, NY, USA); γH2AX (Upstate Biotechnology, Lake Placid, NY, USA); actin and β-tubulin (Sigma, St. Louis, MO, USA); HIF-1α (SC-13515, Santa Cruz Biotechnology, Santa Cruz, CA); BNIP3 (Abcam Ltd., Cambridge, United Kingdom).

2.3. Cell culture and antiproliferative activity studies

The human tumour cell lines used in the study included an ovarian carcinoma (A2780), six squamous cell carcinoma (SiHa, FaDu, A253, A431, CaSki and KB) and a prostate carcinoma (DU145). Cells were maintained in different culture medium supplemented with 10% FCS (Invitrogen, Gaithersburg, MD): RPMI 1640 (BioWhittaker, Verviers, Belgium) for A2780, KB, SiHa, Caski and A431; McCoy's medium (BioWhittaker) for A253 cells; EMEM (BioWhittaker) for FaDu cells. All cell lines were maintained in a humidified atmosphere with 5% CO_2 at 37 °C.

Cells in the logarithmic phase of growth were seeded in duplicate in six-well plates and, 24 h after seeding, were exposed to ST1968 for 1 h. Cells were then washed and incubated in drug-free medium for 72 h. Adherent cells were trypsinised and counted by using a cell counter (Coulter Electronics, Luton, United Kingdom). IC_{50} was defined as the concentration required for 50% cell growth inhibition compared with control cells.

2.4. Western blot analysis

Cells or tumour samples were processed for total protein extraction and analysed by immunoblotting as previously described.[6] Tumour protein lysates were prepared from frozen tumour samples. Whole-cell lysates (40 μg) were resolved by SDS–polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose filters. Membranes were incubated with primary antibody overnight and with peroxidase-conjugated anti-mouse or -rabbit antibody to reveal immunoreactive bands using the enhanced chemiluminescence detection system from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

2.5. Cell cycle analysis

After drug treatment, adherent cells were trypsinised, fixed and stained with a propidium iodide (PI) solution (Sigma–Aldrich, St. Louis, MO) (10 μg/ml PI and 66 U/ml RNaseA in PBS) for 18 h. At least 40,000 cells were collected and analysed by FACScan flow cytometry. Cell cycle distribution was calculated by ModFit software (Becton Dickinson).

2.6. Assessment of apoptosis

Apoptosis was detected at different times after treatment by morphological analysis of PI-stained cells and by TUNEL assay (Roche, Germany). At least 100 cells stained with propidium solution as described above, in two different smears, were examined for nuclear morphology by a fluorescence microscope and the results were expressed as percentage of apoptotic cells over the cell number of the whole population. In the TUNEL assay, the samples were prepared as described[6] according to the manufacturer's instructions and analysed by flow cytometry.

2.7. In situ staining of SA β-Gal activity

Cells were washed in PBS, fixed for 5 min (room temperature) in 2% formaldehyde and 0.2% glutaraldehyde, washed and incubated overnight at 37 °C with fresh senescence associated β-Gal (SA-β-Gal) stain solution containing 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml (stock = 20 mg of dimethylformamide per ml)/40 mM citric acid/sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM $MgCl_2$. After 18 h of the incubation, cells were examined at 100× magnification (at least 200 cells/sample). The results were expressed as percentage of SA-β-Gal positive cells over the SA-β-Gal negative or unlabelled cells.

2.8. Cellular pharmacokinetics and in vivo distribution

Cells were plated in six-well plates at a subconfluent density (around 50,000 cells/cm^2 for Caski and SiHa cells; 100,000 cells/cm^2 for A431 cells). Cells (3 wells/sample) were then washed with serum-free medium and exposed for 30 min,

1 h or 2 h to 1 μM of ST1968. Treated cells were washed three times with PBS to remove the extracellular drug and, for retention studies, maintained in a drug-free medium for 5 or 24 h. At different times, washed cells were resuspended in 150 μl of PBS and subjected to 5 cycles of freezing in liquid nitrogen and thawing in thermostated bain at 37 °C. Each cycle lasted 5 min. Cell lysates were frozen at –20 °C.

For *in vivo* tumour distribution, tumours were established in CD1 nude mice as described for the antitumour activity studies. When tumours reached a size of ~400 mm^3, animals (3 mice/group) were treated with ST1968 at the dose of 30 mg/kg and sacrificed at different times (0.5–1–2–4–8–24–48 h) after intravenous injection.

Samples were processed by adding cold 0.1% acetic acid/methanol (1:5, v:v). Analysis was performed using the 32Karat Software (Beckman-Coulter) with a fluorescence detector (Jasco or Shimadzu) fixed at 370 nm as excitation wavelength and 510 nm as emission wavelength. ST1968 was eluted on a Discovery HS F5 column (5 μm, 100 × 4.6 mm, Supelco) kept at room temperature (r.t.) in isocratic condition (0.1 M acetic acid: (0.1% TEA:CAN, 75:25, v:v) with a flow rate of 1 mL/min.

2.9. Antitumour activity in vivo

All experiments were carried out using female athymic Swiss nude mice, 6–8 weeks-old (Charles River, Calco, Italy). Mice were maintained in laminar flow rooms keeping temperature and humidity constant. Mice had free access to food and water. Experiments were approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale dei Tumori of Milan according to institutional guidelines and to the UK Coordinating Committee on Cancer Research Guidelines.[7]

Exponentially growing tumour cells (10^7 cells/mouse) were subcutaneously (s.c.) injected into mice flank. Tumour lines were achieved by serial s.c. passages of fragments (about 2 × 2 × 6 mm) from growing tumours into healthy mice, as previously described[8] Groups of 5–8 mice bearing s.c. tumours implanted in one or both flanks were employed. Tumour fragments were implanted on day 0 and tumour growth was followed by biweekly measurements of tumour diameters with a Vernier caliper. Tumour volume (TV) was calculated according to the formula: TV (mm^3) = $d^2 \times D/2$, where d and D are the shortest and the longest diameter, respectively. Drugs were delivered i.v. every fourth day for four times (q4d×4) starting when tumours were just palpable.

The efficacy of the drug treatment was assessed as

(i) Tumour volume inhibition percentage (TVI %) in treated versus control mice, calculated as: TVI % = 100 – (mean TV treated/mean TV control × 100).
(ii) Complete responses (CR), i.e. complete tumour regression for at least 10 days.
(iii) Cure was defined as no evidence of tumour regrowth following treatment-induced regression at the end of the experiment (at least 3 months).

The toxicity of the drug treatment was determined as body weight loss and lethal toxicity. The highest body weight loss percentage induced by treatments is reported in Section 3. Deaths occurring in treated mice before the death of the first control mouse were ascribed to toxic effects. Student's *t*-test (two-tailed) and Fisher's exact test were used for statistical comparison of tumour volumes and CR, respectively, in mice.

3. Results

3.1. Antitumour activity studies

The study was performed in a panel of squamous cell carcinoma (SCC) xenografts derived from various organs (head and neck, 3; uterine cervix, 2; cutis, 1) (Table 1 and Fig. 1). For the purpose of comparison, we also included in the panel an ovarian carcinoma model A2780, a well-known responsive tumour, in which apoptosis is a major determinant of sensitivity to camptothecins[9] and the prostate carcinoma model DU145, a model refractory to camptothecin treatment. Irinotecan (CPT11) was used as a reference drug of this class. Both agents (ST1968 and irinotecan) were delivered by i.v. administration with the intermittent q4d×4 schedule, i.e. a standard schedule used for comparative evaluation of antitumour activity of camptothecins.[8] Irinotecan was used at its optimal dose (MTD) (in the range of 50–60) in each tumour model. The MTD of ST1968 was in the range of 30–35 mg/kg. The dose of 30 mg/kg caused an acceptable toxicity (7–12% body weight loss), without lethal toxicity. The dose of 35 mg/kg caused some toxic death and should be considered approximately the LD_{10}. ST1968 exhibited an outstanding efficacy against all tested SCC models, producing a TVI in the range of 96–100%. As observed in the ovarian carcinoma A2780, the treatment with ST1968 produced a 100% complete response rate in 3 SCC models (FaDu, A431 and KB). In these tumours, the complete responses achieved in the range of optimal doses (30–35 mg/kg) can be considered cures, because no tumour regrowth was observed at the end of the experiments. The antitumour effect of suboptimal doses (15–17.5 mg/kg) was still marked and comparable to that of the MTD in the A2780 and FaDu tumours. In the most responsive tumours (A2780, FaDu and A431) also irinotecan exhibited an excellent activity; however, the efficacy of ST1968 was clearly superior against KB tumour, in terms of complete response rate, and against A2780 and A431 (Fig. 1), because the complete responses induced by irinotecan were not maintained as a consequence of partial regrowth of regressed tumours. CaSki tumour was very responsive only to ST1968, because 9/12 tumours were cured. Irinotecan was substantially less effective without evidence of tumour regression. In the less responsive A253 and SiHa tumours (Fig. 1), the treatment outcome was less impressive, although in contrast to irinotecan, ST1968 produced an appreciable number of complete responses in both tumour models. As expected, DU145 prostate carcinoma model exhibited a low responsiveness.

3.2. Cellular response to drug treatment

In an attempt to understand the cellular basis of the hypersensitivity of SCC models to ST1968, we have investigated some aspects of cellular response to *in vitro* treatment, with particular reference to apoptosis, perturbation of cell cycle and DNA damage response. The SCC cell lines examined in

Table 1 – Antitumour activity of ST1968 and irinotecan

Tumour model[a]	Drug	Dose[b] (mg/kg)	TVI (%)	CR[c]	NED[d]	Median Time to CR[e]	BWL[f] (%)
A2780 (3)	ST1968	30	100	7/7	6/7§	5	11
		15	100	8/8	8/8§§		7
	CPT11	60	100	7/7	1/7		9
FaDu (3.9)	ST1968	35	100	7/7	7/7	10	12 (1/8)
		30	100	8/8	8/8		7
		15	100	8/8	8/8		4
	CPT11	60	100	8/8	8/8		6
A431 (4)	ST1968	30	100	12/12	12/12	16	2
	CPT11	50	100	12/12	9/12		7
KB (5)	ST1968	35	100*	7/7	7/7§§	20	9 (1/8)
		17.5	99*	4/8	4/8§		4
	CPT11	60	92	0/8	0/8		4
CaSki (6.9)	ST1968	30	99**	9/12	9/12§§	43	8
	CPT11	50	75	0/12	0/12		2
SiHa (7.1)	ST1968	30	99**	4/12	4/12§	22	7
	CPT11	50	73	0/12	0/12		0
A253 (8.5)	ST1968	35	96*	2/7	2/7	20	13
		30	90*	1/8	1/8		9
	CPT11	60	79	0/8	0/8		6
DU145 (10)	ST1968	30	75	0/8	–	–	13
	CPT11	60	82	0/8	–	–	2

a In parentheses, doubling time (days).
b Irinotecan was used at a single dose equivalent to MTD in each model (q4d×4, i.v.).
c CR, complete response at the end of treatment.
d NED, no evidence of disease at the end of experiment.
e Time required to achieve complete tumour regression following the start of treatment.
f BWL, body weight loss (in parentheses, toxic deaths).
* $P < 0.05$ vs CPT11.
** $P < 0.01$ vs CPT11, by Student's t-test.
§ $P < 0.05$ vs CPT11.
§§ $P < 0.01$ vs CPT11, by Fisher's exact test.

this study were characterised by different p53 status, i.e. mutations (A431, FaDu), lack of expression (A253) or presence of the human papilloma virus 16 or 18 E6 protein resulting in reduced p53 protein expression (Caski, SiHa and KB). No precise correlation could be found between p53 status and cell sensitivity or tumour response (Table 2). With one exception (SiHa cells), the antiproliferative effects of ST1968 in the tested SCC cell lines were comparable to that found in the reference sensitive ovarian carcinoma A2780 cells. SiHa cells exhibited a reduced sensitivity similar to that of the resistant prostate cancer DU145 cells.

The analysis of cell death processes activated following treatment evidenced a quite different pattern of response amongst the examined cell lines (Table 2). As observed in A2780 cells, the percentage of apoptotic cells produced by equitoxic drug concentrations at 72 h after drug treatment in the most sensitive cells (A431 and FaDu) was substantially higher (around 50%) than in other SCC cell lines. Under these conditions, A431 and FaDu cells showed an early cell death already detectable at 24 h associated with caspase 3 activation and cleavage of PARP-1 (Fig. 2A). In CaSki and KB cells the appearance of apoptotic cells was found only at a prolonged time (144 h), indicating that the apoptosis induction was a somewhat delayed event. We observed that drug treatment caused an increase of cell volume in KB, CaSki and SiHa cells. This morphology change and the low susceptibility to apoptosis suggested the induction of a senescence-like state as a response to DNA damage. Indeed a large number of cells positive for β-galactosidase activity were detected in these cell lines, particularly in SiHa cells in which a marginal apoptotic response was found. Thus modality of cell death in KB, CaSki and SiHa cells appeared to be a complex phenomenon implicating not only apoptosis, but also senescence. Relevant to this point is the observation that only cells with wild-type p53 (KB, CaSki and SiHa) exhibited the ability to activate the senescence programme. Indeed the induction of senescence has previously reported to be p53-dependent.[10] In these cell systems, the presence of the viral protein E6 caused a low level of drug-induced up-regulation of the p53 protein, associated to induction of p21 (most evident at 48 h) (Fig. 2B). A253 cells exhibited a slow and late apoptotic response (a weak activation of caspase 3 associated with cleavage of PARP-1 was barely detectable only at 48 h after treatment (Fig. 2B)) without evidence of senescence. The pattern of response in SiHa and A253 cells characterised by a lower susceptibility to apoptosis was consistent with a

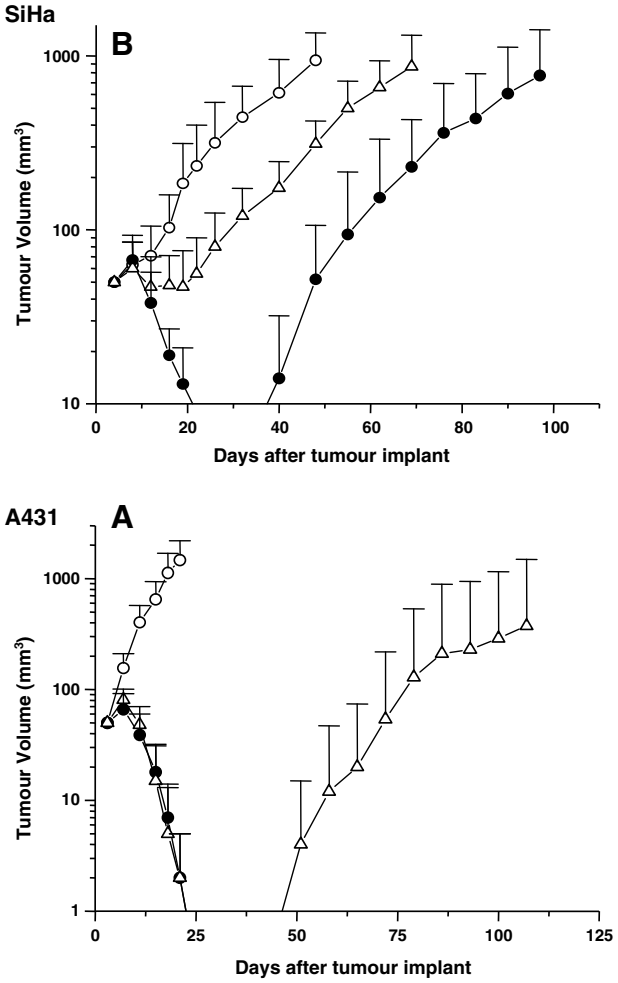

Fig. 1 – Antitumour activity of ST1968 and irinotecan against A431 (A) and SiHa (B) tumour xenografts. ST1968 (30 mg/kg) and irinotecan (50 mg/kg) were administered i.v. at their respective MTD with an intermittent treatment schedule (q4d×4). In parentheses, number of regrowing tumours. ○, untreated control; △, irinotecan; ●, ST1968.

substantially reduced *in vivo* responsiveness as compared to other models.

The analysis of the cell cycle was performed at equitoxic (IC_{50} or IC_{80}) and equimolar concentrations (Fig. 3). Exposure to ST1968 caused a partial S phase arrest in FaDu and CaSki cells or G2/M cell accumulation in other cells, which is a common response to camptothecins and other DNA damaging agents. The response of the most sensitive cell lines (A2780, A431 and FaDu) was characterised by an early appearance of a sub-G1 peak, according to the induction of apoptosis detected by the TUNEL assay and the activation of CPP32 (Table 2 and Fig. 2A). The perturbation of cell cycle was persistent in the other SCC cell lines (up to 144 h in SiHa cells). This persistent cell cycle arrest is in keeping with the senescence state described above (Table 2).

Amongst the DNA damage response factors, we have examined the phosphorylation of the H2AX histone (γ-H2AX), p53 (serine 15) and RPA-2 (detected by the shift in the electrophoretic mobility, as previously described (9)) (Fig. 2A and B), which are implicated as early events in the DNA damage signalling pathway. At equitoxic concentrations (IC_{80}), all treated cells exhibited to a variable extent phosphorylation of these proteins. The low extent of p53 phosphorylation in KB, CasKi and SiHa was consistent with the low protein expression. DNA damage response was observed at equimolar concentrations (<1 μM) in all tested cell lines, except in A253 cells in which it was barely detectable. RPA-2 phosphorylation was persistent in cells characterised by a late apoptosis or senescence (KB, Caski, SiHa and A253), supporting a permanent maintenance of a DNA damage response state.

3.3. *Biochemical analysis of in vivo response*

A comparative analysis of *in vivo* response was performed in the tumour model A2780, in which hypersensitivity was associated with an early and massive apoptotic cells death, in the model KB, characterised by slow apoptotic response and occurrence of senescence, and in the resistant prostate carcinoma DU145 (Fig. 2C). In the A2780 tumour, an early DNA

Table 2 – Antiproliferative activity, apoptosis and senescence induction by ST1968

Cell line	p53 status	IC_{50} (μM)[a]	Apoptosis[b]		β-GAL positive cells[c] (%)
			72 h	144 h	
A2780	wt	0.12 ± 0.02	52 ± 7[d]		15 ± 2
FaDu	mutant	0.076 ± 0.019	66 ± 6		n.d.
A431	mutant	0.18 ± 0.11	46 ± 4		0
KB	wt/HPV18	0.30 ± 0.09	6 ± 2	48 ± 6	49 ± 3
CaSki	wt/HPV16	0.53 ± 0.08	25 ± 5	54 ± 5	54 ± 8
SiHa	wt/HPV16	1.30 ± 0.13	4 ± 1	15 ± 3	73 ± 4
A253	null	0.24 ± 0.08	18 ± 2	28 ± 2	0
DU145	mutant	1.57 ± 0.02	n.d.		0

n.d., not determined.
a IC_{50} values were determined by dose–response curves at 72 h following 1 h-exposure to the drug.
b Apoptosis level determined by TUNEL assay following 1 h-exposure to 2.3 μM (around $IC_{80–90}$), except in CaSki and SiHa cells exposed to 6.9 μM.
c β-Galactosidase positive cells were determined at 144 h following exposure to 2.3 μM, except in CaSki and SiHa cells exposed to 6.9 μM.
d Apoptosis was determined at 24 h.

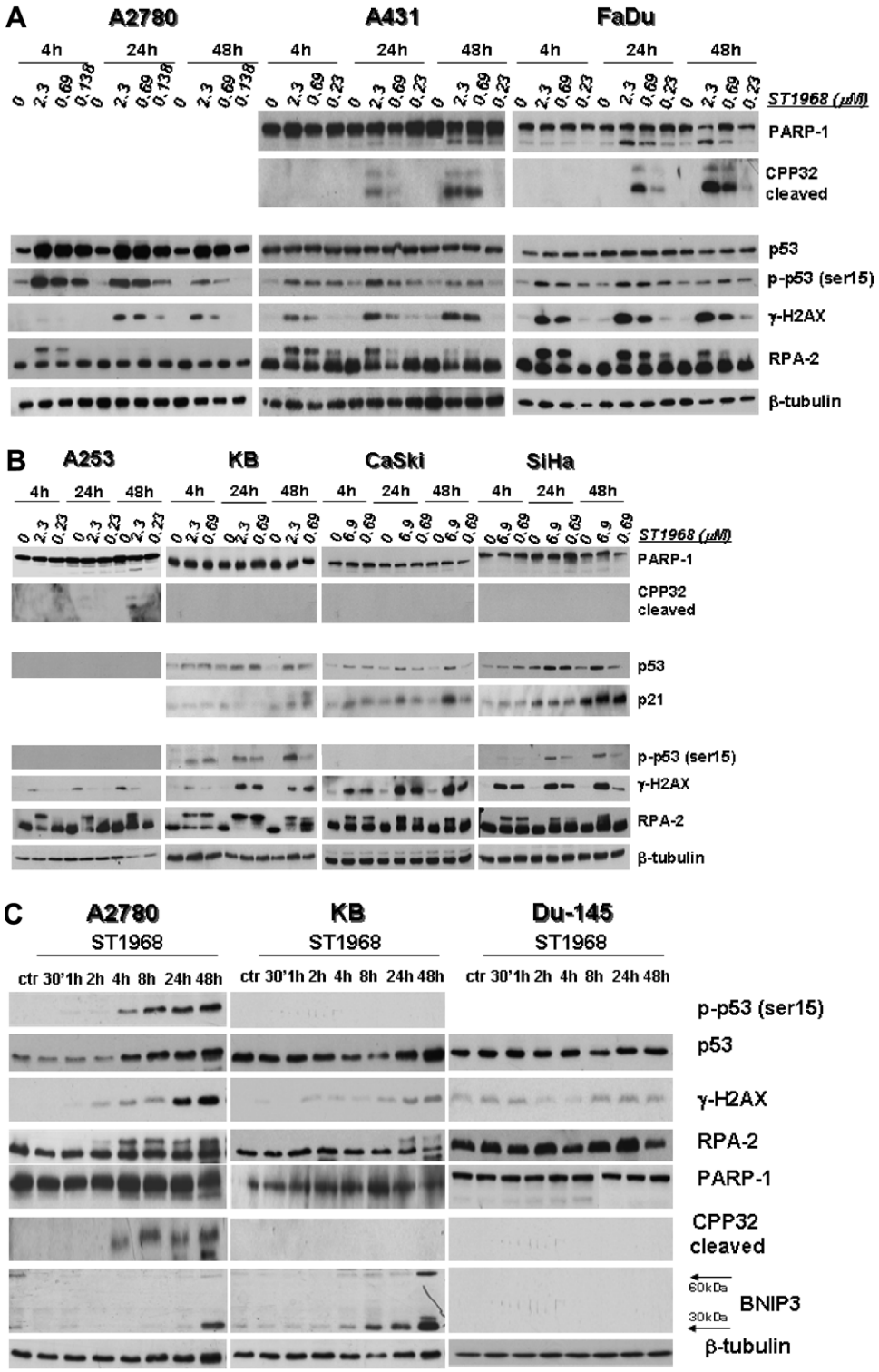

Fig. 2 – (A) and (B) Analysis of CPP32 and PARP-1, and of DNA damage response factors in ST1968-treated cells at various times after treatment. Cells were exposed to eqitoxic (IC_{80} and IC_{50}) and equimolar concentration of ST1968, respectively, for 1 h and harvested at 4, 24 and 48 h after treatment. (C) Western blot analysis of proteins from A2780, KB and DU145 tumours, xenografted in nude mice, after i.v. treatment with ST1968 (30 mg/kg). Tumours were processed at various times after drug treatment as described in Section 2. Western blot was performed as described in Section 2. β-Tubulin is shown as control of the loading.

damage response was detected, with evidence of p53 induction and phosphorylation, phosphorylation of histone H2AX and RPA-2 at 4 h after treatment. As observed in *in vitro* treated A2780 cells, early manifestations of apopotic response

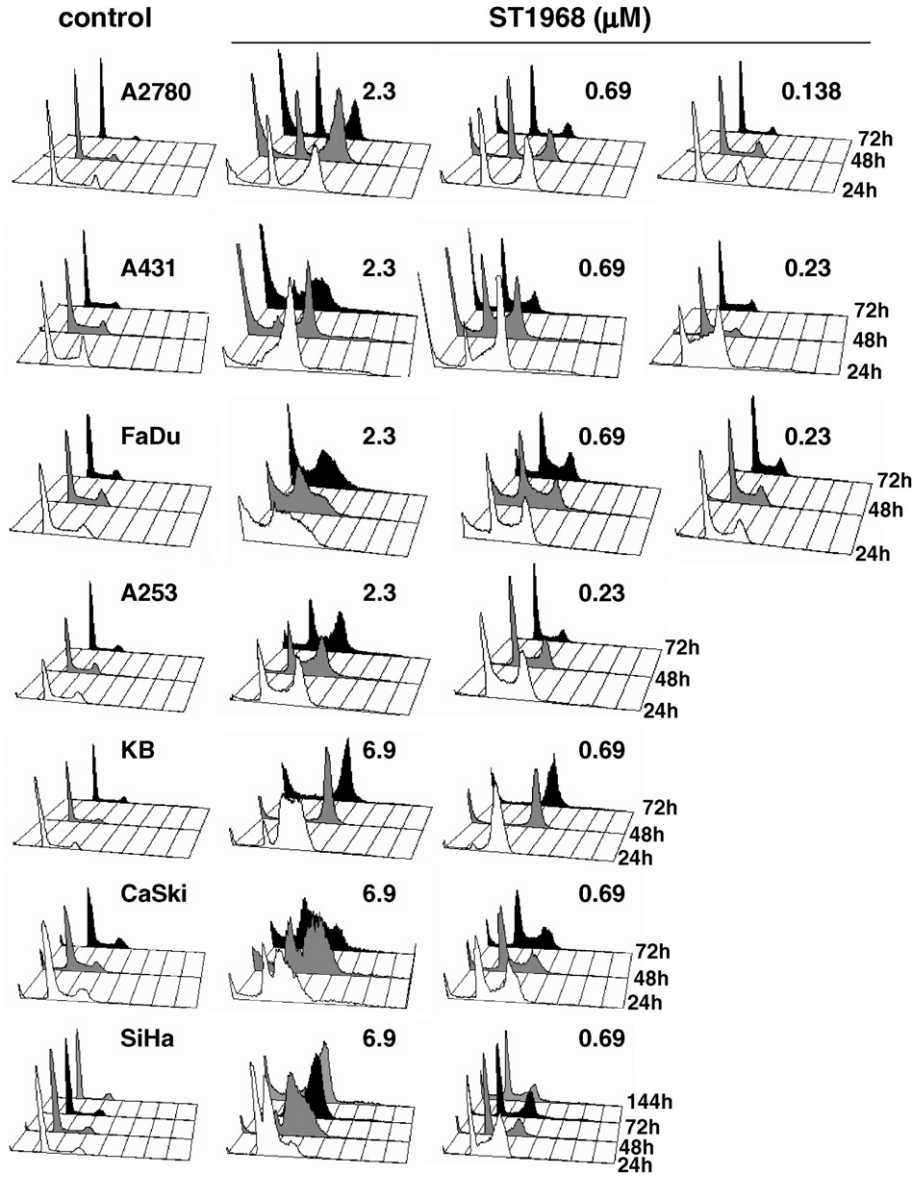

Fig. 3 – Cell cycle analysis of ST1968-treated cells at various times after drug treatment. Cells were treated for 1 h with equitoxic (IC_{80} and IC_{50}) or equimolar concentration and FACS analysis was performed on PI-stained cells.

were detected by caspase activation. In contrast, in KB tumour only a marginal induction of p53 was found at 48 h associated with phosphorylation of RPA-2 and H2AX. No caspase activation was evidenced up to 48 h in KB tumour. This pattern of biochemical effects was consistent with a delayed response to drug treatment (Table 1). Although no change of expression of HIF-1α could be detected in both cell lines (not shown), an early expression of BNIP3, a protein known to be under the control of HIF-1α and involved in a necrosis-like cell death of hypoxic tumours[11], was evidenced only in KB tumours. No manifestation of DNA damage response was detected in the resistant tumour DU145.

3.4. *Cellular pharmacokinetics and in vivo distribution*

In an attempt to investigate if the different cellular response to drug treatment was related to cellular pharmacokinetic behaviour, the cellular accumulation of ST1968 after 2 h exposure and drug retention following 5 h or 24 h-incubation of cells in a drug-free medium were studied in all tested cell lines (Fig. 4A). Following exposure to 1 μM, drug uptake reached a plateau in the interval between 1 and 2 h. All tested SCC and the ovarian carcinoma A2780 cells exhibited a substantial higher intracellular drug accumulation than the resistant DU145 prostate carcinoma cells. Drug retention at 5 or 24 h was measurable in all these cells but not in DU145 cells. SiHa and A253 cells exhibited a lower intracellular drug accumulation and retention than other SCC cell systems.

A favourable accumulation of the drug by the responsive tumours (A2780 and KB) was also observed *in vivo* (Fig. 4B). Indeed these tumours exhibited an appreciably higher concentration of the drug than DU145 tumour 30 min following i.v. treatment. DU145 tumour exhibited a marked reduction of drug content at 24–48 h following treatment. In contrast, substantial drug

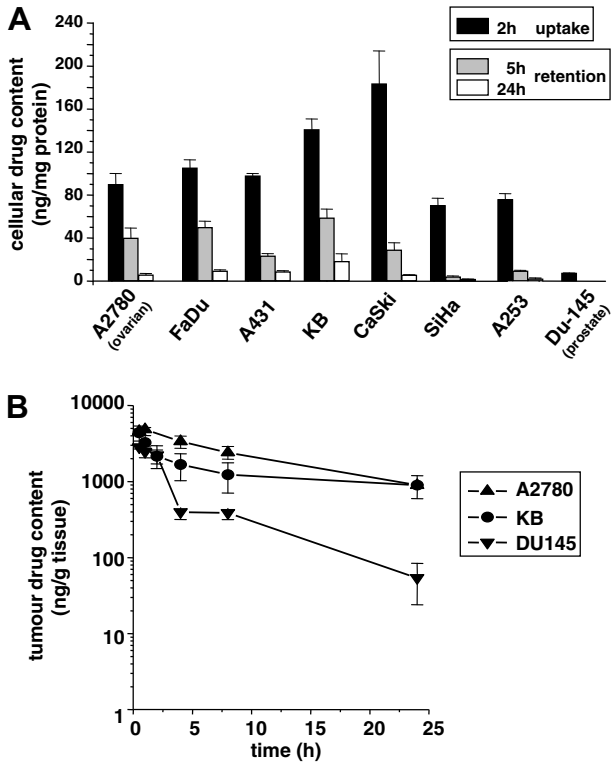

Fig. 4 – (A) Cellular pharmacokinetics of ST1968. Drug uptake was determined after 2 h-exposure to 1 μM. Drug retention was measured 5 or 24 h after drug removal and incubation in drug-free medium. (B) *In vivo* tumour distribution of ST1968. Tumours were established in CD1 nude mice as described for the antitumour activity studies. When tumours reached a size of ~400 mm^3, animals (3 mice/group) were treated with ST1968 at the dose of 30 mg/kg and sacrificed at different times (0.5–1–2–4–8–24–48 h) after i.v. injection.

retention was found in the two responsive tumours (A2780 and KB). Indeed at these times the drug content in A2780 and KB tumours was at least 10 times higher than in DU145 (around 1000 versus 60 ng/g tissue).

4. Discussion

Our recent efforts to identify novel camptothecin analogues with favourable pharmacological profile have focused on the influence of hydrophilic substituents at the 7-position. We have found that, amongst the derivatives of the 7-oxyiminomethyl series, the presence of a free amino group in the side chain confers a remarkable ability to stabilise the cleavable complex.[5] This finding is consistent with the stabilization of the cleavable complex observed with other CPT analogues containing hydrophilic groups at the 7-position.[12] The topoisomerase I–DNA complex stability is recognised to play a critical role in drug activity.[13]

Selected hydrophilic compounds, including ST1968, were chosen for *in vivo* evaluation of antitumour activity in a large panel of human tumour models ([5] and manuscript in preparation). The results presented in this study indicate that ST1968 was extremely effective against squamous cell carcinoma xenografts, because i.v. treatment achieved a high rate of complete responses and tumour cures in most tested models. With one exception (FaDu tumour), ST1968 was substantially more effective than irinotecan at least in terms of cure rate. The limited efficacy of irinotecan against squamous cell carcinoma xenografts is consistent with the marginal activity observed in clinical treatment of tumours with this histotype.[14,15]

The results of our study support that a critical determinant of the exquisite sensitivity of SCC models to ST1968 is a favourable cellular pharmacokinetics characterised by high level of intracellular accumulation and by a substantial drug retention, as compared to a model, prostate carcinoma tumour DU145, which exhibited a low responsiveness. This behaviour is expected to ensure prolonged drug effects and enhanced efficacy. Indeed, this interpretation is consistent with a persistent DNA damage response detected in SCC cells.

The hypersensitivity to camptothecin treatment of A2780, FaDu and A431 tumours likely reflected multiple factors, including the susceptibility to fast apoptosis, because an early caspase activation was detected in treated cells. The susceptibility to CPT-induced apoptosis was clearly independent of the p53 functional status, because the tested SCC tumours are characterised by inactivation or other defects of p53 function.

In *in vitro* analysis, we have found a reduced sensitivity to apoptosis of cells derived from other tumours (e.g. KB, CaSki and SiHa) as indicated by slow and late onset of apoptosis. However, under the same treatment conditions these tumour cells exhibited multiple manifestations of delayed cell death, including the appearance of cell senescence, mainly in SiHa cells. The response of KB tumour, which retained an *in vivo* responsiveness comparable to that of FaDu and A431 tumours at the MTD, was characterised by the activation of BNIP3, a protein up-regulated in hypoxic perinecrotic regions of tumours[16] and involved in cell death by apoptosis caspase independent, necrosis or autophagy.[17] In contrast, a low apoptotic level, associated with marginal manifestations of DNA damage response (γ-H2AX) and no evidence of senescence, was observed in the A253 tumour, which exhibited a less impressive response as compared to other tumour models of our panel.

Thus, our study on SCC tumour models has identified two distinct modalities of cell/tumour response to campthothecin-induced DNA damage. Like the response of A2780, in FaDu and A431 cells, the early apoptotic response reflected a fast-acting DNA damage response and probably resulted in a fast tumour regression. In contrast, the modality of tumour response in KB, Caski and SiHa models appeared to be a more complex phenomenon involving not only a late onset of apoptosis, but also senescence. Cellular senescence could be regarded as a stress-induced response, likely as the consequence of a persistent cell-cycle arrest.[10] Indeed, these cell lines exhibited a persistent G2 arrest. All these models express low levels of wild-type p53 protein, presumably as a result of its rapid degradation by the human papillomavirus 16 or 18 E6 proteins present in these cells. A tentative explanation of the peculiar response of the cells, which activate the senescence programme, is that the low expression of p53 may have a protective rather than pro-apoptotic function. The p53/p21 axis has been reported to be critical for drug-inducible senescence.[10] This interpretation is also consistent with a lack

of senescence in the p53 null A253 cells. The persistence of DNA damage response, which may reflect stability of the cleavable complex or slow drug release by tumour cells, may favour activation of senescence as a stress-inducible program in the absence of a fast apoptotic response or defects in the pro-apoptotic signalling cascades. Indeed, a peculiar feature of the SCC cells, which activate cellular senescence in response to CPT treatment, is the slow release of the drug observed in both *in vitro* cultured cells and *in vivo* growing tumours.

In conclusion, the novel camptothecin analogue ST1968 exhibited an outstanding efficacy, superior to that of irinotecan, against human tumour xenografts characterised by the squamous cell carcinoma histotype. Although activation of different cell death processes likely contribute to the responsiveness of SCC, a favourable cellular/tumour pharmacokinetics appeared to be a critical determinant of antitumour efficacy of ST1968. These preclinical results provide a solid rationale for clinical development of the novel agent. Relevant to this point is the clinical evidence of efficacy of camptothecins in the clinical therapy of cervical carcinoma.[18] The clinical efficacy of camptothecins in SCC of other origin remains unknown. Our observations provide a rational basis for evaluation of ST1968 in tumours with the SCC histotype, in particular head and neck carcinoma. The radiosensitising effect of CPT further supports the interest of ST1968 in combined chemoradiation, which has become a standard approach for unresectable head and neck tumours.[19]

Conflict of interest statement

Claudio Pisano, Loredana Vesci, Rosanna Fodera, Federica Bucci, Concetta Aulicino, Sergio Penco, Paolo Carminati are a body of researchers employed in Sigma-tau, which is the proprietary Company of ST1968.

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