

James L. Flowers · Robert M. Hoffman
Timothy A. Driscoll · Monroe E. Wall
Mansukh C. Wani · Govindarajan Manikumar
Henry S. Friedman · Mark Dewhirst
O. Michael Colvin · David J. Adams

The activity of camptothecin analogues is enhanced in histocultures of human tumors and human tumor xenografts by modulation of extracellular pH

Received: 16 December 2002 / Accepted: 25 March 2003 / Published online: 3 June 2003
© Springer-Verlag 2003

Abstract Background: Most solid human tumors exist in an acidic microenvironment, due in part to inefficient vasculature and a higher intrinsic rate of glycolysis. This leads to a tumor-selective pH gradient, which can be exploited therapeutically with antitumor agents such as the camptothecins (CPTs). Previous work in this laboratory has shown that camptothecin activity is enhanced 40- to 60-fold in monolayer cell culture by reducing the extracellular pH to 6.8. Three-dimensional histoculture has been shown to be a technique that allows human tumor tissue to grow in an in vivo-like way with maintenance of tissue histology and function and drug sensitivity for long periods of time. **Purpose:** In the current study, we utilized these features of histoculture to study new analogues of camptothecin that have superior pharmacological properties. **Methods:** We evaluated six CPT analogues in histocultures of human brain, neuroblastoma, breast, colon, and prostate tumors. Fragments were exposed to 10,11-methylenedioxy-CPT (MDC), 7-chloromethyl-MDC, SN-38, topotecan (TPT), 9-amino-CPT, 10-amino-CPT, paclitaxel, 5-fluorouracil, 4-hydroperoxycyclophosphamide and doxorubicin, and antitumor activity was assessed. For in vivo tumor outgrowth studies, fragments were treated in parallel, implanted into

nude mice, and monitored for development of tumors. **Results:** Against 15 of 16 tumor xenografts and all primary tumor samples tested, all compounds were cytotoxic at pH 7.4 (IC_{50} range 13–921 μM). MDC, SN-38, TPT, and 9-amino-CPT achieved an average 5-fold increase in activity (range 3–14) at pH 6.8, while 7-chloromethyl-MDC was enhanced 8-fold (range 6–14). The most potentiated analogue was 10-amino-CPT at 27-fold (range 17–49). In contrast, the other agents were active against one or more tumor types but were not enhanced by acidic pH. Importantly, the toxicity of MDC in histoculture of D54 glioma xenografts strongly correlated with the outgrowth of treated fragments subsequently implanted in vivo. **Conclusion:** Evaluation of anticancer drug activity in native-state histoculture supports the concept that pH modulation may be an important approach to improve the selectivity and antitumor effectiveness of camptothecin-based chemotherapy.

Keywords Camptothecin · Histoculture · pH modulation · In vitro drug screening

J. L. Flowers · T. A. Driscoll · H. S. Friedman
M. Dewhirst · O. M. Colvin · D. J. Adams (✉)
Duke University Medical Center, Durham,
NC 27710, USA
E-mail: adams041@mc.duke.edu
Tel.: +1-919-6844383
Fax: +1-919-6683925

R. M. Hoffman
AntiCancer, Inc., San Diego, CA 92111, USA

M. E. Wall · M. C. Wani · G. Manikumar
Research Triangle Institute, Research Triangle Park,
NC 27709, USA

J. L. Flowers
Department of Medicine, Duke University Medical Center,
DUMC-3843, Durham, NC 27710, USA

Introduction

Camptothecins (CPTs) are potent antitumor agents and irinotecan (CPT-11) and topotecan (TPT), two water-soluble analogues of CPT, have shown significant activity and utility in the clinic in colon, ovarian and small-cell lung carcinomas [30, 42]. CPTs act by inhibiting topoisomerase I, a nuclear enzyme involved in DNA replication and transcription [26, 27]. Inhibition of this enzyme results in an accumulation of DNA strand breaks and ultimately, cell death [28, 37]. Recently, it has been demonstrated in our laboratory that reducing the extracellular pH in vitro enhances activity of the CPTs up to 65-fold (Adams et al., submitted for publication; [1]). This approach was designed to mimic the tumor microenvironment

in situ, where inefficient vasculature combined with a higher intrinsic rate of glycolysis results in a buildup of waste products, such as lactate, and reduction of the extracellular pH.

Due to the unique chemistry of the CPT molecule, this physiology results in environmental conditions that may provide tumor site-specific enhancement of CPT activity. CPT has a labile E-ring lactone that opens to the inactive hydroxy acid at physiological pH (Fig. 1). In principle, this reaction is reversible at acidic pH. Hence, the tumor-specific pH gradient [10, 38, 48] can potentially enhance the proportion of the active lactone at the tumor site. In addition, since CPT is a weak acid, this tumor-specific pH gradient may promote intracellular concentration of the CPTs [20]. While the acidic extracellular environment of the tumor will help stabilize the E-ring lactone, the pharmacodynamics in whole blood is more complex since the CPT carboxylate avidly binds to human serum albumin and shifts the equilibrium towards the inactive form [3, 5]. At the same time, the highly lipophilic CPT molecule also partitions into red blood cell membranes and serum lipids, which protects the lactone [4, 33]. Thus, multiple pharmacodynamic equilibria regulate CPT action at the tumor site.

A critical parameter in determining drug sensitivity is the state in which the cells are maintained. It is well established that cells cultured in a two-dimensional monolayer have a very different chemosensitivity profile from those that are maintained in the native state [49, 51]. The cell-to-cell and cell-to-stroma contacts of intact, three-dimensional tumor architecture are maintained in histoculture and are known to have an impact on gene expression [16, 23, 47, 52] as well as cellular metabolism and replication [2, 12, 25, 49, 50]. In addition, diffusion of the antitumor agents, as well as nutrients and oxygen, will be limited in cells located in the interior of the fragment, and this setting closely mimics tumor biology in vivo. Thus, a more realistic profile of in vivo chemosensitivity can be obtained through three-dimensional histoculture.

To rule out artifacts of monolayer cell culture as being responsible for the potentiation of activity previously reported, we evaluated CPT analogue antitumor activity as a function of extracellular pH in three-dimensional histoculture of several tumor types. We found that the rank order potency and pH modulation of the CPT analogues assessed in three-dimensional histoculture correlated with that observed in monolayer assays. Furthermore, we demonstrated that the chemosensitivity of D54 glioma fragments to 10,11-methylenedioxy-CPT (MDC) in histoculture was highly

correlated with the subsequent ability of the tumor fragment to grow when reimplanted into a mouse.

Materials and methods

Chemicals, reagents and antitumor agents

Six camptothecin analogues were used in this study. MDC, 7-chloromethyl-MDC, 9-amino-CPT, 10-amino-CPT and 7-ethyl-10-hydroxy-CPT (SN-38) were synthesized according to established methods [32, 45, 53, 55]. TPT was obtained from Smith Kline Beecham (Philadelphia, Pa.). Paclitaxel (TXL; Bristol Meyers Squibb, Princeton, N.J.), 5-fluorouracil (5-FU; Pharmacia, Kalamazoo, Mich.), and doxorubicin (DOX; Pharmacia & Upjohn, Kalamazoo, Mich.) were purchased as clinically formulated agents. 4-Hydroperoxycyclophosphamide (4-HC) was a generous gift from Dr. Susan Ludeman of the Duke University Medical Center. The CellTiter 96 Aqueous metabolic assay (MTS) is a tetrazolium-based metabolic indicator, purchased as a kit from Promega (Madison, Wis.). Sodium chloride, sodium bicarbonate and glucose were purchased from Sigma.

Human tumor xenograft lines

Female BALB/c nu/nu mice were obtained from a Duke University in-house colony. Human tumor xenografts were transplanted into animals that were 4 to 6 weeks old and weighed 20 to 25 g. We evaluated xenografts of hormone-sensitive, hormone-insensitive and refractory human breast and prostate carcinomas (MDA-MB-231, MCF-7 and MCF-7tamR [8] and PC-3, CWR22 and CWR22R, an androgen-resistant variant, respectively). Other tumor types evaluated were colon cancer (AdCo), a low-passage colon carcinoma xenograft line established in this laboratory from patient tissue, neuroblastoma (IMR-32, n-MYC amplified, and SK-N-SH, n-MYC unamplified), adult high-grade glioma (D54, D245, and D245PR, a procarbazine-resistant variant), pediatric high-grade glioma (D212), medulloblastoma (D341 and D487), and ependymoma (D612 and D528). All tumor lines were maintained through serial passage into nude mice.

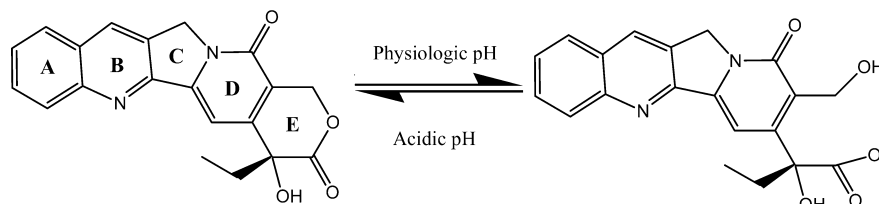
Primary human tumors

Two neuroblastoma (NB1 and NB2), one ovarian (Ov1) and one colon carcinoma (used to generate AdCo) patient tumor samples were acquired postoperatively, while one colon carcinoma (from liver metastasis) was acquired immediately post-mortem (Co1). Specimens were placed in sterile containers and submitted to the surgical pathology department at Duke University Medical Center. Excess tissue was supplied to the laboratory for analysis after being stripped of all patient identifiers. This work was exempt from IRB review according to 45CFR46.102(f).

Processing of tumor samples

The primary human tumor tissue was placed in ice-cold transport medium comprising Hank's balanced salt solution (Hank's BSS,

Fig. 1 The camptothecin molecule contains a labile E-ring lactone that hydrolyzes to an inactive hydroxy acid at physiological pH. The reaction is reversible under acidic conditions, regenerating the active compound



GIBCO, Gaithersburg, MD), 10% fetal bovine serum (FBS; Hyclone, Logan, Utah, or Sigma, St. Louis, Mo.), nonessential amino acids (NEAA, 1% v/v of 100× stock; GIBCO) and 0.2 mg/ml gentamicin sulfate. Xenograft tumors were removed from nude mice and placed in ice-cold transport medium. In either case, the tissue was immediately transported on ice to the laboratory for processing. The tumors were placed in Petri dishes where connective tissue, fat, and necrotic sections were removed. The remaining tumor was minced into approximately 1-mm³ fragments that were washed three to five times in growth medium comprising low-glucose DMEM (Sigma) supplemented with 1 g/l glucose, 10% FBS, 1% NEAA and 0.2 mg/ml gentamicin sulfate. For colon carcinoma samples, the medium also contained 240,000 U penicillin G, 250 mg streptomycin, 400 mg amphotericin B, 10 mg tetracycline, 100 mg amikacin, and 150 mg chloramphenicol (Sigma) per liter. After the antibiotic wash, the fragments were placed in 24-well plates containing 2 ml growth medium (as above, except that the medium for colon samples was supplemented with 50,000 U penicillin G and 1.26 mg amphotericin B) to which was added 200 μ l MTS reagents for 1 h. Since viable cells, but not connective tissue or fat, metabolize MTS to a purple formazan product, viable tumor fragments were stained purple. Fragments stained less than half purple by visual examination were excluded. Assessment of viability and composition before inclusion into the assay was necessary to reduce interassay variation. The selected fragments were placed in a humidified incubator overnight at 37°C in an atmosphere containing 5% CO₂ for acclimation to culture conditions and to allow diffusion of the formazan product out of the fragments. Reducing the concentration of sodium bicarbonate to 205 mg/l, and osmotically balancing the medium with 2.4 g/l sodium chloride, adjusted the pH of the histoculture medium from 7.4 to 6.8. The pH of the medium was measured inside the incubator after 3 h equilibration time with a Beckman Φ 10 portable pH meter. Due to natural variation in bicarbonate concentration in FBS, the sodium bicarbonate and sodium chloride concentrations were re-established with each new lot of FBS to ensure proper pH. Aseptic conditions were maintained at all times during these procedures.

Histoculture assay

Two or three fragments of tissue (4–8 mg) were placed into each well of a 24-well plate containing 2 ml fresh medium at pH 7.4 or 6.8 with various concentrations of chemotherapeutic agents and incubated for 72 h. The medium was changed and the fragments were replaced in the incubator for an additional 24 h to allow washout of the drug. Fragment metabolic viability was then assessed by MTS assay. Briefly, 5% v/v MTS reagent was added to each of the wells containing fragments or medium blanks. The plates were incubated for 3 h at 37°C, shaken and aliquots of the medium measured for absorbance at 490 nm using an EL-340 microplate reader (Bio-Tek, Winooski, Vt.). After subtraction of the appropriate blank, the results were reported as percent of untreated control absorbance. Where possible, the IC₅₀, which is defined as the concentration of agent which reduces metabolic viability by 50% as compared to control, was calculated using TableCurve 2D (SPSS, Chicago, Ill.). The potentiation of antitumor activity by pH modulation is defined as (IC₅₀ at pH 7.4)/IC₅₀ at pH 6.8).

In vivo tumor outgrowth assay

Tumor fragments from a parallel histoculture assay were washed and incubated in drug-free medium for 24 h. The fragments were then implanted subcutaneously into the flank of female BALB/c nu/nu mice. The mice and tumors were monitored for development of tumors and measurements were taken three or four times per week with vernier calipers until the tumor had expanded to a volume five times the initial volume. Tumor volume was calculated using the formula (width)² × (length)/2. All animal work was performed under an approved IACUC protocol.

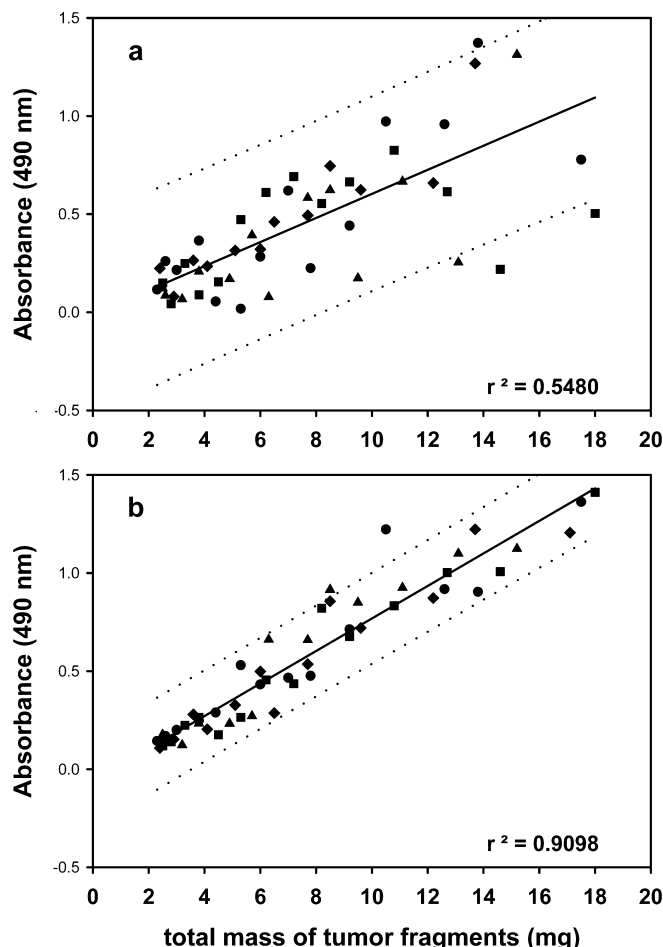


Fig. 2a, b Selection of tumor fragments with an MTS prescreen results in more uniform production of formazan per fragment. Between one and eight fragments of MCF-7 (■), D54 (●), AdCo (▲), or IMR-32 (◆) xenografts were weighed and assayed for metabolism of MTS substrate. The tested fragments were either randomly selected (**a**) or chosen by MTS prescreen (**b**). After incubation for 1 h in histoculture medium with 5% v/v MTS substrate, aliquots of the medium were taken and analyzed for absorbance at 490 nm. Each point represents a single experiment. The dashed lines are the 95% prediction intervals for the data

Results

Prescreening tumor fragments reduces experimental variation

Prescreening the tumor fragments with MTS to select only > 50% viable samples reduced the interexperimental variation intrinsic to the histoculture assay as originally developed. As seen in Fig. 2a, the variation in MTS metabolism of randomly selected fragments, measured as absorbance at 490 nm, was high, due to the heterogeneous nature of the tumor. This variation occurred irrespective of the tumor type or number of fragments analyzed. The absorbance per milligram of tumor became more uniform and predictable with fragment preselection (Fig. 2b), allowing an assay of two or three fragments per well.

While the metabolism of MTS was minimally reduced at pH 6.8, the linearity of the response was conserved under the acidic conditions (data not shown).

CPT analogues have antitumor activity in histoculture at physiological pH

All of the CPT analogues evaluated in this study were cytotoxic to the tumor fragments in histoculture (Table 1). For purposes of this study, an exposure to a CPT analogue that resulted in greater than 25% reduction in viability of tumor fragments, based on the MTS endpoint, constituted cytotoxicity. The IC_{50} values generated in histoculture were generally in the micromolar range, whereas in monolayer cell culture assays, the same compounds are active at nanomolar levels [1].

CPT activity is potentiated by acidic pH in histoculture

Each of the analogues exhibited increased cytotoxicity at acidic pH against all but one of the tumors tested. As shown in Table 1, the degree of enhancement by acidic pH was less dependent on tumor type than on the specific analogue. On average, MDC, SN-38, TPT and 9-amino-CPT were potentiated 4.8-fold (range 3–14). 7-Chloromethyl-MDC was enhanced 8.3-fold (range 6–15) and 10-amino-CPT was the most potentiated analogue at 26-fold (range 17–49). At both physiological and acidic pH, the primary tumor fragments and the

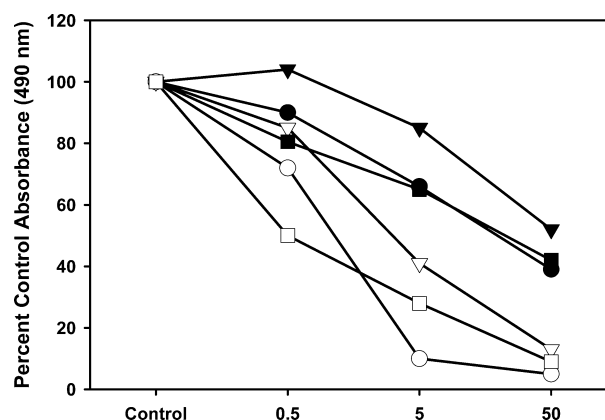


Fig. 3 Primary patient tumors are sensitive in histoculture to CPTs and exhibit modulation of CPT activity by reduction of culture pH. An abdominal adrenal primary neuroblastoma (NB1; squares), along with IMR-32 (triangles) and SK-N-SH (circles) xenografts, were exposed to 10-amino-CPT for 72 h at physiological (closed symbols) and acidic (open symbols) extracellular pH. The primary tumor and the xenografts were similarly sensitive to 10-amino-CPT and the magnitude of potentiation at pH 6.8 was also comparable. Data points represent the average of two experiments, each measuring the collective conversion of MTS substrate to formazan by two to four tumor fragments

corresponding xenografts exhibited similar sensitivity profiles to the CPT analogues tested (Fig. 3; Table 1). However, of the tumor-types tested, the metastasis-derived prostate line, PC-3, was the only tumor, xenograft or primary, which did not exhibit increased sensitivity in histoculture to CPT analogues at pH 6.8 (Table 1) nor

Table 1 The activity of camptothecin analogues in histoculture is enhanced at acidic extracellular pH. The activity of the agents, expressed as IC_{50} (μM), was measured following a 48-h exposure to agents and 24 h in drug-free medium, and was determined by viability assay as described in Methods. The ratio was determined

by the formula, IC_{50} at pH 7.4/ IC_{50} at pH 6.8. Unless otherwise indicated, IC_{50} values are the average of three to five experiments, each measuring the collective conversion of MTS substrate to formazan by two to four tumor fragments (ND not done)

Tumor line	CMMDC			MDC			SN38			10-Amino-CPT			TPT			9-Amino-CPT		
	7.4	6.8	Ratio	7.4	6.8	Ratio	7.4	6.8	Ratio	7.4	6.8	Ratio	7.4	6.8	Ratio	7.4	6.8	Ratio
MDA-MB-231	85	6	14	35	5	7	332	68	5	77	3	24	ND	ND	ND	ND	ND	ND
MCF-7/tamR	56 ^a	4 ^a	13	30 ^a	9 ^a	3	103 ^a	24 ^a	4	ND	ND	ND	ND	ND	ND	ND	ND	ND
MCF-7	103	9	11	59	15	4	224	47	5	41	2	17	ND	ND	ND	ND	ND	ND
D54	36	5	7	ND	ND	ND	201	39	5	65	2	27	797	194	4	ND	ND	ND
D245	48	6	8	ND	ND	ND	312	104	3	85	3	28	623	201	3	ND	ND	ND
D245PR	61	7	9	ND	ND	ND	133	22	6	ND	ND	ND	921	242	4	ND	ND	ND
D341	59	10	6	ND	ND	ND	253	39	7	ND	ND	ND	349	87	4	ND	ND	ND
D487	24	3	8	ND	ND	ND	523	38	14	ND	ND	ND	552	162	3	ND	ND	ND
D528	78	11	7	ND	ND	ND	160	42	4	ND	ND	ND	613	136	5	ND	ND	ND
D212	45	5	9	ND	ND	ND	136	26	5	ND	ND	ND	817	157	5	ND	ND	ND
D612	15	2	6	ND	ND	ND	254	40	6	ND	ND	ND	756	210	4	ND	ND	ND
AdCo	163	22	7	13	3	5	439	68	7	ND	ND	ND	ND	ND	ND	375	70	5
Co1	203	33	6	21	7	3	222	58	4	ND	ND	ND	ND	ND	ND	288	42	7
CWR22	97	12	8	40	12	3	269	46	6	ND	ND	ND	ND	ND	ND	ND	ND	ND
PC-3	201	251	1	85	77	1	334	334	1	ND	ND	ND	ND	ND	ND	413	376	1
CWR22R	123	19	7	71	17	4	201	47	4	ND	ND	ND	ND	ND	ND	301	70	4
IMR32	143	18	8	57	15	4	217	45	5	66 ^a	4 ^a	17	573	185	3	ND	ND	ND
SK-N-SH	89	11	8	31	7	5	189	36	5	71 ^a	3 ^a	26	434	124	4	ND	ND	ND
NB1	201 ^a	35 ^a	6	29 ^a	5 ^a	6	332 ^a	60 ^a	6	70 ^a	1 ^a	49	678 ^a	226 ^a	3	ND	ND	ND
NB2	59 ^a	6 ^a	10	41 ^a	7 ^a	6	190 ^a	31 ^a	6	95 ^a	3 ^a	30	409 ^a	100 ^a	4	ND	ND	ND

^aAverage of two experiments

was activity enhanced at acidic pH against the parental cell line in monolayer cell culture assays [11].

Although the individual tumor groups responded with varying degrees of sensitivity to the four clinically established agents, 4-HC (the preactivated form of cyclophosphamide), DOX, 5-FU and TXL, none of these agents was significantly potentiated at pH 6.8 (Table 2). In fact, sensitivity to DOX, which is a weak base and preferentially accumulates in the extracellular fluid [21, 39], was diminished in the breast and neuroblastoma tumors (Table 2). These results are consistent with the patterns of activity previously seen in monolayer cell culture [1, 39].

In vitro metabolic endpoint correlates with in vivo growth delay

To validate the relevance of the MTS endpoint used to measure the activity of the CPTs in the histoculture assay, we performed parallel experiments in vivo. After treatment with varying concentrations of MDC, one set of D54 glioma fragments were reimplanted into nude mice. These fragments re-established tumors that grew to, and beyond, five times the initial tumor volume (Fig. 4a). The remaining fragments were assessed by MTS assay as described above. The calculated time for the tumor to reach five times the initial volume was compared to the absorbance value generated by the corresponding concentration of MDC in the histoculture assay (Fig. 4b), and a significant linear correlation (Pearson $r^2=0.941$) was found between the in vivo regrowth endpoint and the in vitro metabolic assay.

Discussion

Previous work in this laboratory has demonstrated that the antitumor activity of CPTs in monolayer cell culture

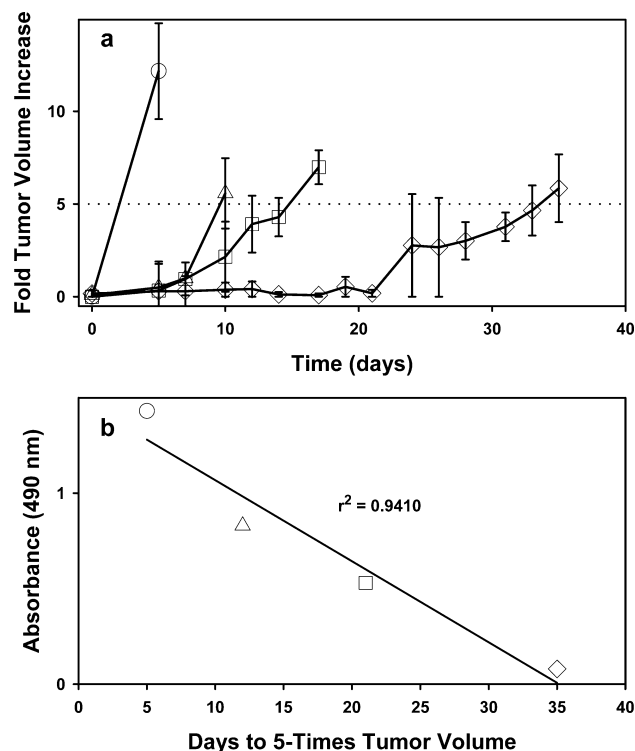


Fig. 4 **a** Treatment of D54 glioma fragments with MDC in histoculture delays regrowth in nude mice. Tumor fragments exposed to various concentrations of MDC (○ untreated control, △ 1 μ M, □ 10 μ M, ◇ 100 μ M) were reimplanted into nude mice and monitored for tumor development. Increasing concentrations of the compound resulted in proportionately greater delay in tumor development. **b** The metabolic endpoint used in the histoculture assay directly correlates with tumor growth delay in vivo. The in vitro endpoint used in the histoculture assay was validated by comparison to tumor development in living mice, confirming that the results of the assay have physiological relevance

assays is enhanced by a reduction in extracellular pH [1]. This was shown to be due, in part, to increased stability of the lactone form of the molecule. In addition,

Table 2 The activity of doxorubicin (DOX), 4-hydroperoxycyclophosphamide (4HC, the preactivated form of cyclophosphamide), 5-fluorouracil (5-FU) and paclitaxel (TXL) as measured by IC_{50} (μ M) in histoculture was not enhanced by acidic extracellular pH. The activity of these clinically established agents, expressed as IC_{50} (μ M), was measured following a 48-h exposure to drugs and 24 h in

drug-free medium, and was determined by viability assay as described in Methods. The ratio was determined by the formula IC_{50} at pH 7.4/ IC_{50} at pH 6.8. Unless otherwise indicated, IC_{50} values are the average of three to five experiments, each measuring the collective conversion of MTS substrate to formazan by two to four tumor fragments (ND not done)

Tumor line	DOX			4HC			5-FU			TXL		
	7.4	6.8	Ratio	7.4	6.8	Ratio	7.4	6.8	Ratio	7.4	6.8	Ratio
MDA-MB-231	25	36	1	214	243	1	ND	ND	ND	53	55	1
MCF-7tamR	16 ^a	20 ^a	1	312 ^a	289 ^a	1	ND	ND	ND	42 ^a	40 ^a	1
MCF-7	23	19	1	275	300	1	ND	ND	ND	59	61	1
AdCo	ND	ND	ND	196	214	1	102	99	1	ND	ND	ND
Co1	ND	ND	ND	173	167	1	96	94	1	ND	ND	ND
IMR32	51	47	1	342	302	1	ND	ND	ND	ND	ND	ND
SK-N-SH	18	21	1	276	216	1	ND	ND	ND	ND	ND	ND
NB1	22 ^a	27 ^a	1	201 ^a	246 ^a	1	ND	ND	ND	ND	ND	ND
NB2	41 ^a	46 ^a	1	255 ^a	288 ^a	1	ND	ND	ND	ND	ND	ND

^aAverage of two experiments

reduction in extracellular pH also results in decreased intracellular glutathione, which appears to confer additional sensitivity to the CPTs [1]. Neither of these factors, even when taken together, could account for the remarkable potentiation seen with 7-chloromethyl-MDC and 10-amino-CPT in monolayer assays of human breast cancer cells (Adams et al., submitted for publication; [1]). In order to ensure that this phenomenon was not merely an artifact of monolayer cell culture, we utilized the histoculture assay to screen a panel of CPT analogues whose activities were enhanced to varying degrees in monolayer assays done at pH 6.8. We did this using both xenograft tumor lines and primary patient tumor samples.

In histoculture, the three-dimensional structure of a tumor is more conserved, thus gene expression, cellular metabolism and replication rates, as well as sensitivity to antitumor agents, more closely mimic the native state of the tumor [24]. These parameters are critical considering recent work that has shown that the gene expression profiles of breast and prostate cancer cell lines are vastly different from primary breast and prostate tumor samples [35, 43]. Furthermore, work in spheroids has demonstrated a mechanism of drug resistance present only in cells cultured in three-dimensions [22, 29]. This approach to evaluating antineoplastic agents may be more predictive of the clinical effect of the compounds.

Novel compounds, including CPTs, have previously been evaluated by histoculture in prostate, colon and gastric cancers and hormone sensitivity has been assessed in ovarian, endometrial and prostate carcinomas [6, 15, 17, 18, 19, 34, 36, 54]. Additionally, clinical trials have been done in head and neck, non-small-cell lung, gastric and advanced colorectal cancers in which the histoculture assay was highly predictive of clinical outcome, including survival. These studies involved samples from hundreds of patients and evaluated sensitivity and resistance to cisplatin, mitomycin C, DOX, etoposide, 5-FU and UFT, a 1:4 mixture of tegafur and uracil [13, 31, 40, 41, 46, 56]. The predictive accuracy of these studies ranged from 44.4% to more than 90%. Other characteristics of patient tumors can also be revealed using this approach. For instance, Yoshimasu et al. have demonstrated a high incidence of multidrug resistance among the cohort of patients enrolled in a non-small-cell lung cancer trial [56].

However, the histoculture assay is less practical than monolayer cell assays, particularly for high-throughput applications. The quantity of tumor available for evaluation is limiting and interexperimental variation necessitates multiple repeats to achieve a statistically significant result. For example, to overcome the biological heterogeneity of a tumor sample, histoculture experiments evaluating homocamptothecin required the use of ten randomly selected tumor pieces per compound, limiting the scope of the assay to one to three compounds on any one tumor sample [36]. We modified the assay to include a preselection step based on metabolic activity of the fragment, which significantly

decreased variation in the assay and allowed screening of compounds using only two or three fragments, thus increasing the number of compounds that can be screened against a single, unique tumor sample.

We evaluated MDC, 7-chloromethyl-MDC, SN-38, TPT, 9-amino-CPT and 10-amino-CPT, along with 4-HC, TXL, DOX and 5-FU, using the histoculture assay and determined that all of the compounds possessed at least minimal antitumor activity. Under physiological conditions, the agents were cytotoxic, based on our operational definition, in the range of 10^{-4} to 10^{-9} M. For the CPT analogues, the rank order of potency was similar to that seen in monolayer cell culture at the same pH [1], but the relative potency in histoculture was as much as three orders of magnitude lower than in monolayer culture. This is not unexpected as the mechanism most commonly associated with CPT toxicity, namely topoisomerase I-mediated DNA replication, requires cells to be in S phase. Relatively few cells are actually undergoing replication in a three-dimensional, histotypic structure, which more accurately reflects the rate in vivo. Compared to the high fraction of dividing cells in monolayer cell culture, this can lead to the disparity in observed antitumor activity. In non-cycling cells, CPTs may cause cell death via inhibition of RNA synthesis through blockage of DNA transcription. For example, in B-CLL, a non-replicating cell type against which CPTs are cytotoxic, treatment with SN-38 resulted in inhibition of RNA synthesis and apoptosis. Similar to histoculture, this effect occurred at higher doses than that required for cycling leukemia cells [7].

Two other investigations have examined CPT activity in histoculture [36, 56] using tritium-labeled thymidine incorporation following 48 h of continuous exposure. CPT, TPT, and SN-38 were evaluated as reference agents for two novel homocamptothecins, a class of seven-membered E-ring CPT analogues. In these experiments, SN-38 and TPT were active at 10^{-7} to 10^{-9} M, much lower concentrations than we determined. This discrepancy is likely due to differences in the respective growth inhibition assays. For example, our assay utilized a metabolic endpoint and incorporated a 24-h recovery time in drug-free medium. This protocol permits full expression of apoptosis in sensitive cells, and allows insensitive cells time to repair any damage and resume metabolic activity. A thymidine incorporation endpoint does not take into account this potential of the cell to survive treatment and assumes that lack of tritium incorporation is equivalent to cell death. However, the endpoint we used to measure activity of the CPT analogues in vitro correlated well (Pearson $r^2=0.941$) with the ability of the treated fragments to grow in vivo when reimplanted into the flanks of nude mice (Fig. 4b).

In this study, we showed that the potentiation of CPT antitumor activity previously observed in monolayer cell culture at acidic extracellular pH is observed in histoculture of tissue fragments in vitro. Each of the analogues evaluated in this study was more potent when the assay was conducted at pH 6.8. The activities of MDC,

SN-38, 9-amino-CPT and TPT were enhanced by nearly 5-fold, while 7-chloromethyl-MDC and 10-amino-CPT were potentiated 8.3- and 26-fold, respectively. The relative increase in activity is similar to that seen in monolayer assays [1]. In general, a minimal enhancement is seen with all analogues, which is partially explained by pH-dependent lactone-carboxylate kinetics. However, the chloromethyl substitution at the seven-position results in more than twice the improvement expected from the threefold increase in active lactone species at pH 6.8 [1]. At pH 6.8, 10-amino-substituted CPTs also exhibited a greater degree of enhancement than expected. While the reason for this is unclear, the amino moiety may influence tissue distribution kinetics and cell permeability. This amino substitution is an electron-donating group and results in enhanced fluorescence of the conjugated ring system of CPT. It is conceivable that the electronic influence of this group also serves to stabilize the E-ring lactone. The amino substitution along with a 7-butyl moiety enhances lipophilicity 7-fold over 7-butyl alone (Wall, unpublished observations), potentially increasing the amount of drug partitioning into red blood cell membranes and serum lipids and thus more readily available for pharmacological activity. Indeed, peptide prodrug forms of SN-38 [9] and 7-butyl,10-amino-CPT (Driscoll and Flowers, unpublished observations) show antitumor activity in a highly aggressive neuroblastoma xenograft under conditions where CPT-11 is inactive.

Screening CPT analogues in this system, taking into account the physiological characteristics of the tumor microenvironment and the non-replicative state of most of the tumor, revealed a rank order of potency which differs from that seen at physiological pH. Thus, this approach may allow discovery of more selective agents that take advantage of the tumor-specific pH gradient. In this study, we also confirmed that the modulation of antitumor activity in histoculture at pH 6.8 was specific to the CPTs and was not shared by the other classes of compounds tested.

We demonstrated that pH modulation of CPT activity was not dependent on the developmental stage of the tumor. The xenograft models we evaluated included hormone-sensitive, hormone-insensitive and refractory tumors, tumors with and without amplification of oncogenes, and tumors which had developed drug resistance. All of these tumors, with the exception of PC-3, were more sensitive to CPT analogues when cultured under acidic conditions. In monolayer assays, PC-3 is the only cell line tested in this laboratory that does not exhibit increased sensitivity to the CPTs at pH 6.8 [11]. Previous work has shown that culture at acidic pH results in a decrease in intracellular glutathione and that treating with buthionine sulfoximine or ethacrynic acid at physiological pH confers some increased sensitivity to the CPTs [1, 14]. This correlation between cellular redox state and CPT sensitivity is a possible explanation for the lack of modulation of activity in PC-3. The reduction in intracellular glutathione observed in cells cultured at pH 6.8 is not observed

in PC-3 cells at that pH (Flowers, unpublished observation). This may be due in part to the normally acidic environment of the prostate. PC-3 has also been found to overexpress HIF-1 α , hypoxia-inducible factor, which plays an important role in pH regulation [44]. Since overexpression of this gene was exceedingly rare in 117 primary and metastatic human prostate tumors, PC-3 was characterized as not being truly representative of prostate tumors, and perhaps not an entirely appropriate model [44]. However, PC-3 cells may serve to help elucidate the true mechanism of pH modulation of CPT antitumor activity in two- and three-dimensional culture.

Finally, by reimplanting fragments treated with CPTs, we correlated the metabolic endpoint of the MTS assay with in vivo regrowth potential. This relationship demonstrates the importance of this approach to drug screening and could help minimize animal testing, which is time consuming, expensive and labor intensive. By screening with histoculture, only candidate compounds which have shown the potential to act under in vivo-like conditions would be subsequently tested in an animal system.

In summary, we demonstrated pH modulation of CPT analogue activity in cells cultured at pH 6.8 in a histoculture system, and have demonstrated that the mechanism of this enhancement is a complex physiological phenomenon based on analogue-specific and cell type-specific degrees of enhancement. By screening for compounds which take advantage of the pH gradient present in most tumors, potentially selective compounds may be more readily identified than when screened in traditional assays at physiological pH.

Acknowledgements This work is dedicated to the memory of Dr. Monroe E. Wall. The authors wish to acknowledge Alan Proia and Duke Surgical Pathology for invaluable assistance with procuring the primary human tissue samples and Stacey Snyder of Duke Radiation Oncology Department, Ray Liao and Dr. David Price of Duke Urology Department and Steve Keir of Duke Neurooncology Department for expert technical assistance and providing xenografts for histoculture experiments. This work was supported by NIH grant U01 CA68697-02.

References

1. Adams DJ, Dewhirst MW, Flowers JL, Gamcsik MP, Colvin OM, Manikumar G, Wani MC, Wall ME (2000) Camptothecin analogues with enhanced antitumor activity at acidic pH. *Cancer Chemother Pharmacol* 46:263
2. Baibakov B, Frank GA, Sergeeva N, Novikova I, Youngkin T, Connors KM, Hoffman RM, Margolis LB (1991) In vivo growth patterns of human lung tumors in three-dimensional histoculture. *In Vitro Cell Dev Biol* 27A:897
3. Burke TG, Mi Z (1993) Preferential binding of the carboxylate form of camptothecin by human serum albumin. *Anal Biochem* 212:285
4. Burke TG, Mishra AK, Wani MC, Wall ME (1993) Lipid bilayer partitioning and stability of camptothecin drugs. *Biochemistry* 32:5352
5. Burke TG, Munshi CB, Mi Z, Jiang Y (1995) The important role of albumin in determining the relative human blood stabilities of the camptothecin anticancer drugs. *J Pharm Sci* 84:518

6. Chang SG, Jung JC, Rho YS, Huh JS, Kim JI, Hoffman RM (1996) Efficacy of the platinum analog [Pt(cis-dach)(DPPE)-2NO₃] on histocultured human patient bladder tumors and cancer cell lines. *Anticancer Res* 16:3423
7. Cohen DP, Adams DJ, Flowers JL, Wall ME, Wani MC, Manikumar G, Colvin OM, Silber R (1999) Pre-clinical evaluation of SN-38 and novel camptothecin analogs against human chronic B-cell lymphocytic leukemia lymphocytes. *Leuk Res* 23:1061
8. Connor CE, Norris JD, Broadwater G, Willson TM, Gottardis MM, Dewhirst MW, McDonnell DP (2001) Circumventing tamoxifen resistance in breast cancers using antiestrogens that induce unique conformational changes in the estrogen receptor. *Cancer Res* 61:2917
9. Driscoll TA, Flowers JL, Colvin OM (2002) Potentiation of camptothecin-based anti-neuroblastoma activity by selective tumor acidification. In: *Proceedings of the 93rd Annual Meeting of the American Association of Cancer Research*, San Francisco
10. Engin K, Leeper DB, Cater JR, Thistlethwaite AJ, Tupchong L, McFarlane JD (1995) Extracellular pH distribution in human tumours. *Int J Hyperthermia* 11:211
11. Flowers JL, Hoffman RM, Manikumar G, Wani MC, Wall ME, Colvin OM, Adams DJ (2002) Activity of camptothecin analogues against human prostate cancer cells in monolayer and in 3-dimensional histoculture as a function of extracellular pH. In: *Proceedings of the 93rd Annual Meeting of the American Association of Cancer Research*, San Francisco
12. Freeman AE, Hoffman RM (1986) In vivo-like growth of human tumors in vitro. *Proc Natl Acad Sci U S A* 83:2694
13. Furukawa T, Kubota T, Hoffman RM (1995) Clinical applications of the histoculture drug response assay. *Clin Cancer Res* 1:305
14. Gamcsik MP, Kasibhatla MS, Adams DJ, Flowers JL, Colvin OM, Manikumar G, Wani MC, Wall ME, Kohlhagen G, Pommier Y (2001) Dual role of glutathione in modulating camptothecin activity: depletion potentiates activity, but conjugation enhances the stability of the topoisomerase I-DNA cleavage complex. *Mol Cancer Ther* 1:11
15. Geller J, Sionit LR, Connors K, Hoffman RM (1992) Measurement of androgen sensitivity in the human prostate in vitro three-dimensional histoculture. *Prostate* 21:269
16. Geller J, Sionit LR, Connors KM, Youngkin T, Hoffman RM (1993) Expression of prostate-specific antigen in human prostate specimens in in vitro three dimensional histoculture. In *Vitro Cell Dev Biol* 29A:523
17. Geller J, Sionit LR, Baird A, Kohls M, Connors KM, Hoffman RM (1994) In vivo and in vitro effects of androgen on fibroblast growth factor-2 concentrations in the human prostate. *Prostate* 25:206
18. Geller J, Partido C, Sionit L, Youngkin T, Nachtsheim D, Espanol M, Tan Y, Hoffman R (1997) Comparison of androgen-independent growth and androgen-dependent growth in BPH and cancer tissue from the same radical prostatectomies in sponge-gel matrix histoculture. *Prostate* 31:250
19. Geller J, Sionit L, Partido C, Li L, Tan X, Youngkin T, Nachtsheim D, Hoffman RM (1998) Genistein inhibits the growth of human-patient BPH and prostate cancer in histoculture. *Prostate* 34:75
20. Gerweck LE, Seetharaman K (1996) Cellular pH gradient in tumor versus normal tissue: potential exploitation for the treatment of cancer. *Cancer Res* 56:1194
21. Gerweck LE, Kozin SV, Stocks SJ (1999) The pH partition theory predicts the accumulation and toxicity of doxorubicin in normal and low-pH-adapted cells. *Br J Cancer* 79:838
22. Green SK, Frankel A, Kerbel RS (1999) Adhesion-dependent multicellular drug resistance. *Anticancer Drug Des* 14:153
23. Guadagni F, Roselli M, Hoffman RM (1991) Maintenance of expression of tumor antigens in three-dimensional in vitro human tumor gel-supported histoculture. *Anticancer Res* 11:543
24. Hoffman RM (1998) Three-dimensional sponge-gel matrix histoculture of human tumors: methods and applications, 1st edn. Academic Press, New York
25. Hoffman RM, Connors KM, Meerson-Monosov AZ, Herrera H, Price JH (1989) A general native-state method for determination of proliferation capacity of human normal and tumor tissues in vitro. *Proc Natl Acad Sci U S A* 86:2013
26. Hsiang YH, Hertzberg R, Hecht S, Liu LF (1985) Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J Biol Chem* 260:14873
27. Hsiang YH, Lihou MG, Liu LF (1989) Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. *Cancer Res* 49:5077
28. Jaxel C, Kohn KW, Wani MC, Wall ME, Pommier Y (1989) Structure-activity study of the actions of camptothecin derivatives on mammalian topoisomerase I: evidence for a specific receptor site and a relation to antitumor activity. *Cancer Res* 49:1465
29. Kerbel RS, Rak J, Kobayashi H, Man MS, St Croix B, Graham CH (1994) Multicellular resistance: a new paradigm to explain aspects of acquired drug resistance of solid tumors. *Cold Spring Harb Symp Quant Biol* 59:661
30. Kollmannsberger C, Mross K, Jakob A, Kanz L, Bokemeyer C (1999) Topotecan—a novel topoisomerase I inhibitor: pharmacology and clinical experience. *Oncology* 56:1
31. Kubota T, Sasano N, Abe O, Nakao I, Kawamura E, Saito T, Endo M, Kimura K, Demura H, Sasano H, et al (1995) Potential of the histoculture drug-response assay to contribute to cancer patient survival. *Clin Cancer Res* 1:1537
32. Luzzio MJ, Besterman JM, Emerson DL, Evans MG, Lackey K, Leitner PL, McIntyre G, Morton B, Myers PL, Peel M, et al (1995) Synthesis and antitumor activity of novel water soluble derivatives of camptothecin as specific inhibitors of topoisomerase I. *J Med Chem* 38:395
33. Mi Z, Burke TG (1994) Differential interactions of camptothecin lactone and carboxylate forms with human blood components. *Biochemistry* 33:10325
34. Olbina G, Miljkovic D, Hoffman RM, Geller J (1998) New sensitive discovery histoculture model for growth-inhibition studies in prostate cancer and BPH. *Prostate* 37:126
35. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. *Nature* 406:747
36. Philippart P, Harper L, Chaboteaux C, Decaestecker C, Bronckart Y, Gordover L, Lesueur-Ginot L, Malonne H, Lavergne O, Bigg DC, da Costa PM, Kiss R (2000) Homocamptothecin, an E-ring-modified camptothecin, exerts more potent antiproliferative activity than other topoisomerase I inhibitors in human colon cancers obtained from surgery and maintained in vitro under histotypical culture conditions. *Clin Cancer Res* 6:1557
37. Pommier Y, Pourquier P, Fan Y, Strumberg D (1998) Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. *Biochim Biophys Acta* 1400:83
38. Prescott DM, Charles HC, Poulson JM, Page RL, Thrall DE, Vujaskovic Z, Dewhirst MW (2000) The relationship between intracellular and extracellular pH in spontaneous canine tumors. *Clin Cancer Res* 6:2501
39. Raghunand N, He X, van Sluis R, Mahoney B, Baggett B, Taylor CW, Paine-Murrieta G, Roe D, Bhujwala ZM, Gillies RJ (1999) Enhancement of chemotherapy by manipulation of tumour pH. *Br J Cancer* 80:1005
40. Robbins KT, Varki NM, Storniolo AM, Hoffman H, Hoffman RM (1991) Drug response of head and neck tumors in native-state histoculture. *Arch Otolaryngol Head Neck Surg* 117:83

41. Robbins KT, Connors KM, Storniolo AM, Hanchett C, Hoffman RM (1994) Sponge-gel supported histoculture drug-response assay for head and neck cancer: correlations with clinical response to cisplatin. *Arch Otolaryngol Head Neck Surg* 120:288
42. Rosen LS (1998) Irinotecan in lymphoma, leukemia, and breast, pancreatic, ovarian, and small-cell lung cancers. *Oncology (Huntingt)* 12:103
43. Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, Iyer V, Jeffrey SS, Van de Rijn M, Waltham M, Pergamenschikov A, Lee JC, Lashkari D, Shalon D, Myers TG, Weinstein JN, Botstein D, Brown PO (2000) Systematic variation in gene expression patterns in human cancer cell lines. *Nat Genet* 24:227
44. Saramaki OR, Savinainen KJ, Nupponen NN, Bratt O, Visakorpi T (2001) Amplification of hypoxia-inducible factor 1 α gene in prostate cancer. *Cancer Genet Cytogenet* 128:31
45. Sawada S, Okajima S, Aiyama R, Nokata K, Furuta T, Yokokura T, Sugino E, Yamaguchi K, Miyasaka T (1991) Synthesis and antitumor activity of 20(S)-camptothecin derivatives: carbamate-linked, water-soluble derivatives of 7-ethyl-10-hydroxycamptothecin. *Chem Pharm Bull (Tokyo)* 39:1446
46. Singh B, Li R, Xu L, Poluri A, Patel S, Shaha AR, Pfister D, Sherman E, Goberdhan A, Hoffman RM, Shah J (2002) Prediction of survival in patients with head and neck cancer using the histoculture drug response assay. *Head Neck* 24:437
47. Theodorescu D, Connors KM, Groce A, Hoffman RM, Kerbel RS (1993) Lack of influence of c-Ha-ras expression on the drug sensitivity of human bladder cancer histocultured in three-dimensions. *Anticancer Res* 13:941
48. Thistlethwaite AJ, Leeper DB, Moylan DJ 3rd, Nerlinger RE (1985) pH distribution in human tumors. *Int J Radiat Oncol Biol Phys* 11:1647
49. Vescio RA, Redfern CH, Nelson TJ, Ugoretz S, Stern PH, Hoffman RM (1987) In vivo-like drug responses of human tumors growing in three-dimensional gel-supported primary culture. *Proc Natl Acad Sci U S A* 84:5029
50. Vescio RA, Connors KM, Youngkin T, Bordin GM, Robb JA, Umbreit JN, Hoffman RM (1990) Cancer biology for individualized therapy: correlation of growth fraction index in native-state histoculture with tumor grade and stage. *Proc Natl Acad Sci U S A* 87:691
51. Vescio RA, Connors KM, Kubota T, Hoffman RM (1991) Correlation of histology and drug response of human tumors grown in native-state three-dimensional histoculture and in nude mice. *Proc Natl Acad Sci U S A* 88:5163
52. Wachsbarger PR, Landry J, Storck C, Davis K, O'Hara MD, Owen CS, Leeper DB, Coss RA (1997) Mammalian cells adapted to growth at pH 6.7 have elevated HSP27 levels and are resistant to cisplatin. *Int J Hyperthermia* 13:251
53. Wall ME, Wani MC, Nicholas AW, Manikumar G, Tele C, Moore L, Truesdale A, Leitner P, Besterman JM (1993) Plant antitumor agents. 30. Synthesis and structure activity of novel camptothecin analogs. *J Med Chem* 36:2689
54. Wallen JW, Cate RL, Kiefer DM, Riemen MW, Martinez D, Hoffman RM, Donahoe PK, Von Hoff DD, Pepinsky B, Oliff A (1989) Minimal antiproliferative effect of recombinant mulberian inhibiting substance on gynecological tumor cell lines and tumor explants. *Cancer Res* 49:2005
55. Wani MC, Nicholas AW, Manikumar G, Wall ME (1987) Plant antitumor agents. 25. Total synthesis and antileukemic activity of ring A substituted camptothecin analogues. Structure-activity correlations. *J Med Chem* 30:1774
56. Yoshimasu T, Oura S, Maebeya S, Tanino H, Bessho T, Arimoto J, Sakurai T, Matsuyama K, Naito Y, Furukawa T, Yano T, Suzuma T, Hirai I (2000) Histoculture drug response assay on non-small cell lung cancer (in Japanese). *Gan To Kagaku Ryoho* 27:717