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Efficacy of sulofenur and a second generation diarylsulfonylurea, *N*-[5-(2,3-dihydrobenzofuryl) sulfonyl]-*N'*-(3,4-dichlorophenyl)urea (LY295501), against colon adenocarcinoma xenografts

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Sulofenur and a second generation diarylsulfonylurea (DSU), *N*-[5-(2,3-dihydrobenzofuryl)sulfonyl]-*N'*-(3,4-dichlorophenyl)urea (LY295501), were evaluated against a panel of eight colon adenocarcinoma xenografts. Of these tumors, four were derived from adult patients and four from young patients (age range 11–26 years). Both drugs were administered twice daily by oral gavage, 5 days each week for two or three consecutive weeks. The maximum tolerated dose for sulofenur was 300 mg/kg/dose for three courses and 200 mg/kg/dose for LY295501. Against 'adult' derived tumors, sulofenur caused a high proportion of objective regressions of advanced xenografts in two of four lines, with significant inhibition of growth in three tumor lines. Colon adenocarcinomas from young patients were similarly sensitive to sulofenur with a high proportion of complete and partial responses in two of three lines. LY295501 demonstrated a very similar spectrum of activity against this panel of xenografts. Tumors intrinsically resistant to sulofenur were resistant to LY295501, although this agent was slightly more active than sulofenur against tumors from younger patients. In addition, xenografts were established from a cloned colon adenocarcinoma line (GC_3/c1) and its derivative (GC_3/LYC5) selected *in vitro* for resistance to sulofenur. GC_3/c1 xenografts were highly responsive to both sulofenur and LY295501, whereas GC_3/LYC5 xenografts were completely resistant to both agents administered at the maximum tolerated dose and schedule. These results indicate that the second generation DSU, LY295501, demonstrates a similar spectrum of activity against colon tumors as does sulofenur, and that the mechanism of action and/or resistance to the two drugs is probably similar.

Key words: Adenocarcinoma, colon, LY295501, sulofenur, xenograft.

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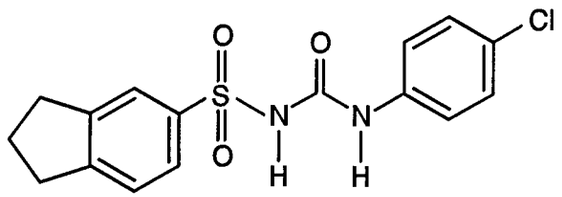
Introduction

The diarylsulfonylureas (DSU) represent a new class of antitumor compound with a potentially novel mechanism of action.[1–4] Although the mechanism by which DSU exert their cytotoxic action is unknown, there is no inhibition of nucleic acid or protein synthesis except at very high, non-pharmacological, concentrations. Work from several groups has indicated that DSU enter cells by passive diffusion, which is greater at low extracellular pH,[1] and concentrate intracellularly.[2] Concentrative accumulation can be inhibited by uncoupling mitochondria or by ionophores such as nigericin that collapse the pH gradient across the mitochondrial membrane.[2] High concentrations of DSU uncouple oxidative phosphorylation in isolated mitochondria and in intact cells;[3] however, the concentrations of drug required indicate that this is unlikely to be the primary mechanism of cytotoxicity at drug concentrations achieved *in vivo*.[4,5]

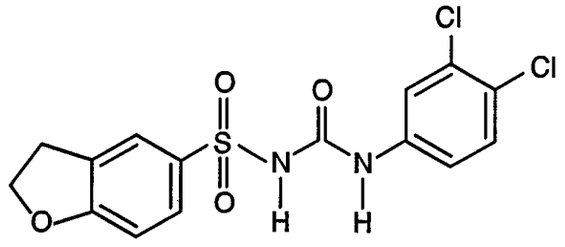
In vivo, sulofenur and several other DSU have demonstrated very significant activity against rodent and human xenograft models, but little activity against leukemia models.[6] Notably, sulofenur demonstrated good activity against both early and advanced colon adenocarcinomas,[6,7] and very significant activity against a panel of rhabdomyosarcoma models.[8] Both *in vitro* and *in vivo* there is no evidence for DSU being part of the P-glycoprotein-mediated multidrug resistance phenotype.[8] Sulofenur has undergone phase I/II clinical evaluation, but demonstrated limited activity. Toxicity in humans (and non-human primates) was predominantly anemia and methemaglobinemia, whereas toxicities more frequently manifest in patients treated with cytotoxic drugs, myelosuppres-

sion and gastrointestinal toxicities, were not frequent or limiting.[9–11] In part the discrepancy between the marked preclinical antitumor activity and limited clinical utility may be a consequence of differential metabolism between rodents and man. Thus, plasma concentrations of free drug achieved at limiting toxicity were approximately three times greater in rodents. The toxic effects of sulofenur have been ascribed to its major metabolites as well as to parent drug, since they all may lead to hydrolysis and the generation of *p*-chloroaniline, thought to be responsible for methemaglobinemia.[12,13] Further, the levels of free (active) sulofenur may be greater in rodents where protein binding is slightly less than in human plasma (unpublished results). Plasma protein binding is extensive for DSU.[14] In serum, protein binding to albumin is greater than 99.9% and the extent of such binding seems to correlate with the potency of DSU. Thus, DSU that bind with greatest avidity to albumin are the most potent cytotoxic analogs under serum-free conditions.[15]

To overcome some of the problems associated with sulofenur, an analog, *N*-[5-(2,3-dihydrobenzofuryl)sulfonyl]-*N'*-(3,4-dichlorophenyl) urea (LY295501), where the indanyl ring was replaced by a furanose (Figure 1) was synthesized in the anticipation that this analog would not be metabolized by *ortho*-hydroxylation. Consequently, this would reduce the formation of *p*-chloroaniline and, potentially, the toxicity associated with sulofenur. Here we report the evaluation of sulofenur and LY295501 against a panel of colon tumor xenografts derived from typical adult patients and from younger patients. Part of the work has been presented in preliminary form previously.[7]



sulofenur



LY295501

Figure 1. Chemical structure of sulofenur and LY295501.

Materials and methods

Immune deprivation of mice

Female CBA/CaJ mice (Jackson Laboratory, Bar Harbor, ME), 4 weeks of age, were immune-deprived by thymectomy, followed 3 weeks later by whole-body irradiation (950 cGy) using a ^{137}Cs source. Mice received 3×10^6 nucleated bone marrow cells within 6–8 h of irradiation.[8]

Tumor lines

Each of the independently derived lines from previously untreated colon adenocarcinoma have been described previously.[16] For chemotherapy studies, all tumors were used within 32 passages of their engraftment in mice. Each tumor grew routinely in over 90% of recipient mice, and all are human as determined by karyotype and species-specific isoenzyme patterns. Colon adenocarcinomas used (HC_1, GC_3, VRC_5 and ELC_2) were derived from elderly patients (61–83 years) and have been characterized extensively.[16–18] SJC2 is a moderately differentiated adenocarcinoma from a 14 year old female, SJC_3A and SJC_3B tumors were independent primaries in a 26 year old male, and SJC8 is a well differentiated adenocarcinoma from an 11 year old male.

Growth inhibition studies

Mice bearing bilateral subcutaneous tumors each received administration of agent when tumors were approximately 0.4–1 cm in diameter. Tumor response was determined at 7 day intervals using digital calipers (maxcal) interfaced to a Dell 486/50 microcomputer. Two perpendicular diameters were used to compute volumes. Growth delay was calculated from the difference in days required for treated tumors to grow to 4-fold their volume at the time of starting treatment compared with vehicle-treated controls. For each treatment group, six or seven tumor-bearing mice each with bilateral tumors were used. Relative tumor volumes were

calculated from the formula RTV = (V_X/V_0), where V_X is the volume on day X and V_0 is the volume of tumor at time of initiating treatment. The definition of partial response (50% or greater regression) or complete response required that the tumor at some time point after treatment demonstrated such a reduction in volume.

Formulation and administration

Stock suspensions of sulofenur or LY295501 were prepared in 5% Emulphor EL-620 (GAF, Wayne, NJ), and, after vigorous resuspension of the agent, given by oral gavage (0.1 ml/20 g body weight). All protocols used twice-daily administration (b.i.d), the doses being spaced by approximately 8 h. For the studies reported, tumor bearing mice were treated 5 days per week, with courses repeated on two or three consecutive weeks.

Statistical analysis

The results of individual tumor inhibition studies were analyzed with one-way analysis of variance, using the number of days to reach four times the original tumor volume as the dependent variable. Only tumors from mice that survived the entire study were included in the analyses and any tumor that did not reach four times the original volume was assigned a default value of the maximum duration of the study.

To compare the efficacy of various courses of treatment, data were collapsed across studies, within a tumor line. The percent of tumors showing partial and/or complete regression and any regrowth were calculated for the individual tumor lines as described previously.[8]

Results

Sulofenur was tolerated at dose levels up to 300 mg/kg b.i.d for two (adult colon) or three (juvenile colon) courses by tumor bearing mice. For mice bearing HC_1, GC_3 and ELC_2 tumors (derived from older patients), the maximal tolerated dose level was approximately 200 mg/kg b.i.d given for two consecutive 5 day courses (two of 35 deaths). Higher dose

Table 1. Activity of sulofenur against adult derived colon adenocarcinomas

Tumor	Dose (b.i.d. mg/kg)	Days to 4× ± SE	Growth delay (days)	PR (%)	CR (%)	MCR (%)	Survival (%)
HC_1	control	16.7 ± 1.6					
	(100 × 5)2	27.4 ± 5.6	10	0	0	0	100
	(200 × 5)2	29.4 ± 2.2	12	0	0	0	100
	(100 × 5)3	30.7 ± 5.4	14	0	0	0	100
	(300 × 5)2	toxic					
GC_3	control	20.1 ± 4.5					
	(200 × 5)2	63.6 ± 4.1	43	33	0	0	83
	(300 × 5)2	64.1 ± 5	44	92	8	0	80
	(400 × 5)2	>84	>64	41	42	17	86
	(100 × 5)3	67.2 ± 3.7	47	16	0	0	100
	(200 × 5)3	>84	>64	30	30	20	80
VRC_5	control	10.9 ± 0.5					
	(100 × 5)2	59.9 ± 4.0	49	21	79	14	100
	(200 × 5)2	68.8 ± 9.9	58	7	93	14	100
	(400 × 5)2	71.2 ± 5.6	61	43	50	29	50
	(600 × 5)2	toxic					
ELC_2	control	36.5 ± 4.6					
	(200 × 5)2	67.9 ± 3.7	31	0	0	0	100
	(300 × 5)2	79.0 ± 2.2	43	7	0	0	86
	(400 × 5)2	82.9 ± 1.2	46	0	0	0	86
	(200 × 5)3	>84	>47	0	0	0	100
GC_3/c1	control	49.9 ± 6.4					
	(200 × 5)2	74.1 ± 4.7	24	50	33	33	100
	(400 × 5)2	76.1 ± 1.7	26	75	25	25	57
GC_3/LYC5	control	38.5 ± 2.6					
	(200 × 5)3	41.1 ± 3.6	2	0	0	0	100
	(400 × 5)3	34.1 ± 0.4	−4	0	0	0	100

PR, partial regression (>50%); CR, complete regression; MCR, maintained CR at 12 weeks.

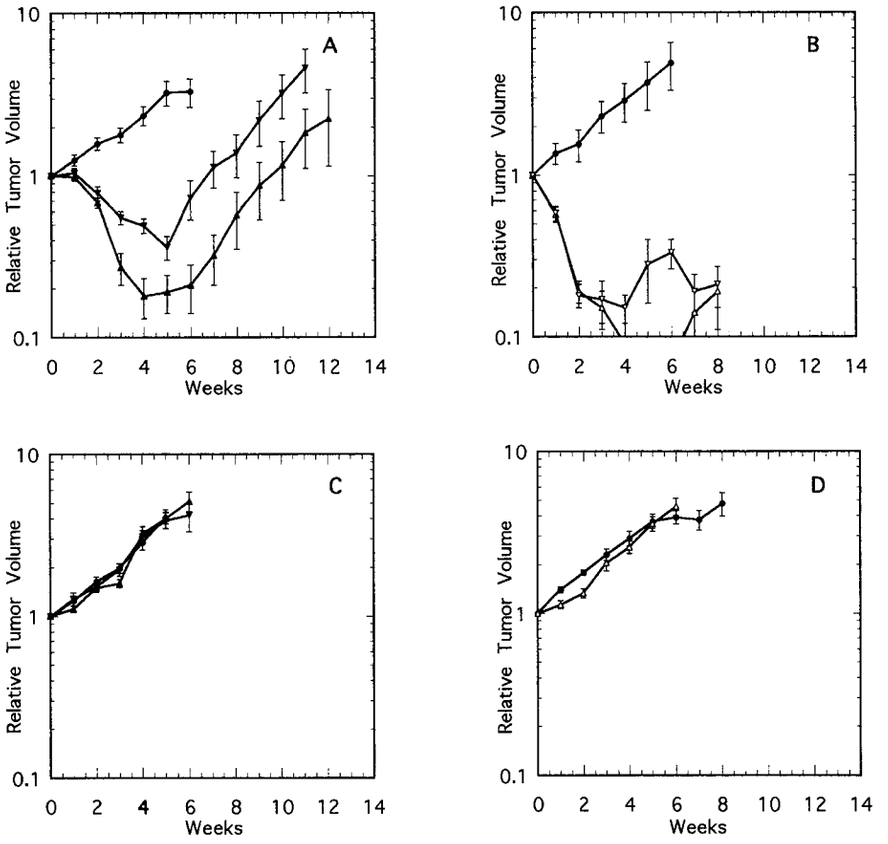


Figure 2. Responses of GC_3/c1 (A and B) or GC_3/LYC5 (C and D) xenografts in mice treated with sulofenur (A and C) or LY295501 (B and D). Specific treatments were: (▲) 300; (▼) 200 mg/kg/ dose sulofenur; (△) 200; (▽) 100 mg/kg/ dose LY295501. (●) Control. Agents were administered b.i.d 5 days per week for three consecutive weeks. Each curve is the relative tumor growth and represents the mean growth of 12 or 14 tumors in a treatment group.

Table 2. Activity of sulofenur against colon adenocarcinoma derived from young patients

Tumor	Dose (b.i.d. mg/kg)	Days to 4× ± SE	Growth delay (days)	PR (%)	CR (%)	MCR (%)	Survival (%)
SJC_2	control	28.6 ± 1.9					
	(100 × 5)3	44.7 ± 6.4	16	0	0	0	100
	(200 × 5)3	72.6 ± 2.1	44	33	42	33	100
	(300 × 5)3	74.3 ± 4.1	46	29	64	43	100
SJC_3A	control	29.8 ± 2.4					
	(100 × 5)3	63.0 ± 4.4	33	43	39	28	100
	(200 × 5)3	73.3 ± 3.3	43	29	72	7	100
	(300 × 5)3	78.2 ± 2.8	48	8	92	42	100
SJC_8	control	25.0 ± 1.6					
	(100 × 5)3	79.5 ± 5.6	54	7	0	0	100
	(200 × 5)3	>84	>59	57	0	0	100
	(300 × 5)3	>84	>59	79	0	0	100

levels or extending the number of courses resulted in variable toxicity. Tumor lines GC_3 and VRC_5 were relatively sensitive to sulofenur, demonstrating a high frequency of partial or complete responses. The growth of ELC_2 xenografts was significantly inhibited, but sulofenur did not induce regressions at any dose or schedule. HC_1 tumors were intrinsically resistant to sulofenur (Table 1). A clone derived from the GC_3 xenograft (GC_3/c1) and LYC5, a clone selected *in vitro* for resistance to sulofenur, were re-established as xenografts, and their sensitivity to sulofenur evaluated.[4] As shown in Figure 2, GC_3/c1 tumors were highly sensitive to sulofenur whereas GC_3/LYC5 tumors were resistant. Compar-

ison at 200 mg/kg showed that 83% of parental tumors demonstrated partial or complete regressions with a growth delay of more than 44 days compared with no growth inhibition or regressions in GC_3/LYC5 tumors.

Responses to sulofenur of colon tumors derived from young patients are presented in Table 2. Sulofenur demonstrated significant activity in each of three lines. The maximal tolerated dose for three courses of therapy was 300 mg/kg (zero of 21 deaths). Sulofenur demonstrated dose-dependent activity, causing significant growth inhibition at each dose level, with increasing frequency of partial and complete responses as the dose was increased.

The second generation analog, LY295501, was also evaluated against this panel of colon adenocarcinomas (Table 3). The maximal tolerated dose was 200 mg/kg given for three consecutive 5 day courses. As shown in Table 3, LY295501 demonstrated significant activity causing partial and complete regressions of advanced VRC_5, SJC_3A, SJC_3B and SJC_8 xenografts. Of note was that given at the maximum tolerated dose level, LY295501 was more efficacious than sulofenur in treating SJC_3A xenografts (Figure 3). Growth of ELC_2 tumors was significantly inhibited, whereas HC_1 xenografts were resistant to this agent. GC_3/c1 xenografts were also highly responsive to LY295501 whereas GC_3/LYC5 tumors, selected *in vitro* for resistance to sulofenur, were resistant (Figure 2).

Discussion

Although some progress in the treatment of colon carcinoma has been achieved by rational combination of 5-fluorouracil with leucovorin,[19] this malignancy remains refractory to most chemotherapeutic agents. Consequently, new, novel agents are required if significant impact on survival by chemotherapeutic agents is to be achieved. Sulofenur, a novel DSU, was found to have significant activity against rodent solid tumors and human tumor xenografts including colon adenocarcinomas. However, despite these promising preclinical data, clinically sulofenur was disappointing, yielding few objective responses in phase II trials. In man, limiting toxicities (anemia and methemaglobinemia) are limiting at plasma concentrations of drug that are below those shown to be associated with significant antitumor activity in mice. In part this may relate to more extensive metabolism in man yielding *p*-chloroaniline, thought to be responsible for toxicity.[12,13] Consequently, LY295501 was synthesized with the intent of blocking the initial metabolic conversion observed with sulofenur, specifically hydroxylation

Table 3. Activity of LY295501 against adult and juvenile derived colon cancers

Tumor	Dose (b.i.d. mg/kg)	Days to 4× ± SE	Growth delay (days)	PR (%)	CR (%)	MCR (%)	Survival (%)
HC_1	control	28.3 ± 2.7					
	(100 × 5)3	33.3 ± 2.0	5	0	0	0	100
	(200 × 5)3	41.8 ± 3.8	13	0	0	0	100
	(300 × 5)3	toxic	–	–	–	–	57
VRC_5	control	16.3 ± 1.6					
	(100 × 5)3	>84	>67	20	80	80	100
	(200 × 5)3	74.0 ± 2.8	58	50	43	43	100
ELC_2	control	43.5 ± 4.4					
	(200 × 5)3	80.6 ± 1.2	37	0	0	0	100
	(300 × 5)3	toxic	–	–	–	–	57
SJC_3A	control	33.5 ± 3.2					
	(100 × 5)3	>84	>50	17	83	83	86
	(200 × 5)3	>84	>50	0	100	100	71
SJC_3B	control	29.3 ± 3.3					
	(200 × 5)3	78.4 ± 2.0	49	54	15	0	100
SJC_8	control	34.3 ± 3.8					
	(100 × 5)3	83.8 ± 1.1	49	36	14	14	100
	(200 × 5)3	81.8 ± 2.3	47	84	8	0	100
GC_3/c1	control	40.3 ± 7.2					
	(100 × 5)3	>84	>44	0	100	83	81
	(200 × 5)3	>84	>44	0	100	92	71
GC_3/LYC5	control	44.0 ± 3.5					
	(200 × 5)3	45.5 ± 6.3	1	0	0	0	84

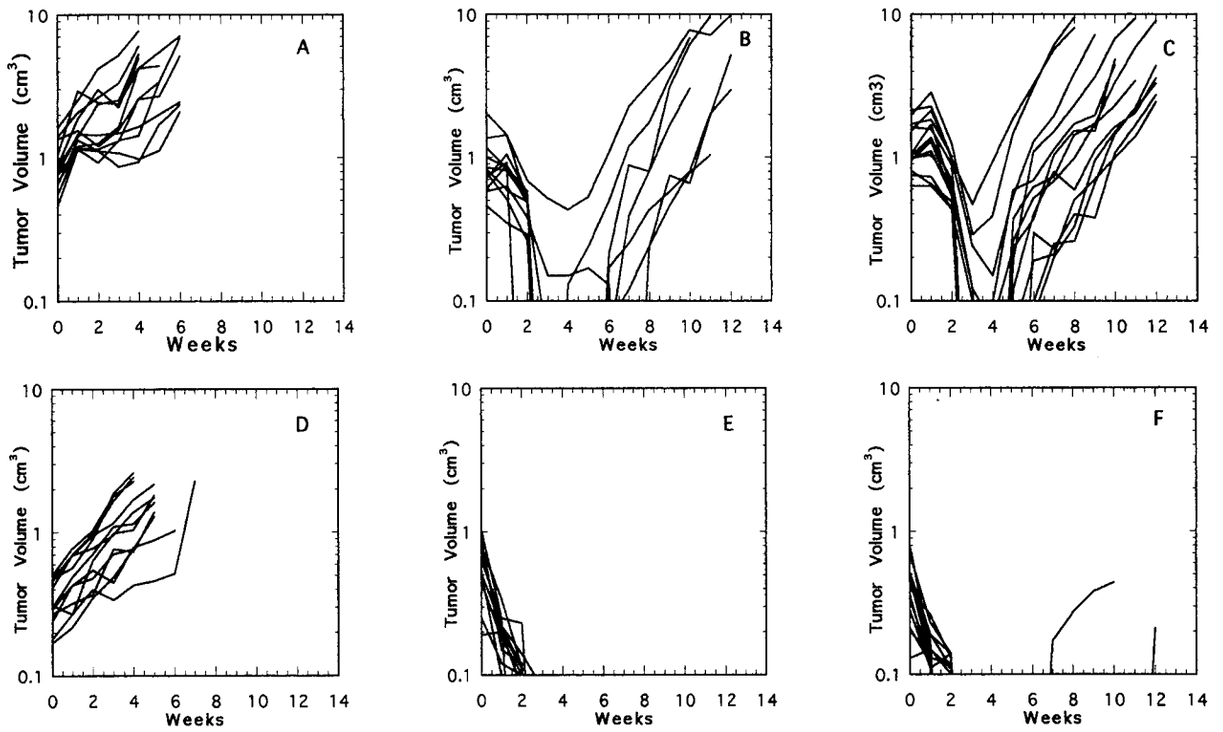


Figure 3. Responses of SJC_3A human colon adenocarcinoma xenografts to sulofenur (A–C) or LY295501 (D–F). Mice were treated with vehicle control (A and D) or sulofenur: (B) 300; (C) 200 mg/kg per dose b.i.d. or LY295501: (E) 200; (F) 100 mg/kg per dose b.i.d. All mice received drug by oral gavage 5 days per week for three consecutive weeks. Each curve represents the growth of an individual tumor.

at the 1-position of the indanyl ring (Figure 1). Preclinical toxicology indicates that in rodents and non-human primates the pattern of toxicity is different from sulofenur, and is predominantly gastrointestinal and myelosuppressive in nature.[20]

Sulofenur and LY295501 have been evaluated against a panel of eight colon adenocarcinoma xenografts. Both agents demonstrate a similar spectrum of activity. Tumors intrinsically sensitive to sulofenur respond equally well to LY295501, whereas tumors, such as HC_1, that are resistant to sulofenur are also resistant to LY295501. Sulofenur caused a high frequency of objective responses in VRC_5, SJC_3A, SJC_2 and SJC_8 xenografts. LY295501 showed slightly greater activity against SJC_3A and $GC_3/c1$ xenografts than did sulofenur, but otherwise appeared similar in efficacy.

We have also examined the activity of DSU against a clone of GC_3 and its derivative selected *in vitro* for resistance to sulofenur.[4] *In vitro*, the resistant mutant (GC_3/LYC5) is approximately 4-fold resistant relative to parental $GC_3/c1$ cells and resistance was stable in the absence of drug selection for more than 2 years. Interestingly, GC_3/LYC5 cells are resistant only under conditions of continuous exposure at low drug concentration, but not when cells are exposed for short periods (4 h) to high concentrations of DSU.[21] We have proposed that the mechanisms of cytotoxicity exerted by DSU are quite distinct under different conditions of exposure and have proposed a two-site model of action.[4] Thus, LYC5 cells appear to be resistant to low concentrations of DSU when exposed to drug for 7 days, hence are resistant to the pharmacologically important action of antitumor DSU. To test this, GC_3/LYC5 cells were re-established as xenografts and their sensitivity to sulofenur and LY295501 determined. GC_3/LYC5 xenografts were completely resistant to both DSUs tested and thus confirm that the mechanisms of action defined as 'site 1' is indeed the pharmacologically important mechanism of DSU activity *in vivo*.[4]

In conclusion, both sulofenur and LY295501 demonstrate significant activity against a panel of human colon adenocarcinoma xenografts. This level of activity and the altered pattern of toxicity

from sulofenur make LY295501 a potentially interesting compound for evaluation in human malignancies.

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