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ORIGINAL ARTICLE

R-etodolac (SDX-101) and the related indole–pyran analogues SDX-308 and SDX-309 potentiate the antileukemic activity of standard cytotoxic agents in primary chronic lymphocytic leukaemia cells

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Abstract
Objective SDX-101 is the non-cyclooxygenase 2-inhibiting *R*-enantiomer of the non-steroid anti-inflammatory drug etodolac, and has anti-tumour activity in chronic lymphocytic leukaemia (CLL). SDX-308 and SDX-309 are more potent, structurally related indole–pyran analogues of SDX-101. The current study was performed to investigate and quantify the cytotoxic potentiating effects resulting from a combination of either SDX-101, SDX-308 or SDX-309 with standard cytotoxic agents used in the CLL treatment today.
Methods The lymphoma cell line U937-gtb was used, together with primary tumour cells isolated from seven CLL patients. Combinations between chlorambucil and each one of the agents etodolac, SDX-101, SDX-308 and SDX-309 were studied. In addition, SDX-309 was combined with fludarabine, doxorubicin or vincristine. Both simultaneous and sequential exposures were explored using the median-effect method.
Results Most combinations were additive, which could be of clinical benefit since SDX-101 has been shown to be well tolerated. At the 70% effect level, synergy was observed between SDX-308 and chlorambucil in U937-gtb cells and in two-third of the CLL samples. Since chlorambucil is the most important drug in CLL therapy today and SDX-308 is presently targeted as the lead clinical candidate, this combination would be interesting for further studies. Vincristine and SDX-309 were synergistic in two-fourth of CLL samples.
Conclusions To conclude, the non-COX-inhibiting etodolac-derivatives SDX-101, SDX-308 and SDX-309 are potential candidates for combination treatment of CLL. Especially, SDX-308 in combination with chlorambucil warrants further evaluation.

Keywords Combination · Etodolac · CalcuSyn · SDX-101 · SDX-308

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Introduction

Many non-steroid anti-inflammatory drugs (NSAID) have been shown to be able to inhibit carcinogenesis in colon cancer, as well as to induce apoptosis in a variety of tumour cell lines. Some of the anti-tumour effects of NSAIDs are thought to be due to their cyclooxygenase 2 (COX-2) inhibitory activity, but several lines of evidence now claim that COX-2 independent mechanisms are important [15].

Etodolac is a NSAID used in many countries as an analgesic and anti-inflammatory drug in the treatment of arthritis. A case report from 2002 described an antileukaemic activity of etodolac, given for neck pain, in a patient suffering from B-chronic lymphocytic

leukaemia (CLL) [14]. Commercially available etodolac is provided as a racemate, but only the S-enantiomer possesses potent COX-inhibitory activity [5]. The *R*-isoform (*R*-etodolac, SDX-101), on the other hand, lacks significant COX-inhibiting activity, but has been shown to have similar antitumoural properties as the S-isoform, suggesting that the mechanism of cytotoxicity could be independent of inhibition of COX [1]. One potential cytotoxic mechanism which has been proposed is the inhibition of β-catenin activity, thereby inhibiting the up-stream Wnt signalling pathway [2, 13]. SDX-101 also reduces the intracellular levels of the anti-apoptotic protein myeloid-cell leukaemia sequence 1 (Mcl-1), a member of the Bcl-2 family [1, 2, 16]. In addition, SDX-101 has shown in vivo activity in a prostate cancer model via its effect on the PPAR-γ and RXR-α pathway [9]. SDX-101 is currently in clinical development for the treatment of chronic lympocytic leukaemia, and is undergoing evaluation in a Phase II controlled unblinded trial comparing chlorambucil (2 mg/kg) biweekly versus chlorambucil biweekly and SDX-101 (1,200 mg) twice daily for up to 6 months total treatment.

A series of more potent analogues of SDX-101 has been synthesized, including the preclinical lead candidates SDX-308 and SDX-309. These molecules share the indole–pyran ring system of SDX-101, with various alkyly or halide substitutents appended to the aromatic ring. Additionally SDX-308 and 309 are reduced to the primary alcohol from the acetate attached to the chiral centre of the pyran ring of etodolac. Reduction of the carboxylate is associated in these two molecules with loss of potent COX-inhibitory activity (Fig. 1). Antileukemic activity of these compounds have previously only been presented as conference reports [7, 8, 11, 12].

In the preclinical oncology drug development process, the use of primary tumour cells from cancer patients has gained interest. Primary tumour cells have been shown to retain their original properties in a better way than cell lines, and are better predictors of the disease-specific activity of antitumoural agents [6]. Another important aspect of early cancer drug development is the preclinical in vitro evaluation of drug combinations for potential synergism, since cancer chemotherapy today is mainly based on combination therapy rather than single agent treatment. A commonly used approach for evaluating efficacy potentiation using drug combinations is the median effect analysis method of Chou and Talalay [4]. This method allows evaluation of antagonistic, additive and synergistic interaction of drug combinations. To be able to optimally suggest interesting combination regimens for evaluation in clinical trials, the sequence of drug administration in combination regimens should also be investigated.

In this study, SDX-101 (*R*-etodolac) and the indole–pyran structural analogues SDX-308 and SDX-309 were each studied in combination with traditional cytotoxic drugs commonly used in the treatment of CLL today. A lymphoma cell line as well as primary patient CLL samples was used, and both simultaneous and sequential drug exposure regimens were evaluated.

Materials and methods

Cell lines and primary tumour cells

The lymphoma cell line U937-gtb was maintained in RPMI-1640 culture medium (Sigma, St Louis, MO) supplemented with 10% heat-inactivated foetal calf serum (FCS, Sigma), 2 mM L-glutamine (Sigma), 100 µg/ml streptomycin and 100 unit/ml penicillin (Sigma), at 37°C in 5% carbon dioxide. Primary tumour cells from seven patients with CLL collected between 1999 and 2005 were used (Table 1). Sample collection was approved by the local ethics committee at Uppsala University Hospital.

Mononuclear cells from peripheral blood or bone marrow were isolated by Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation [10]. The tumour cells were cryo-preserved in FCS containing 10% dimethyl sulphoxide (DMSO, Sigma) at –150°C. Before use, the cells were thawed and washed twice with culture medium. Cell viability was determined using the Trypan blue exclusion test. The proportion of tumour cells was assessed by May–

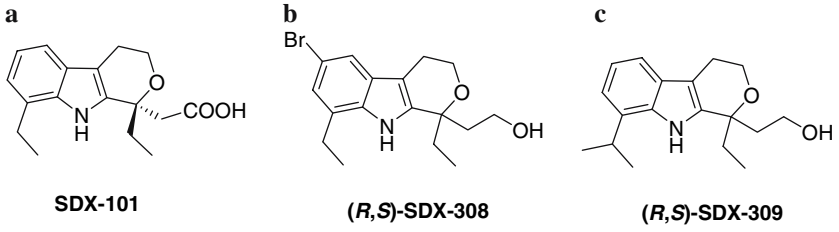

Fig. 1 Chemical structure of **a** *R*-etodolac, **b** racemic SDX-308 and **c** racemic SDX-309

Table 1 Characteristics of the CLL patients sampled to obtain primary tumor cells for the study

Patient number	Sex	Age	Treatment status at sampling time
1	F	71	Untreated
2	M	57	Untreated
3	M	59	Untreated
4	M	59	Treated
5	M	84	Untreated
6	M	85	Untreated
7	M	81	Not known

Grünwald–Giemsa staining of cytocentrifugate preparations.

Drugs and plate preparation

SDX-101 (*R*-etodolac), SDX-308 (racemic mixture) and SDX-309 (racemic mixture) were kindly provided by Salmedix Inc (San Diego, CA, USA), dissolved in DMSO to 250 mM (SDX-101) or 100 mM (SDX-308 and SDX-309) and further diluted in sterile water. Etodolac and chlorambucil were purchased from Sigma. Fludarabine (Fludara), doxorubicin (Adriamycin) and vincristine (Oncovin) were supplied by the local pharmacy.

The 384-well microtiter plates (Nunclon surface, NUNC Brand Products, Denmark) were pre-prepared with 5 μl drug solution in duplicate at ten times the desired final drug concentration. Serial drug dilution and preparation of 384-well microtiter plates were performed by the pipetting robot BIOMek 2000 (Beckman Coulter, USA). The plates were stored at –70°C until use and protected from light during all experimental steps. Twofold dilutions were used. For the investigation of sequence dependency, two different types of mother plates with ten times the desired concentrations of the respective component drugs were prepared.

Fluorometric microculture cytotoxicity assay

The fluorometric microculture cytotoxicity assay (FMCA) is a total cell kill assay, based on the ability of cells with intact cell membranes to convert non-fluorescent fluorescein diacetate (FDA) to fluorescent fluorescein [10]. Cell suspension was seeded into the drug-prepared microtiter plates, using the pipetting robot Precision 2000 (Bio-Tek Instruments Inc, USA) or BIOMek 2000, to a total volume of 50 μl in each well. The resulting cell density was 50×10^3 cells/well for CLL and 5×10^3 for cell lines. Twelve wells with culture medium served as blank and six wells with cells but no drug served as controls. The plates were divided into 4–6 sections with separate control areas for increased precision. The plates were incubated at 37°C in 5% carbon dioxide for a total incubation time of 72 h.

After incubation, the FMCA was performed using the automated Optimized Robot for Chemical Analysis (Orca, Beckman Coulter, Fullerton, CA) programmed through the software SAMI (Beckman Coulter). Medium and drug were aspirated from the plates and the cells were washed twice with phosphate buffered saline (PBS), with a 50 min sedimentation period between each step. Fifty microlitres buffer was added before addition of 1 μl FDA-solution (0.5 mg/ml; Sigma) to a final concentration of 10 μg/ml in each well. The plates were incubated for 50–70 min and the fluorescence was measured in the Fluostar Optima (BMG Technologies, Germany) at 485/520 nm.

Cell survival was presented as survival index (SI) calculated as the mean fluorescence in duplicate drug-treated wells divided with the fluorescence in control wells with the blank value subtracted. Low SI values indicate a high cytotoxic effect. A successful assay required a ratio of >5 between the signal in the control wells and the blank wells, a coefficient of variation (CV) of <30% in the control wells, and ≥70% tumour cells in the cell preparation. Only assays which met these criteria were included in the results.

Combination studies

The combination studies were designed as suggested in the CalcuSyn software manual, using a fixed ratio of the drugs across a concentration gradient. Single-drug potency in CLL was determined in pilot experiments, and fixed concentration ratios of the drugs were used with twofold serial dilutions in nine steps for combination and for dilution of single drug containing wells. The concentration range for the individual drugs, as well as their molar ratios, is shown in Table 2. The first study investigating etodolac or its analogues in combination with chlorambucil, was performed on the U937-gtb cell line and on CLL samples 1, 2 and 3. The second study using SDX-309 in combination with the cytotoxic drugs chlorambucil, fludarabine, doxorubicin and vincristine was performed using CLL samples 2, 4, 5 and 6.

To investigate the potential influence of sequence on the combination effect, SDX-309 in combination with either chlorambucil, fludarabine, doxorubicin or vincristine was investigated both added simultaneously and sequentially. Empty microtiter plates were prepared with cells, and drug was added from mother-plates containing drug solution using BioMek 2000. In

Table 2 Chemotherapeutic agents used in the combination experiments

Chemotherapeutic agent	Concentration range tested (μM)	Chlorambucil-to-drug ratio
(a) SDX-analogs + chlorambucil		
Chlorambucil	1.9–480	
Etodolac	9.4–2,400	1:5
SDX-101	9.4–2,400	1:5
SDX-308	1.9–480	1:1
SDX-309	3.8–960	1:2
		SDX-309-to-drug ratio
(b) SDX-309 + standard agents		
SDX-309	3.8–960	
Fludarabine	0.25–64	15:1
Chlorambucil	1.9–480	2:1
Doxorubicin	0.013–3.2	300:1
Vincristine	0.063–16	60:1

one plate, both drugs were added simultaneously, in one plate SDX-309 was added first, followed by 24 h incubation before addition of the standard drugs, and in one plate the standard drugs were added first and SDX-309 was added after 24 h incubation. All plates were assayed with the FMCA 72 h after addition of the first drug. The resulting experimental plates all had the same drug layout as in previous combination experiments using simultaneous drug exposure. The sequence analysis was performed on CLL sample 3 and 7, and on U937-gtb cells.

Drug combination analyses

To characterize the interactions between the indole–pyran analogues and standard cytotoxic agents, data were analysed using the median-effect method of Chou and Talalay [4] using the software CalcuSyn Version 2 (Biosoft, Cambridge, UK). Each dose–response curve (individual agents as well as combinations) was fit to a linear model using the median effect equation, allowing calculation of a median effect value D (corresponding to the IC50) and slope (m). Goodness-of-fit was assessed using the linear correlation coefficient, r, and only data from analyses with $r > 0.85$ was presented.

The extent of drug interaction between the drugs was expressed using the combination index (CI) for mutually exclusive drugs:

$$\mathrm{CI} = d_1/D_1 + d_2/D_2$$

where D_1 and D_2 represent the concentration of drugs 1 and 2 alone, required to produce a certain effect and d_1 and d_2 are the concentration of drugs 1 and 2 in combination required to produce the same effect. Different CI values are obtained when solving the equation for different effect levels, and 70% effect was chosen for presentation. A CI close to 1 indicates additivity; significantly lower CI was defined as synergy and significantly higher CI as antagonism. Algebraic estimations of the 95% confidence interval of CI were used to determine if the CI differed from 1.

Results

In general, the potentiation effects of chlorambucil with either etodolac, SDX-101, SDX-308 and SDX-309 were modest (Table 3). Most of the interactions observed at 70% effect level were additive, although synergy was observed between SDX-308 and chlor-

Table 3 Combination index at ED_{70} and corresponding effect for chlorambucil combined with etodolac, SDX-101, SDX-308 and SDX-309, in U937-gtb and CLL cells

Combination	U937-gtb			Primary CLL cells		
	n	CI (95% confidence interval[a])	Effect	Patient no.	CI (95% confidence interval[a])	Effect
Chlorambucil + etodolac	3	1.2 (0.9–1.6)	Additive	1	1.3 (0.2–2.3)	Additive
				2	0.9 (0.6–1.1)	Additive
				3	0.9 (0.6–1.3)	Additive
Chlorambucil + SDX-101	3	1.6 (1.3–1.9)	Antagonism	1	1.5 (1.0–2.0)	Antagonism
				2	2.1 (1.1–3.1)	Antagonism
				3	1.4 (0.8–2.0)	Additive
Chlorambucil + SDX-308	3	0.6 (0.4–0.7)	Synergism	1	0.8 (0.4–1.3)	Additive
				2	0.6 (0.5–0.7)	Synergism
				3	0.5 (0.3–0.7)	Synergism
Chlorambucil + SDX-309	3	1.3 (0.7–1.8)	Additive	1	0.8 (0.3–1.2)	Additive
				2	1.4 (0.8–2.0)	Additive
				3	1.5 (1.1–1.9)	Antagonism

Mutual exclusivity is assumed. Synergism and antagonism are defined as a CI statistically significantly lower/higher than 1

[a] 95% confidence intervals are calculated by multiplying the s.d. by 1.96

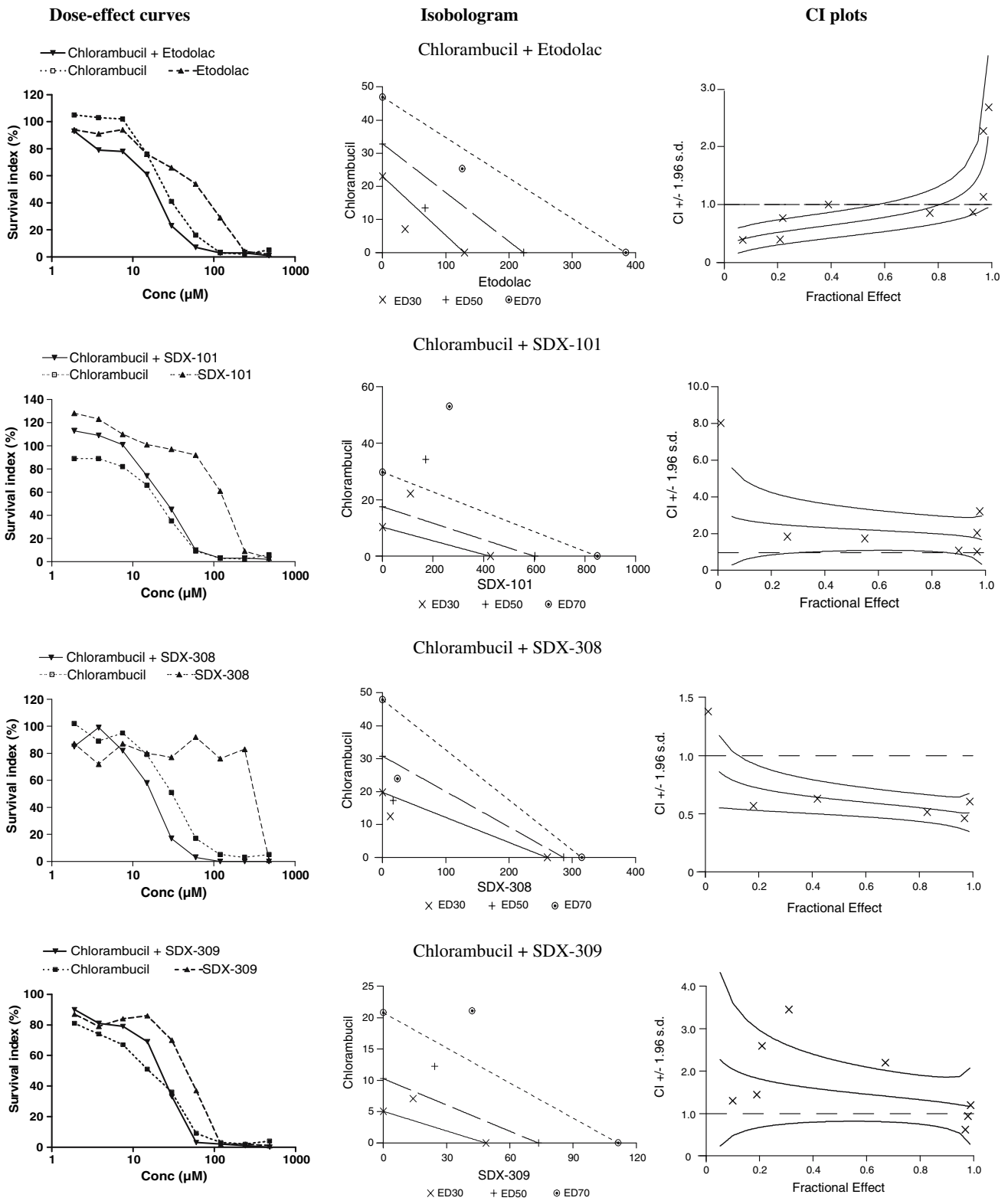

Fig. 2 Combination of chlorambucil with etodolac (*first row*), SDX-101 (*second row*), SDX-308 (*third row*) and SDX-309 (*fourth row*) in CLL cells from patient 2. *First column* shows the concentration–effect curves resulting from the FMCA analysis. *Second column* displays the isobolograms at 30, 50 and 70% effect level, and *third column* the simulated combination index values (with 95% confidence intervals) at all effect levels as calculated by the CalcuSyn software

Table 4 Combination index (CI) at ED_{70} and corresponding effect for SDX-309 combined with fludarabine, chlorambucil, doxorubicin and vincristine, on primary CLL cells

Combination	Primary CLL cells		
	Patient no.	CI (95% confidence interval[a])	Effect
SDX-309 + fludarabine	2	1.2 (0.8–1.5)	Additive
	4	1.0 (0.7–1.4)	Additive
	5	1.8 (1.1–2.6)	Antagonism
SDX-309 + chlorambucil	2	1.9 (1.3–2.5)	Antagonism
	4	1.5 (1.1–1.9)	Antagonism
	5	1.4 (0.8–2.0)	Additive
	6	1.3 (0.0–2.5)	Additive
SDX-309 + doxorubicin	2	1.2 (0.7–1.7)	Additive
	4	1.3 (0.6–2.0)	Additive
	5	1.2 (0.5–2.0)	Additive
	6	1.5 (0.8–2.2)	Additive
SDX-309 + vincristine	2	0.2 (0.1–0.4)	Synergism
	4	0.4 (0.3–0.6)	Synergism
	5	1.5 (0.8–2.3)	Additive
	6	0.8 (0.4–1.2)	Additive

Mutual exclusivity is assumed. Synergism and antagonism are defined as a CI statistically significantly lower/higher than 1

[a] 95% confidence intervals are calculated by multiplying the s.d. by 1.96

ambucil in U937-gtb cells and in 2/3 CLL samples. On the other hand, SDX-101 gave an antagonistic effect with chlorambucil both in the cell line and in two-third of the CLL samples. The same tendency was seen for SDX-309. In Fig. 2, data from one of the CLL patient samples (patient 2) is displayed, and in this sample a synergistic effect of the combination of SDX-308 and chlorambucil can be seen whereas the other analogues give additive or antagonistic effects when combined with chlorambucil. The isobolograms and the CI plots also show a slight tendency towards more pronounced potentiated effects at higher effect levels for three of the four combinations (Fig. 2).

When SDX-309 was combined with standard cytotoxic agents, additive or antagonistic interactions were mostly observed at the 70% effect level (Table 4). In accordance with the data shown in Table 3, the combination between SDX-309 and chlorambucil tended to be unfavourable. Interestingly, vincristine showed a synergistic effect with SDX-309 in 2/4 CLL samples. In Fig. 3, data from one of the CLL patient samples (patient 4) is displayed. No obvious sequence dependency for the effect of the combinations between SDX-309 and either fludarabine, chlorambucil or doxorubicin was observed (Table 5). Most interactions were classified as additive at the 70% effect level independently of schedule used. The results for simultaneous addition of the drugs were well in accordance with previous experiments (Table 4).

Discussion

The current report is the first comprehensive publication presenting data on the indole–pyran analogues SDX-308 and SDX-309, and they are shown to be very active both in a haematological cell line and in primary tumour cells from patients with CLL. Early indications in tumour bearing mice and tumour naive mice suggest that these new chemical entities may prove to have the same beneficial toxicity profile as SDX-101, being well tolerated at doses of 250 mg/kg administered daily for up to 20 days (data not shown, on file at Salmedix, Inc.).

In general, there were additive interaction effects between the SDX-analogues and standard cytotoxic agents. Interestingly, SDX-308 was very active (synergistic) in combination with chlorambucil in this study. Since chlorambucil is the most important drug in CLL therapy today, this combination would be very interesting for future studies. Indeed, additional experiments are planned with SDX-308, presently the lead clinical candidate in the series of indole–pyran molecules tested. On the other hand, SDX-101 and SDX-309 did not look very favourable in combination with chlorambucil, with combination indices above one in almost all samples tested. The difference in interaction pattern between SDX-101 and SDX-309 on one hand, and SDX-308 on the other, suggests a possibility of a difference in mechanism of action. Interestingly, we have also detected a difference in activity pattern in a panel of primary tumor cells from a variety of diagnoses between SDX-308 and the other compounds (Lindhagen et al., manuscript). Also preliminary experiments measuring the global gene expression pattern (Affymetrix gene chip) in prostate cancer cell lines and primary CLL cells exposed to equitoxic concentrations of each drug, indicate a striking difference between SDX-308 and SDX-309 (unpublished data). These results will need to be confirmed, but raise

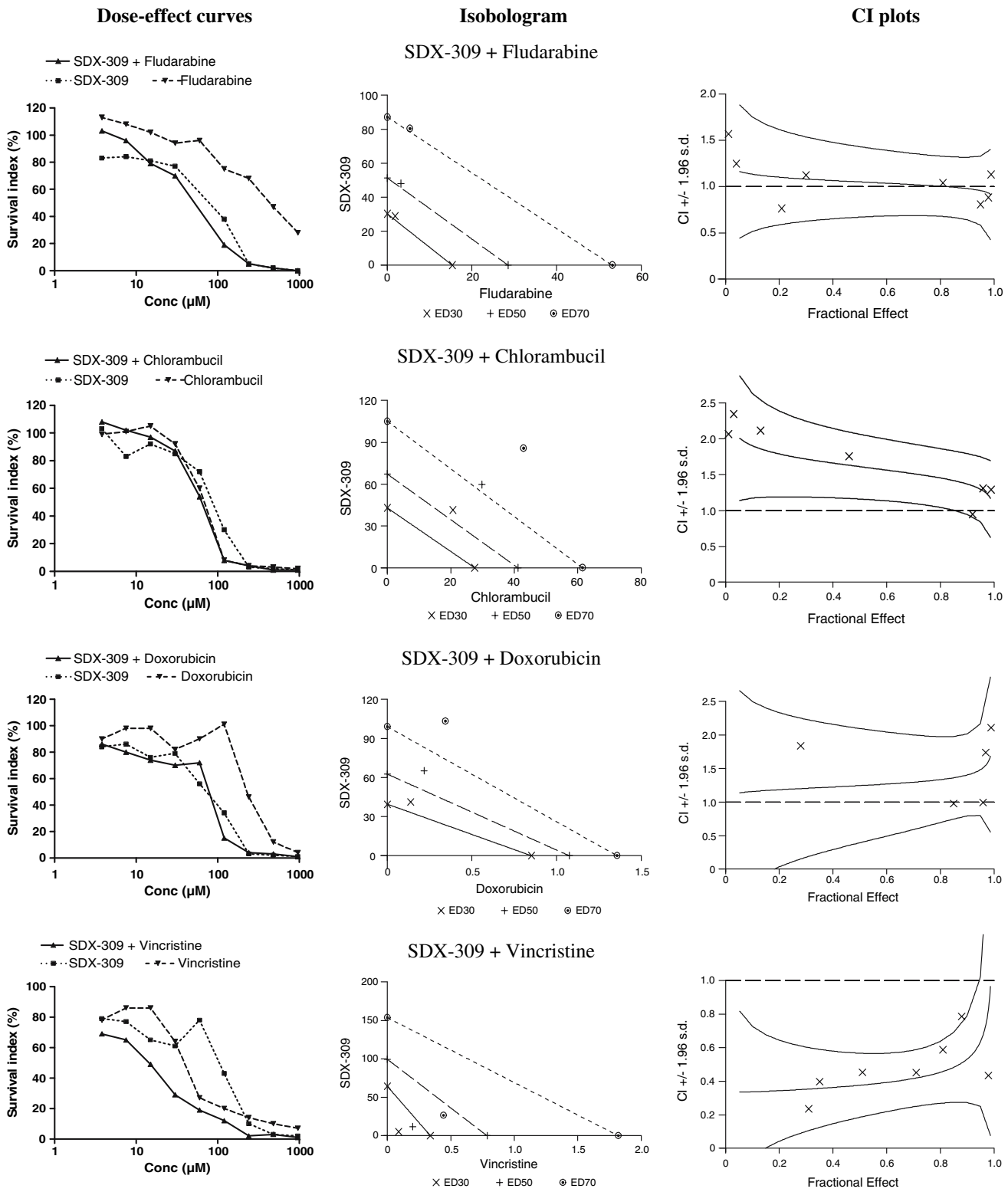

Fig. 3 Combination of SDX-309 with fludarabine (*first row*), chlorambucil (*second row*), doxorubicin (*third row*) and vincristine (*fourth row*) in CLL cells from patient 4. *First column* shows the concentration–effect curves resulting from the FMCA analysis. *Second column* displays the isobolograms at 30, 50 and 70 effect level, and *third column* the simulated combination index values (with 95% confidence intervals) at all effect levels as calculated by the CalcuSyn software

Table 5 Combination index at ED_{70} and corresponding effect for SDX-309 combined with fludarabine, chlorambucil, doxorubicin and vincristine

Schedule	U937-gtb			Primary CLL cells		
	n	CI (95% conf. Interval[a])	Effect	Patient no.	CI (95% conf. interval[a])	Effect
SDX-309 → fludarabine	2	0.9 (0.7–1.2)	Additive	3	1.2 (0.1–2.3)	Additive
				7	1.5 (0.5–2.4)	Additive
Fludarabine → SDX-309	2	1.3 (0.7–2.0)	Additive	3	0.8 (0.2–1.5)	Additive
				7	0.6 (0.4–0.8)	Synergism
SDX-309 + fludarabine	2	1.2 (0.8–1.5)	Additive	7	0.6 (0.2–1.0)	Synergism
SDX-309 → chlorambucil	2	1.0 (0.7–1.3)	Additive	3	1.2 (0.6–1.8)	Additive
				7	1.1 (0.9–1.4)	Additive
Chlorambucil → SDX-309	2	1.3 (1.0–1.6)	Antagonism	7	1.7 (0.7–2.7)	Additive
SDX-309 + chlorambucil	2	1.9 (1.4–2.3)	Antagonism	7	1.4 (0.6–2.2)	Additive
SDX-309 → doxorubicin	2	1.0 (0.6–1.4)	Additive	3	1.2 (0.4–1.9)	Additive
				7	0.7 (0.1–1.4)	Additive
Doxorubicin → SDX-309	2	1.1 (0.4–1.9)	Additive	3	1.4 (0.8–2.1)	Additive
				7	0.7 (0.4–1.1)	Additive
SDX-309 + doxorubicin	2	0.9 (0.4–1.4)	Additive	7	0.8 (0.4–1.1)	Additive

U937-gtb and primary CLL cells were treated with the combinations in sequence or at the same time. No interaction analysis was feasible for vincristine, due to a too high vincristine activity in this material. Mutual exclusivity is assumed. Synergism and antagonism are defined as a CI statistically significantly lower/higher than 1

[a] 95% confidence intervals are calculated by multiplying the s.d. by 1.96

the possibility that SDX-309 could have a different activity profile due to a different mechanism of action.

It is important to remember that additive interactions, not only synergistic, could be of clinical benefit if the drugs have non-overlapping toxicity profiles. Since SDX-101 seems to be very well tolerated, combinations with many cytotoxic agents could be of clinical benefit even if no synergy can be proven. Vincristine was identified as the most promising potential therapeutic partner to SDX-309 in the current study, and vincristine is an important drug in the treatment of many leukaemias and lymphomas today. Considering the limited number of samples tested with this combination, this observation should be verified in further studies. Additional investigation with the individual isomers of SDX-308 and SDX-309 are also planned to confirm any stereochemical preference of the observed cytotoxic effect.

There was no obvious sequence dependency for potentiation in the combinations between SDX-309 and standard agents, suggesting that the order of administration of the drugs would not be of major importance. We did, however, only study one time scheme with a 24-h interval between drug administrations, and other time frames would of course also be interesting. To mimic the clinical situation, longer treatment times could be relevant, but may be difficult to study with this method. One earlier study with etodolac in combination with SN-38 and 5-fluorouracil concludes that etodolac should be administered before the cytotoxic agent [3]. In that publication, the authors speculate in COX-2 related mechanisms, which are not relevant for the analogues used in the present study.

To conclude, SDX-101, SDX-308 and SDX-309 all are novel agents with in vitro activity in primary tumour cells from CLL and have the promise of potentially low clinical toxicity. Studies on primary tumour cells from a variety of other cancer diagnoses, haematological as well as solid, are ongoing at our laboratory. The drugs are likely to be developed for use in combination with cytotoxic agents, a strategy which is supported by the current data. The current combination study shows mostly additive interactions, but identifies SDX-308 + chlorambucil and SDX-309 + vincristine as especially interesting for future studies.

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