HHS Public Access

Author manuscript

Angiogenesis. Author manuscript; available in PMC 2015 August 18.

Published in final edited form as:

Angiogenesis. 2014 July ; 17(3): 661–673. doi:10.1007/s10456-014-9422-9.

Analysis of acquired resistance to metronomic oral topotecan chemotherapy plus pazopanib after prolonged preclinical potent responsiveness in advanced ovarian cancer

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Electronic supplementary material The online version of this article (doi:10.1007/s10456-014-9422-9) contains supplementary material, which is available to authorized users.

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Abstract

An alternative or follow-up adjunct to conventional maximum tolerated dose (MTD) chemotherapy now in advanced phase III clinical trial assessment is metronomic chemotherapy—the close regular administration of low doses of drug with no prolonged breaks. A number of preclinical studies have shown metronomic chemotherapy can cause long term survival of mice with advanced cancer, including metastatic disease, in the absence of overt toxicity, especially when combined with targeted antiangiogenic drugs. However, similar to MTD chemotherapy acquired resistance eventually develops, the basis of which is unknown. Using a preclinical model of advanced human ovarian (SKOV-3-13) cancer in SCID mice, we show that acquired resistance can develop after terminating prolonged (over 3 months) successful therapy utilizing daily oral metronomic topotecan plus pazopanib, an oral antiangiogenic tyrosine kinase inhibitor (TKI). Two resistant sublines were isolated from a single mouse, one from a solid tumor (called KH092-7SD, referred to as 7SD) and another from ascites tumor cells (called KH092-7AS, referred to as 7AS). Using these sublines we show acquired resistance to the combination treatment is due to tumor cell alterations that confer relative refractoriness to topotecan. The resistant phenotype is heritable, associated with reduced cellular uptake of topotecan and could not be reversed by switching to MTD topotecan or to another topoisomerase-1 inhibitor, CPT-11, given either in a metronomic or MTD manner nor switching to another antiangiogenic drug, e.g. the anti- VEGFR-2 antibody, DC101, or another TKI, sunitinib. Thus, in this case cross resistance seems to exist between MTD and metronomic topotecan, the basis of which is unknown. However, gene expression profiling revealed several potential genes that are stably upregulated in the resistant lines, that previously have been implicated in resistance to various chemotherapy drugs, and which, therefore, may contribute to the drug resistant phenotype.

Keywords

Metastasis; Ovarian cancer; Chemotherapy; Topotecan; Pazopanib; Resistance

Introduction

Metronomic chemotherapy is an investigational form of chemotherapy that involves the close, regular and long-term administration of conventional chemotherapeutic drugs at relatively minimally toxic doses without prolonged drug-free breaks [1, 2]. While originally thought to mediate its anti-tumor effects predominantly via antiangiogenic mechanisms, there is a growing body of evidence that metronomic dosing, depending on the chemotherapy drug used, likely involves additional or alternative mechanisms. These include stimulation of the host immune system, e.g. by metronomic cyclophosphamide, targeting of tumor cell associated expression of hypoxia inducible factor-1α (HIF-1α), e.g. by low dose metronomic-like topotecan [3] or adriamycin [4] and direct tumor cell targeting,

including, possibly, the putative cancer stem cell subpopulation [5, 6]. With respect to antiangiogenic mechanisms, direct targeting of activated endothelial cells in the tumor neovasculature [7, 8] as well as blockade of mobilization of pro-angiogenic/vasculogenic bone-marrow derived cell (BMDC) populations (e.g. circulating endothelial progenitor cells) have both been implicated [9, 10]. Suppression of HIF-1α may be another means given its role in promoting angiogenesis [11].

These multiple mechanisms of action may explain why there are circumstances of extremely durable marked suppression of tumor growth in mice, even when advanced metastatic disease is treated [7, 8, 12–14]. However, similar to all other types of anti-cancer drugs or therapies, resistance can eventually develop to metronomic chemotherapy regimens, a finding seemingly reflected in patients receiving this form of therapy [2]. The basis of resistance to metronomic chemotherapy is unknown. However, there are a few published reports showing that resistance to conventionally administered maximum tolerated dose (MTD) chemotherapy, e.g. using cyclophosphamide, does not necessarily lead to ‘cross resistance’ to the same agent given metronomically [7] or vice versa, again using cyclophosphamide [15]. This has potentially important implications for the sequential use of metronomic and MTD chemotherapy using the same agent. Clearly, additional studies are needed to investigate the inter-relationship of acquired resistance mechanisms to metronomic versus MTD conventional chemotherapy. As metronomic chemotherapy is now undergoing evaluation in numerous phase II and at least six randomized phase III clinical trials [2, 16] (www.clinicaltrials.gov and see “Discussion” section), it will be important to gain a better understanding of the nature of response and resistance to this therapeutic regimen. Towards this end we have been studying a model of acquired resistance that develops after terminating a successful long-term metronomic oral topotecan chemotherapy protocol, when used concurrently with the oral antiangiogenic tyrosine kinase inhibitor (TKI) pazopanib to treat advanced ovarian cancer in mice [13].

In the initial study evaluating this treatment combination, SCID mice with established, advanced intraperitoneal ovarian cancer were treated with daily oral low-dose topotecan plus daily oral pazopanib, both given by gavage [13]. The tumor cell line used was an aggressive cloned variant of the SKOV-3 line called SKOV-3-13 which was also luciferase tagged. Based on survival times and whole body bioluminescent imaging, the combination treatment kept 100 % of the mice alive for at least 6 months with no evidence of overt toxicity. In contrast, pazopanib treatment alone had little or no survival impact and mice succumbed to disease 45 days after start of treatment [13]. Metronomic topotecan alone was able to prolong survival by 2 months, which was extended to beyond 6 months when pazopanib was given concurrently with the metronomic topotecan [13]. The efficacy of this combination was confirmed in subsequent experiments reported here, in which continuous and prolonged daily therapy (3 months) succeeded in potent inhibition of metastatic growth, after which therapy was stopped. In this instance, tumors began to relapse after a 6 week break in therapy. These previously dormant but subsequent relapsing tumors then failed to respond to rechallenge with daily therapy using the same topotecan/pazopanib combination. Two such relapsing tumors from a single mouse were isolated in the form of a solid tumor mass or as ascites. Cell lines were established from these tumor sources as a first step to determine the mechanism(s) of resistance to the metronomic topotecan/pazopanib treatment combination.

Herein we report results indicating that the resistance is due to the topotecan component of the two drug treatment combination and that cross resistance to MTD topotecan (and also irinotecan) is expressed by the variant sublines. As such, the results highlight the fact that some metronomic chemotherapy regimens likely exert much or at least some of their antitumor activity by direct effects on the tumor cell population as opposed to, or perhaps instead of, antiangiogenic effects.

Materials and methods

Isolation of ovarian carcinoma acquiring resistance to metronomic topotecan plus pazopanib therapy

All in vivo experiments were performed in female CB17 SCID mice 6–8 weeks of age. Mice were implanted intraperitoneally with 10^6 SKOV-3-13 ovarian cancer cells obtained from the SKOV-3 cell line. This cell line had been previously tagged with luciferase expression vector, cloned and selected for highly aggressive growth in the peritoneal cavity [13]. Two weeks post-tumor cell implantation into the intraperitoneal cavity when multifocal metastatic disease is evident, treatment was initiated with 1 mg/kg topotecan and 150 mg/kg pazopanib, given daily and concurrently by gavage. Mice were monitored for tumor growth by bioluminescence imaging. Treatment was given daily and continuously for 13 weeks, i.e., without any breaks. At the end of this initial but prolonged treatment (prolonged for mice), disease appeared stable and treatment was stopped. Mice were continually monitored. Six weeks after cessation of treatment, mice began to show signs of tumor relapse as indicated by increased bioluminescence. Treatment with the same regimen of topotecan/pazopanib was then re-initiated. Tumor growth nevertheless continued to progress under therapy (as determined by increase in luciferase activity) and mice were sacrificed 10.5 weeks later as a result of extensive metastatic disease. Separate cell lines were established from a solid tumor (7SD) and ascites (7AS) present in the same mouse.

SKOV-3-13, 7AS and 7SD cells were plated in 24-well plates (1×10^3 cells) and treated continuously for 144 h with various concentrations of topotecan (0.00025–30 nM) alone and in combination with pazopanib (0.74 μM), adding fresh solutions with new medium every 24 h. At the end of the experiments, cells were harvested with trypsin/EDTA and viable cells counted with a haemocytometer. Cell viability was assessed by trypan blue dye exclusion. The data are presented as the percentage of the vehicle-treated cells [17]. In separate experiments, tumor cell lines were plated in 96-well plates (6×10^2 cells) and treated were treated continuously, as previously described, with topotecan (0.00025–30 nM), pazopanib (0.001–50 μM), CPT11, gemcitabine and doxorubicin or with their vehicles for 144 h. Proliferation assays were performed using the CellTiter Aqueous One Solution Cell Proliferation Assay (Promega) according to modified manufacturer's instructions. Values are expressed relative to vehicle-treated cells. The concentration of drug that reduced cell proliferation by 50 % (IC50) versus controls was calculated by nonlinear regression fit of themean values of the data obtained in triplicate experiments.

Intracellular accumulation of topotecan

SKOV-3-13, 7AS and 7SD cells (1×10^6) were incubated in RPMI (5 % fetal bovine serum) in 10 cm^2 dishes for 16 h at 37 °C. Topotecan stock solution in DMSO was diluted in pH 3 PBS (37 °C) to convert the compound into their active lactone. Topotecan [1 μM] were diluted in RPMI medium just before the start of the incubation. After incubation for 5 min at 37 °C, the treatment medium was removed; cells were washed twice with cold PBS at pH 5 and scraped immediately. The cells were then ultrasonically lysed (10 s × 3). The lysate (200 μL) was added to 400 μL cold methanol and centrifuged (5 min, 7,500 g, 4 °C) for topotecan extraction to determine the intracellular drug concentration. Proteins in the remaining lysate were quantified by colorimetric assay (Bio-Rad Laboratories) [18]. The concentration of topotecan in the sample was measured using a previously described sensitive high-performance liquid chromatography (HPLC) method [19].

In vivo assessment of resistance to therapy

Cells were injected into mice as above with either SKOV-3-13 or 7SD cell lines (10^6 cells implanted by ip. injection). Two weeks post-tumor cell injection, therapy was initiated. Maximum tolerated dose (MTD) topotecan, was given at a dose 1.5 mg/kg (days 1–5 in a 3 week cycle), MTD CPT11 (40 mg/kg q2dx5, ip), low-dose metronomic CPT11 (10 mg/kg 2× per week, ip). Gemcitabine was given at doses of either 28.5 mg/Kg or 58 mg/kg (2× per week, ip) and doxorubicin at MTD dose of 2 mg/kg (day 1 and 5; 21 day cycles). Mice were sacrificed when signs of distress appeared (e.g. loss of weight, lethargy, scruffiness).

Whole human genome expression detection by microarray

Both metronomic topotecan/pazopanib resistant variants (KHO92-7SD and KHO92-7AS) were compared to the therapy sensitive parental cell line, SKOV3-13, using multiple passage numbers and incorporating a dye swap. RNAs were hybridized on Agilent 4X44K Whole Human Genome Microarray. Total RNA was extracted from samples by using the RNEasy kit mini (Qiagen). Labeled cDNA was synthesized from 25 μg of total RNA using Superscript III (Invitrogen) in the presence of Cyanine 3-dCTP or Cyanine 5-dCTP (Perkin-Elmer Life Sciences). Hybridizations and washes were performed according to manufacturer's instructions. Scanning was done with a ScanArray Lite microarray scanner (Perkin Elmer). QuantArray software (GSI Lumonics) was used to quantify fluorescence intensities.

Differential expression was attributed to genes that were over expressed (≥1.5 fold increase in expression) or under expressed (≤0.67 fold change in expression relative to parental SKOV3-13). For both instances a t-test value (*P* value) <0.05 was taken as significant. Microarray data are analysed using Genespring software (Agilent Technologies), and have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE54621 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54621). (http://www.ncbi.nlmacc=).

RT-PCR confirmation of gene upregulation

RNA was isolated, cDNA generated and QRT-PCR performed as above using multiple in vitro passages of 7SD, 7AS and SKOV-3-13 cells. The following primers were used in conjunction with GAPDH as housekeeping gene.

CYP1B1 F	GCTTTAATCAGAAACCCTCATTGTG
CYP1B1 R	GCAATAACCTGGAGTAAAACTTCTGACT
TKTL1 F	CATGAGTAAAGAAAATGTGGATTGAAGTA
TKTL1 R	AGCAGTAGGCGTCATGGTGTT
CRYAB F	TGGACCAAGGAAACAGGTCTCT
CRYAB R	CGGTGACAGCAGGCTTCTCT
HSPB2 F	CACAGAGGTCAATGAGGTCTACATCT
HSPB2 R	GCAATCAGGGCTCAACTATGG

Statistical analysis

The results (mean ± SD) of all in vitro experiments were subjected to analysis of variance between groups (ANOVA), followed by the Student–Newman–Keuls test. Tumor therapy results are reported as mean ± SD. Survival curves were plotted by the method of Kaplan and Meier and were tested for survival differences with the logrank test. The level of significance was set at $P < 0.05$. Statistical analyses were performed using the GraphPad Prism software package version 4.0 (GraphPad Software, Inc, San Diego, CA).

Results

Acquired resistance to metronomic topotecan/pazopanib combination therapy in a preclinical model of advanced intraperitoneal ovarian cancer metastasis

Previous studies by us and independently by Merritt et al. demonstrated the potent preclinical efficacy of concomitant combination of metronomic topotecan chemotherapy plus pazopanib for the treatment of advanced ovarian cancer metastasis [13, 14]. Using the SKOV-3-13 cell line, this treatment combination resulted in marked inhibition of metastatic disease as assessed by whole body bioluminescence imaging leading to 100 % survival of mice after 6 months of continuous therapy [13]. In order to determine whether mice had been cured as opposed to the presence of occult (dormant) tumor cells that might lead to relapse of disease we repeated this treatment experiment and treated mice as above with topotecan/pazopanib continuously for 13 weeks, and then stopped. At the end of this period, metastatic disease appeared stably negligible, as indicated by unchanged values in luciferase activity. In vivo bioluminescence was then used to monitor mice in the subsequent therapy free-period. Approximately 6 weeks after cessation of treatment we noted that luciferase activity began to increase, indicating that occult tumors were ‘relapsing’. Treatment with combination topotecan/pazopanib therapy was re-initiated at this point and continued for an additional 10.5 weeks. However, unlike the initial potent inhibitory effect caused by the topotecan/pazopanib combination treatment, this treatment now failed to inhibit tumor growth and progression. Mice eventually succumbed to disease (either as extensive ascites

or multiple solid tumors throughout the peritoneal cavity). From these mice, two new ovarian cell line variants were generated: 7SD from a solid tumor and 7AS from ascites. The overall treatment scheme and isolation of the variant sublines is shown in Fig. 1.

We next asked whether the inability to control disease during the second round of treatment could be attributed to the emergence of resistance to the combination of topotecan and pazopanib. To this end, we re-implanted cells from the new ovarian cell subline variants (7SD and 7AS) as well as the parental SKOV-3-13 intraperitoneally. Metronomic topotecan/pazopanib treatment was initiated using the same protocol previously used which caused potent anti-tumor efficacy [13]. Mice were monitored regularly to assess progression of disease, and sacrificed when signs of distress were apparent (i.e. lethargy, scruffiness, distended abdomen). The results showed that mice implanted with parental SKOV-3-13 cells continued to respond to therapy and that progression of disease was successfully controlled (100 % survival, even after 155 days of continuous treatment) (Fig. 2). Conversely, mice implanted with either of the newly isolated variants (7SD and 7AS) began to rapidly succumb to metastatic disease even under therapy as shown in Fig. 2. Progression of disease was manifested as either metastatic tumors in the omentum and diaphragm or as excessive ascites. As a result, mice implanted with either of these cell lines showed a much shorter median survival when compared to SKOV-3-13 (69 days for 7AS, 59 days for 7SD and undefined (100 % survival) for SKOV-3-13; Kaplan–Meier $P < 0.05$ for 7AS and 7SD relative to parental cell line-SKOV-3-13). These results clearly show that the variants had acquired resistance to the topotecan/pazopanib therapy. Of these two variants, 7SD appeared to be more aggressive of the two as all mice succumbed to metastatic disease by day 59 whereas 40 % of the 7AS mice were alive at day 155 (vs. 100 % of the control treated mice).

Characterization of drug treatment resistant variants

To further examine the nature of the apparent resistant phenotype, we conducted in vitro survival assays with the various cell lines. Cells were treated with either topotecan or pazopanib for 144 h and MTS assay used to assess the levels of cell viability under various drug concentrations. The activity of pazopanib on proliferating SKOV-3-13, 7AS and 7SD cell lines was similar (IC_{50}, 0.74 μM; Fig. 3a). In contrast, the 7AS and 7SD variants showed a consistently higher survival when exposed to various concentrations of topotecan relative to the parental SKOV-3-13 cell line (Fig. 3b). Similar results on topotecan were obtained when trypan blue dye exclusion was used to assess cell viability. In vitro topotecan inhibited the cell proliferation of ovarian cancer cell lines in a concentration-dependent manner (Fig. 3c–e); the 144-h topotecan exposure inhibited the proliferation of SKOV-3-13 with an IC50 of 5.842 ± 12.9 nM (Fig. 3a), being lower from those observed in 7AS and 7SD cell lines (11.60 ± 0.906 and 9.804 ± 0.53 nM, respectively; Fig. 3d, e). Collectively, these results suggest that the 7AS and 7SD variants have acquired relative resistance to the anti-tumor effects mediated by exposure to topotecan.

We also examined the in vitro resistance of these cell lines to other agents such as doxorubicin, gemcitabine or the active SN38 metabolite of irinotecan. As noted in Table 1, the 7AS and 7SD variants show an almost doubling of the IC_{50} with respect to the

topoisomerase-I inhibitor, SN38. In contrast the IC_{50} values and thus the cytotoxicity mediated by either gemcitabine or doxorubicin were not affected (Supplementary Fig 1).

We next examined whether the more rapid progression of disease in mice associated with these two variants could be attributed to changes in tumor cell proliferation or invasion. MTS proliferation assays indicated that, at least in vitro, all cell lines proliferated equally (data not shown). Similarly, using transwell invasion assays, we found that 7AS and 7SD cell variants have not acquired an enhanced invasive phenotype when compared to the parental SKOV-3-13 cell line (data not shown).

The higher IC_{50} values noted above for the 7SD and 7AS variants (especially in the case of topotecan) are indicative of increased resistance to the cytotoxic effect mediated by this drug. In addition to this direct effect, topotecan, particularly when given in the metronomic low-dose-like setting, has been shown to inhibit HIF-1 alpha protein expression [14]. Given this finding, it would be expected that under hypoxic conditions, the acquisition of resistance to topotecan could conceivably lead to higher levels of expression of HIF-1 alpha and thus enhance survival under these conditions. Indeed, we noted that 7SD cells cultured under hypoxic conditions and in the presence of topotecan plus pazopanib treatment (administered at their respective IC_{50} values) showed elevated viability relative to normoxic conditions than was the case for the SKOV-3-13 parental line (Fig. 4a). To further elucidate the elements that may contribute to this acquired resistance to topotecan, we examined the intracellular accumulation of topotecan in SKOV-3-13, 7AS and 7SD cell lines. Our results show that at extracellular topotecan concentration of 1 μM, intracellular accumulation in 7AS and 7SD is significantly lower, approximately 50 % lower than in the sensitive cell line (Fig. 4b). After a 5-min incubation with topotecan lactone, the mean ± SD intracellular topotecan lactone concentration was significantly higher in SKOV-3-13 cells than in 7AS and 7SD cells (0.024 ± 0.002 versus 0.012 ± 0.002 and 0.0135 ± 0.002 ng/μg protein, respectively; $P < 0.05$).

In order, to confirm that the apparent resistance detected in 7AS and 7SD was stable, we re-implanted subsequent passages of the more aggressive of these two variant cell lines (i.e. 7SD) as well as the parental SKOV-3-13 cell line. In this case, treatment was initiated 1 week earlier than in previous experiments in order to determine whether resistance to therapy could be circumvented by initiating therapy at an earlier time point. As described previously, the topotecan/pazopanib therapy combination proved highly effective against established SKOV-3-13 metastatic disease. Topotecan/pazopanib treatment (as noted before) resulted in a significant improvement in median survival relative to vehicle control ($P < 0.05$), but despite the earlier initiation of treatment, the therapy was still ineffective against the 7SD variant relative to the results achieved in the parental cell line (median survival of 76 days for 7SD and undefined median survival even at 250 days in parental, Fig. 5, $P < 0.05$). These results suggest that the resistance acquired by this variant cell line is stable and heritable.

Attempts to overcome acquired resistance that develops to the metronomic topotecan/ pazopanib treatment

Emmenegger et al. [15] previously showed that acquired resistance to metronomic cyclophosphamide (CTX) therapy in a prostate carcinoma model where the tumor is highly sensitive initially to the therapy and then relapses after about 2 months of therapy, could be overcome by switching to an maximum tolerated dose (MTD) CTX regimen. Thus, we examined whether we could achieve a similar effect by substituting metronomic topotecan therapy with an MTD topotecan protocol. To this end, we repeated our experiment, in this case using the more aggressive of the two resistant cell lines (7SD). Mice were then treated with metronomic topotecan plus pazopanib or MTD topotecan plus pazopanib. While the regimen incorporating MTD topotecan mediated a statistically significant improvement in survival ($P < 0.05$), this was only a few days longer than that achieved in mice treated with vehicle (34 vs. 29 days, respectively). In fact, a better therapeutic impact was noted with metronomic topotecan plus pazopanib (median survival of 43 days, $P < 0.05$) (Fig. 6a).

We next examined whether resistance could be overcome by switching to another topoisomerase I inhibitor, in this case irinotecan (CPT11). As above, we employed both metronomic or MTD regimens of this topoisomerase inhibitor in combination with pazopanib. The results showed that both regimens induced a positive effect in promoting median survival (statistically significant relative to vehicle control ($P < 0.05$) but this did not reach the degree of efficacy that we had previously noted for metronomic topotecan plus pazopanib or indeed the efficacy of metronomic CPT-11 (35 vs. 91 days for control and metronomic CPT-11 respectively) that had been previously noted for the chemosensitive parental SKOV-3-13 cell line [13] (Fig. 6b). The lack of in vivo efficacy associated with CPT-11 ‘salvage’ therapy is in agreement with the apparent acquired resistance to SN38 noted by our in vitro assays (Table 1) detected in both the 7AS and 7SD variant sublines.

We then examined whether resistance could be overcome by changing the anti-angiogenic drug component of the combination. Thus, we replaced pazopanib with either sunitinib or the DC101 anti-mouse VEGFR-2 antibody (Fig. 6c). Sunitinib is similar but not identical to pazopanib in terms of its receptor tyrosine kinase target profile. In both cases, we maintained the metronomic topotecan backbone. Both topotecan/sunitinib and topotecan/ DC101 showed a beneficial effect in prolonging survival but as before these improvements in median survival were minimal (respectively 7 and 5 days longer than mice in the control group, $P < 0.05$). Furthermore the effect achieved with these combinations was comparable to that of topotecan/ pazopanib. Thus the incorporation of different antiangiogenic agents failed to overcome the resistance noted for these variants nor bring about the same level of efficacy previously noted when treating the parental cell line [13].

Since both 7SD and 7AS appear to remain sensitive to gemcitabine and doxorubicin (Table 1), it is possible that in vivo therapy with these agents might provide an alternative second line treatment for these resistant variants. Previous efforts have shown that treatment with low dose metronomic gemcitabine (58 mg/kg, twice weekly) mediated a significant improvement in median survival in mice implanted with the parental cell line SKOV-3-13 (Francia, Hashimoto & Kerbel, unpublished observation). We repeated this experiment

using a similar dose for the treatment of mice implanted with either SKOV-3-13 or 7SD. As before, gemcitabine treatment mediated an improved survival in the SKOV-3-13 group (median survival of 43 and 57 days for control and treatment group respectively; $P < 0.05$). Similarly, this therapeutic regimen also proved effective against the 7SD variant (Fig. 7) (median survival 47 and 72 days for control and treatment groups respectively; $P < 0.05$). It should be noted, however, that when a lower dose of 28 mg/kg was used, mice implanted with 7SD cell line failed to show any improvement in median survival when compared to control (data not shown). This lower dose regimen did show improved survival in mice implanted with the parental SKOV-3-13 cell line relative to their respective control counterparts. In the case of doxorubicin therapy, treatment of group 7SD showed a minimal improvement of survival which did not reach statistical significance (median survival 47 and 51 days for control and treatment groups respectively; $P > 0.05$). Doxorubicin treatment in SKOV-3-13 similarly showed a minimal improvement in survival (median survival of 43 and 49 days for control and treatment groups), however, in this case this difference reached statistical significance ($P < 0.05$).

Gene expression profile analysis of therapy resistant variants

As noted above, the acquired resistance expressed by the drug resistant 7AS and 7SD cell lines is stable and heritable. This suggests that this phenotype might be mediated by fixed alterations in gene expression in these cell lines. In order to discern the nature of the alterations that may facilitate resistance to metronomic topotecan/pazopanib therapy, we conducted expression profile analysis. The gene expression profiles of cell lines were assessed on the Agilent 4X44K Whole Human Genome Oligo Microarray. Both 7AS and 7SD cell lines were compared to their parental SKOV-3-13 cell line. Each comparison was conducted using two different passage numbers, incorporating a dye-swap. Gene expression >1.5-fold was considered to be upregulated, whereas expression <0.67-fold was considered as downregulated. We were particularly interested in examining whether acquired resistance could be associated with alterations in tumor cell survival (resistance to apoptosis), proliferation, or upregulation in drug efflux pumps (detoxification mechanisms). The results showed significant upregulation as well as downregulation of a number of genes in both 7AS and 7SD cell lines. We decided to initially focus on the group of genes that are commonly altered in both cell lines relative to SKOV-3-13. In this case, we found that 445 genes were commonly upregulated in 7AS and 7SD and whereas 185 were commonly downregulated (Fig. 8, Supplementary Table 1). Based on their potential biological significance in the process of chemotherapy drug resistance, a number of potentially relevant genes were chosen for further confirmation of upregulation using QRT-PCR (Fig. 9). These include: crystallin, alpha B (CRYAB), heat shock 27 kDa protein 2 (HSPB2), transketolase-like 1 (TKTL1) and cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1). All of these genes showed significant upregulation in multiple passages of these cell lines thus indicating stable changes in expression in the resistant variants. Moreover, there is evidence which suggests a potential role for these molecules in acquired resistance. We therefore made use of prognoscan [21]. (http://gibk21.bio.kyutech.ac.jp/(PrognoScan/index.html) to determine whether the upregulation of these particular genes could be associated with poorer survival outcomes. This analysis showed that in a number of studies involving ovarian cancer patients, elevated expression of these genes (CRYAB, HSPB2, TKTL1 and

CYP1B1) is associated with shorter median survival compared to patients with low expression (Table 2). Additional studies have shown that elevated levels of expression of CYP1B1 are associated with a significantly shorter median survival compared to ovarian cancer patients whose tumors are negative or express low levels of this protein [22]. Collectively, these results suggest a potential role for these molecules in progression of ovarian cancer; however, whether any of them are also involved in mediating or contributing to acquired resistance to topotecan/pazopanib chemotherapy is unknown and remains to be determined.

Discussion

The anti-tumor mechanisms implicated for the anti-tumor effects of metronomic chemotherapy are diverse and include a number that do not involve direct tumor cell killing, particularly inhibition of angiogenesis and stimulation of the immune system [1]. Intriguingly, a few studies have also implicated direct tumor cell effects that involve targeting the putative cancer stem cell subpopulation [6, 20]. Hence, it may be anticipated that when resistance develops to a given chemotherapy drug administered in a conventional MTD manner, that switching to a metronomic dose and schedule of the same agent will cause an antitumor effect [7]. Conversely, when resistance develops to a given metronomic chemotherapy regimen, switching to a MTD regimen of the same drug may reverse the resistant phenotype [15]. Clearly these possibilities have potential clinical implications, especially now that metronomic chemotherapy has reached the stage of advanced phase III clinical trial development (www.clinicaltrials.gov) based in part on some encouraging phase II clinical trial results [23–25]. Indeed, one such phase III trial, called CAIRO3, was recently completed and the results were positive. It involved prolonged daily oral low-dose capecitabine, plus bevacizumab, until disease progression as a maintenance therapy (vs observation only) in first line metastatic colorectal cancer patients, after they received induction therapy with higher individual dose capecitabine plus oxaliplatin with bevacizumab. Benefits in progression free survival and adjusted overall survival were reported in the experimental arm [26].

Our results show, for the first time, an instance in which resistance that develops to metronomic chemotherapy regimen also seems to result in cross-resistance to MTD chemotherapy of the same agent. Moreover, the results indicate that the resistance is expressed at the level of the tumor cell population, and to the topotecan component of the drug combination, thus highlighting a non-angiogenic mechanism, at least in part, for the anti-tumor effects of this particular metronomic chemotherapy regimen. Nevertheless, the addition of pazopanib to metronomic topotecan was previously found to remarkably enhance the overall anti-tumor efficacy of metronomic topotecan, despite the fact that pazopanib monotherapy was inactive in either prolonging survival or causing anti-tumor responses based on imaging in our advanced ovarian cancer model [13]. These observations are of particular interest given the failures of numerous phase III clinical trials in many different indications of advanced stage disease in which an oral antiangiogenic TKI was combined with a standard-ofcare MTD chemotherapy [27–34]. For example three phase III trials of concurrent sunitinib plus chemotherapy (paclitaxel, docetaxel, or capecitabine) failed in metastatic breast cancer patients [25–34]. Given our results, and those of others [35] we

suggest that combination with metronomic chemotherapy regimens may be a potential strategy to improve the clinical impact of TKI drugs such as sunitinib when combined with chemotherapy.

The basis of how pazopanib enhances the efficacy of metronomic topotecan—which has also been noted by Merritt et al. [35] in ovarian cancer and by Kumar et al. [36] in several pediatric cancer xenograft models but not in a colorectal cancer xenograft model [37] is unknown, and currently under investigation, as is the basis of acquired resistance to metronomic topotecan. Within the context of the model of ovarian cancer used in our studies, it has been previously noted that the intraperitoneal cavity in mice is hypoxic [38]. As such, progression of disease in this environment may be strongly influenced by the expression or inhibition of HIF-1 alpha and/or HIF-2α. In the case of the 7SD and 7AS variants, the effect of topotecan resistance should facilitate disease progression as manifested by the shorter median survival associated with these variant cell lines, even when under topotecan/pazopanib therapy.

A potential caveat and limitation of our model, and thus the results, concerns the decision to terminate the highly successful metronomic oral topotecan plus pazopanib therapy after 3 months of continuous daily therapy when there was no evidence of disease progression/ relapse or excessive toxicity. Specifically, would continued, indefinite therapy have prevented the onset of resistance? Another concern is the level of topotecan resistance noted in the 7AS and 7AD variants—around twofold. Several considerations are important to note with respect to these concerns. First, the therapy was maintained for about 3 months continuously in mice—about 1/8 of the life span of mice— or about a 10 years equivalent in humans. Ten years of daily therapy in humans with a chemotherapy drug such as a DNA damaging agent or topoisomerase poison is unheard of. The maximum we are aware of is 2 years using minimally toxic metronomic-like 5-FU oral pro-drugs such as UFT [39]. Second there is also the added concern that indefinite therapy using chemotherapy agents such as topotecan could have carcinogenic effects in humans. Third, while it is true that minimally toxic treatments such as metronomic chemotherapy are usually administered until disease progression in clinical trials [40, 41] there are exceptions to this, e.g. where the metronomic chemotherapy (plus an aromatase inhibitor) was given for 6 months. We would also point out that these aforementioned metronomic chemotherapy clinical trials involved concurrent therapy with relatively non-toxic targeted agents such as letrozole or bevacizumab. In contrast, our preclinical study involved an oral TKI, pazopanib. Clinically, such TKI drugs can be quite toxic resulting in patients electing to stop therapy, or necessitating drug holidays or dose reductions. In short, ‘indefinite’ therapy involving a drug such as (metronomic) topotecan in combination with another drug such as pazopanib would likely be problematic in the clinic—in contrast to mice, which can usually much better tolerate therapy because of lesser toxicity.

Regarding the fold differences in resistance to topotecan between the 7AS and 7AD variants and the parental cell line, 1.68- to 1.98-fold resistance may seem small but it is important to note that such modest fold differences in sensitivity/resistance may be highly relevant in vivo, especially *in the clinic*. By way of example, a small cell cancer clinical study by H.H.

Berendsen et al. [42] showed levels of resistance to chemotherapy in the same range—about twofold.

Finally, with respect to these overall issues we would add that if resistance and disease progression were likely precipitated by terminating the therapy when we did, it bolsters the contention that therapy in patients should indeed be maintained until evidence of disease progression, as long as the therapy is safe and tolerable. This is also relevant to the issue of what exactly constitutes “drug resistance”, whether it is stable or reversible, and the concept of “drug rechallenge” of ‘resistant’ disease [43].

In summary, we have noted that despite the excellent efficacy achieved with the combination of metronomic topotecan plus pazopanib in a preclinical model of advanced ovarian cancer, resistance to this regimen can evolve when administered over what are prolonged periods of treatment for mice, especially if therapy is prematurely terminated. This acquired resistance could not be overcome by switching to an MTD regimen of either topotecan or a related topoisomerase inhibitor-irinotecan, or by switching the antiangiogenic component of the combination to either sunitinib or DC101 antibody. However, it appears that this resistance does not necessarily extend to other chemotherapeutic agents such as gemcitabine. We also note that cells that have acquired resistance to topotecan/ pazopanib show significant alterations in gene expression. Some of these alterations have been implicated as potential mediators of chemoresistance. In the case of CYP1B1, for instance, its activity has been previously implicated in selective drug inactivation and resistance [44]. By the same token, CRYAB has been suggested to play a role with respect to cross-resistance to DNA damaging drugs such as etoposide and cisplatin [45], HSPB2 appears to play an antiapoptotic role by inhibiting caspase activation [46] and TKTL1 is strongly expressed in aggressive tumor cells, which are resistant to chemotherapeutics [47]. It remains to be determined whether any of these molecules mediate or contribute to resistance to metronomic oral topotecan.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants to RSK from the Canadian Institutes of Health Research (CIHR) and the National Institutes of Health (NIH), USA (CA-41233) and the Canadian Breast Cancer Foundation (CBCF). RSK is a Canada Research Chair in Tumor Biology, Angiogenesis, and Antiangiogenic Therapy. M.L. Jaramillo, C. Collins, M. Banville and M.D. O’Connor- McCourt were supported by the NRC Genome Health Initiative Cancer Program (NRC publication number NRC5322).

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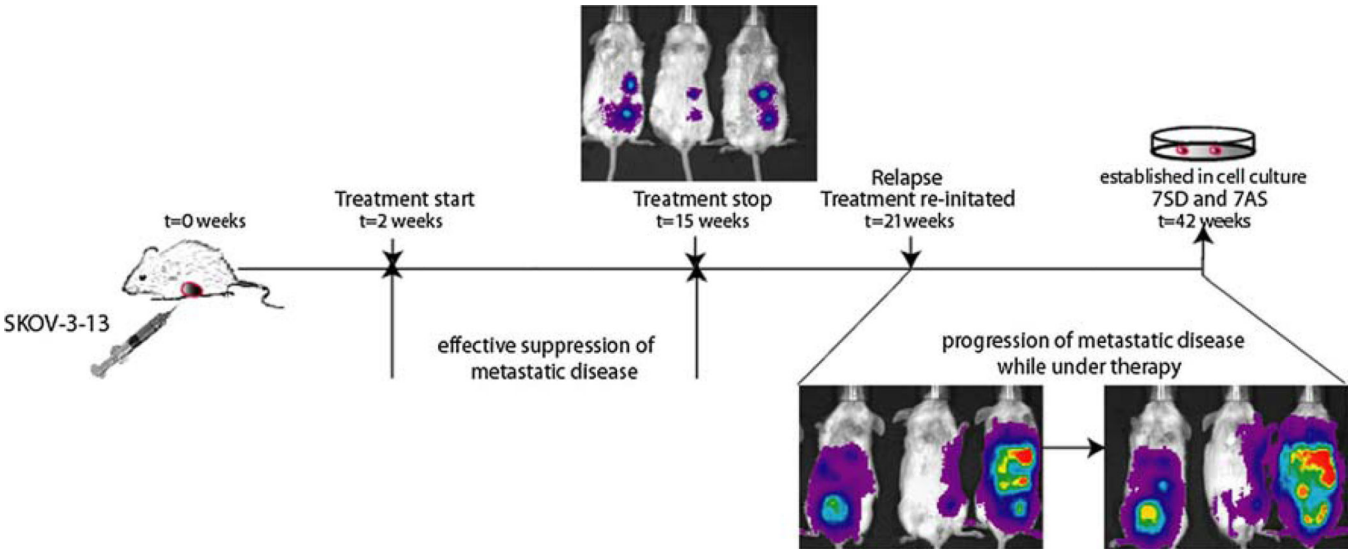

Fig. 1.

Schematic representation of protocol used to derive topotecan/pazopanib resistant ovarian cancer variants 7SD and 7AS using the SKOV-3-13 cell line as a starting point. Mice were treated with metronomic topotecan plus pazopanib for 13 weeks. Six weeks after cessation of treatment, tumor growth began to recur. At this time, treatment was re-initiated but mice failed to respond to the therapy

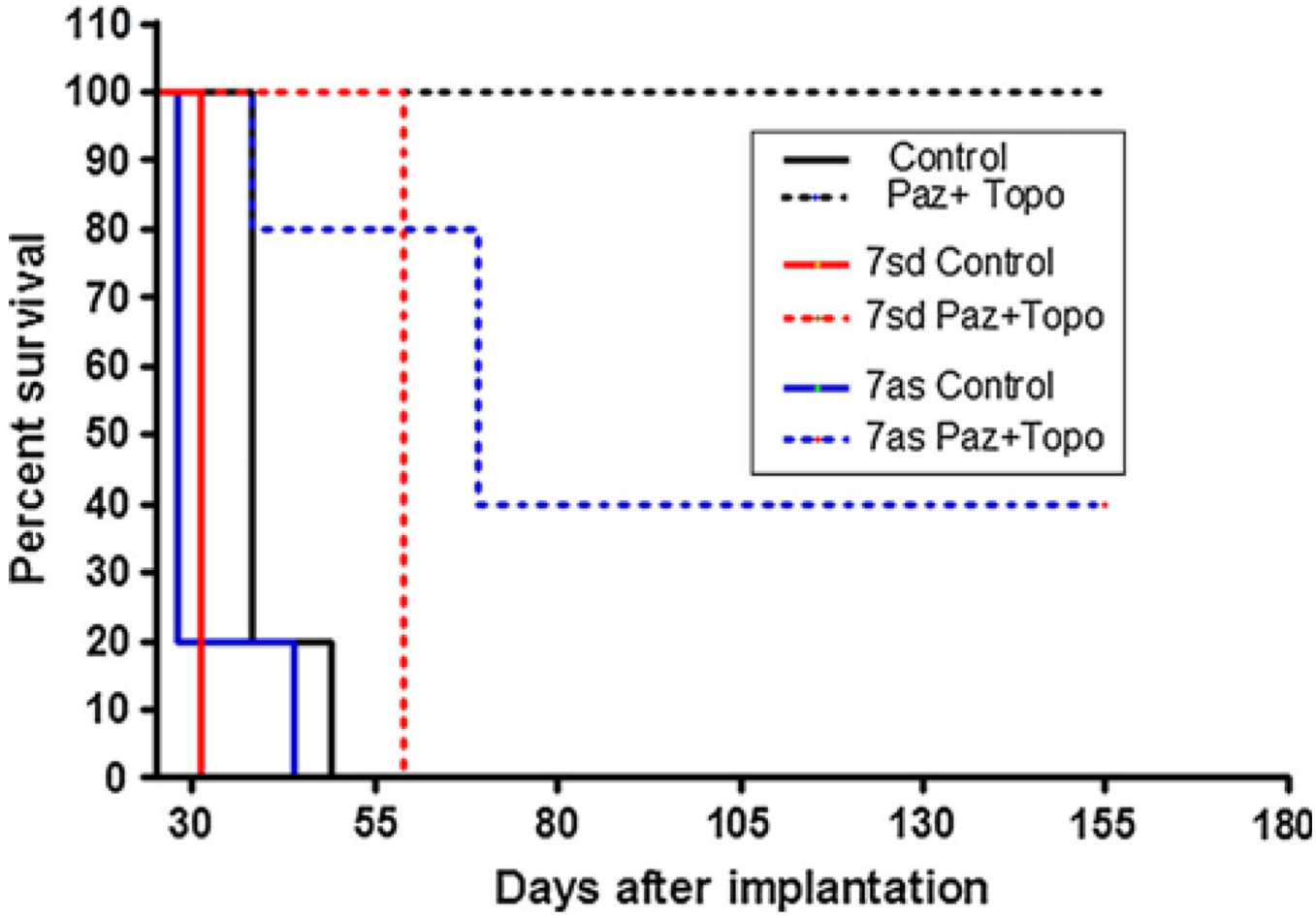

Fig. 2.

As previously reported [13], a combination of metronomic topotecan and pazopanib inhibited progression of metastatic disease in mice implanted with the SKOV-3-13 cell line. However, mice implanted with variants −7SD or −7AS and treated with the same combination chemotherapy, showed a shorter median survival (n = 5 mice for all groups, Kaplan–Meier $P < 0.05$ taken as statistical indication of difference between parental and 7AS and 7SD)

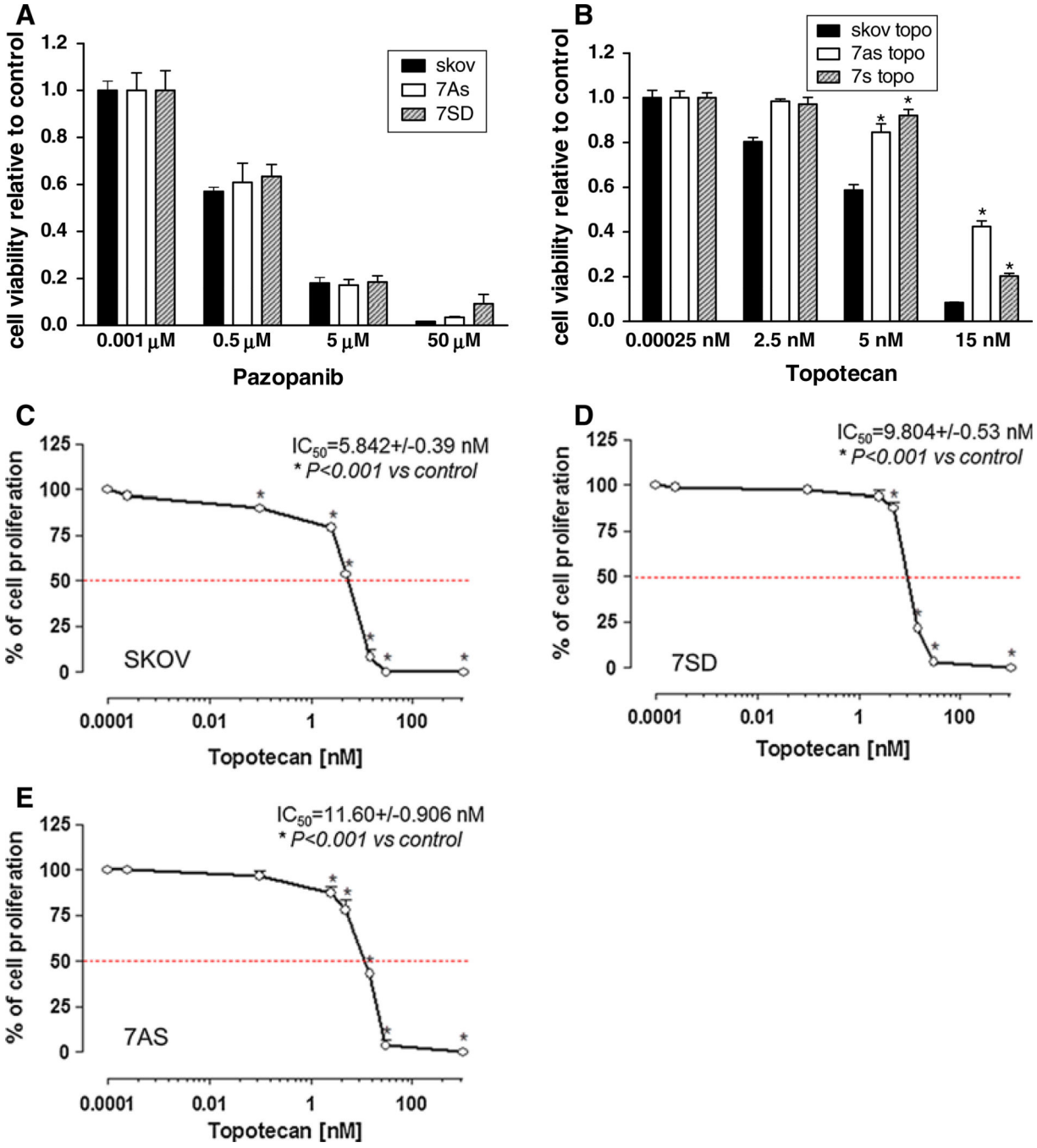

Fig. 3.

Effect of topotecan (**a**) and Pazopanib (**b**), on in vitro cell proliferation by MTS assay. The anti-proliferative effects of the drugs were studied using daily exposures (144 h) on SKOV-3-13 (**c**), 7SD (**d**) and 7AS (**e**) cell lines by trypan blue dye exclusion. Both7AS and 7SD showed significantly higher cell viability in the presence of topotecan than was the case for their parental cell line SKOV-3-13. Symbols and bars indicate mean values ± SD, respectively. **P* < 0.05 and ***P* < .01 versus vehicle-treated controls. IC_{50} indicates the concentration of drug that reduced cell proliferation by 50 %

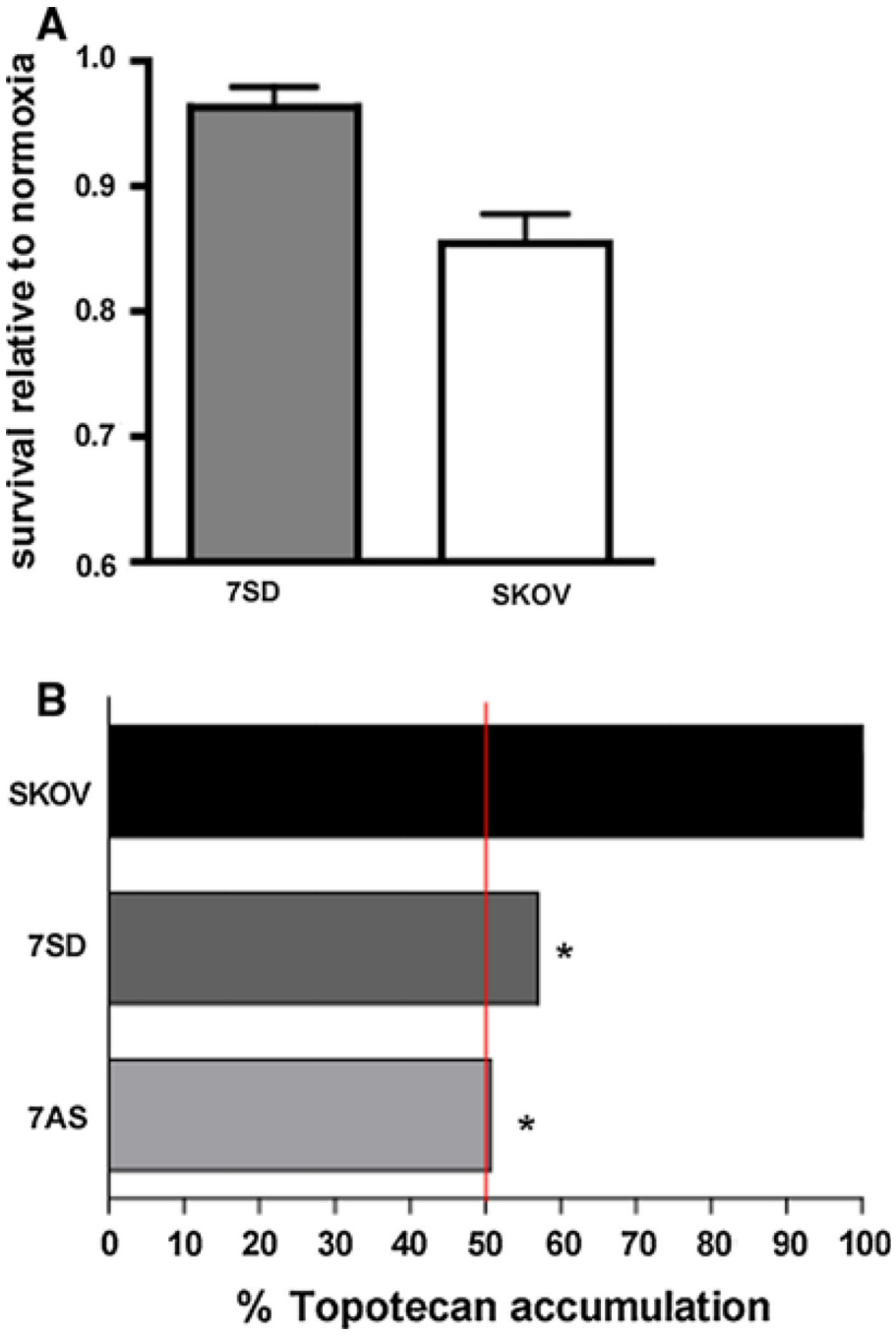

Fig. 4.

a SKOV-3-13 and 7SD cells were cultured in vitro for 144 h in the presence of a combination of topotecan/pazopanib and under either normoxic or hypoxic (1 % O_2) conditions. Cell viability was examined by means of MTS assay. Values for cell viability in hypoxic conditions are expressed relative to their respective values in normoxic conditions. The parental cell line SKOV-3-13 showed poorer cell viability under hypoxia and topotecan/pazopanib treatment than the resistant variant 7SD. **b** Accumulation of topotecan in

SKOV-3-13, 7AS and 7SD cell lines after exposure to 1 μM topotecan. Columns and bars indicate mean values ± SD, respectively. *$P < .001$ versus SKOV cell line

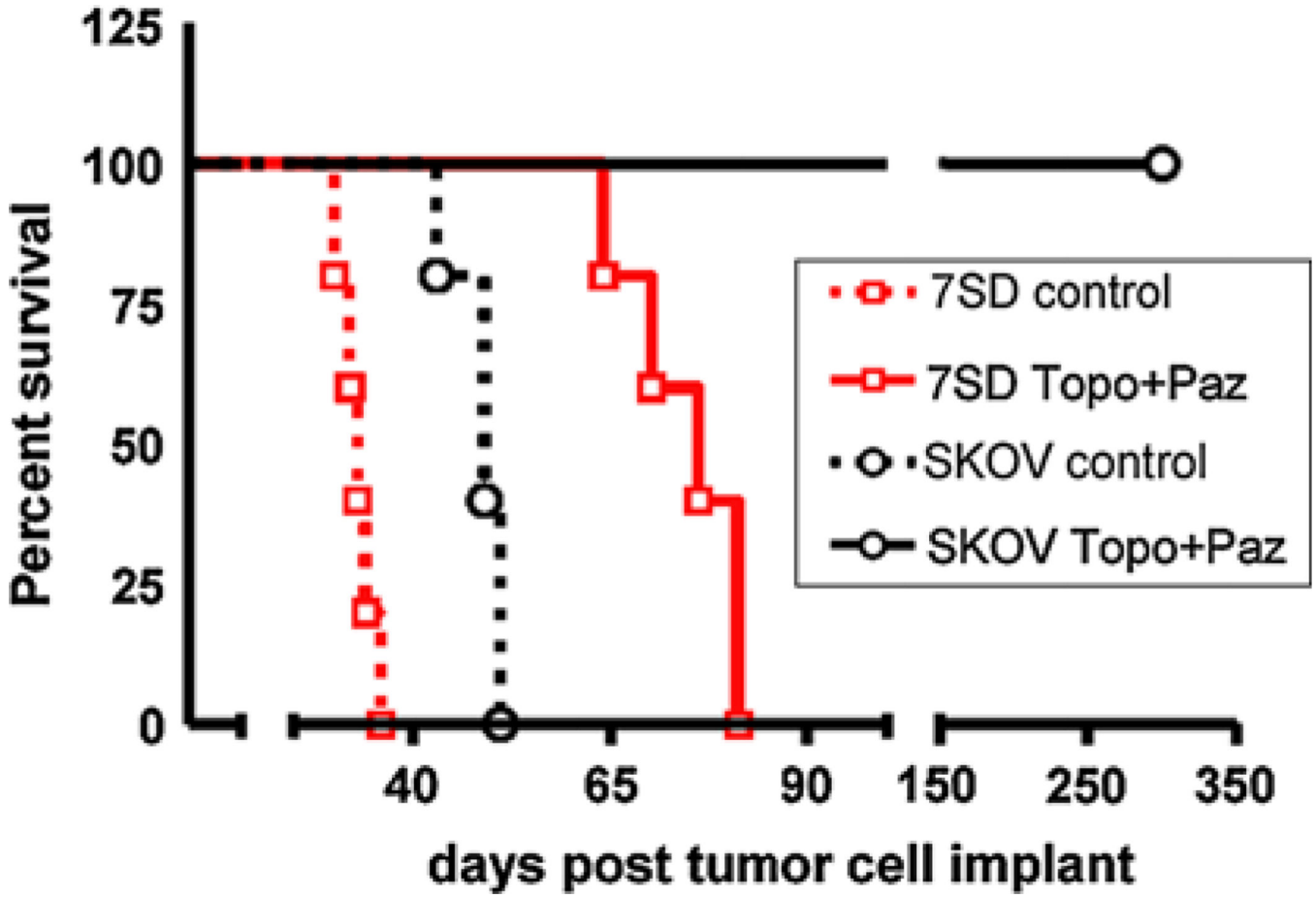

Fig. 5.

Subsequent passages of variant 7SD and parental SKOV-3-13 were re-implanted as described above in SCID mice and treated with the combination of metronomic topotecan and pazopanib. As before, mice implanted with SKOV-3-13 cell line continued to show significant response to the combination resulting in complete survival (even 350 days post-tumor cell implantation) and those implanted with resistant variant 7SD showed poorer response ($P < 0.05$, n = 5 mice for all groups)

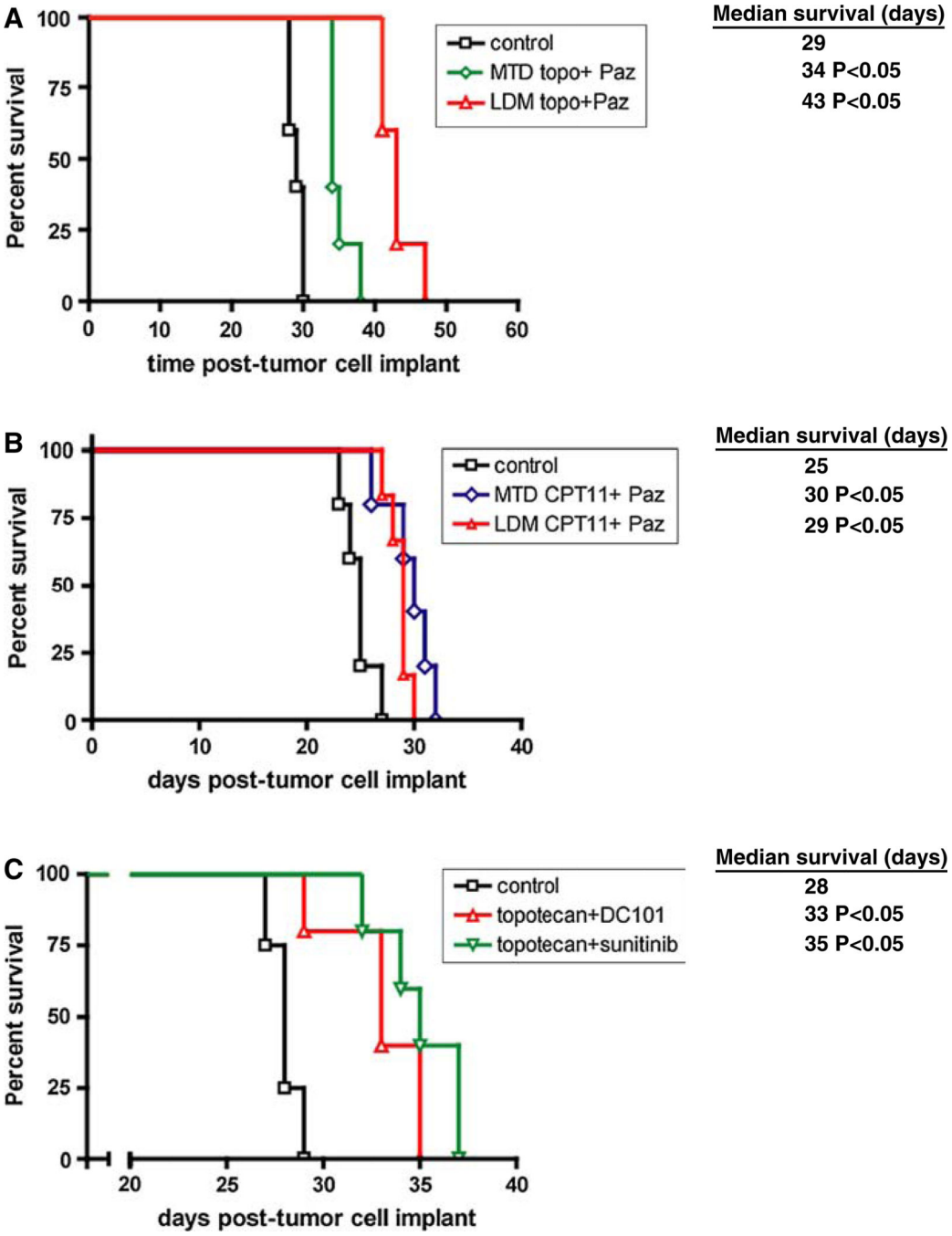

Fig. 6.

Examination of the effect of chemotherapeutic switching on survival of mice implanted with the resistant variant 7SD. **a** The use of MTD topotecan regimen (while maintaining pazopanib) translated into minimal improvement in survival in mice implanted with 7SD variant cell line. **b** Switching to CPT11 either in MTD or metronomic regimen plus pazopanib showed minimal efficacy in the survival. **c** Switching of the antiangiogenic component of the combination therapy to either DC101 or sunitinib in combination with

metronomic topotecan failed to show the significant effect in survival previously noted in the SKOV-3-13 chemo-sensitive model. (n = 5 mice for all groups $P < 0.05$)

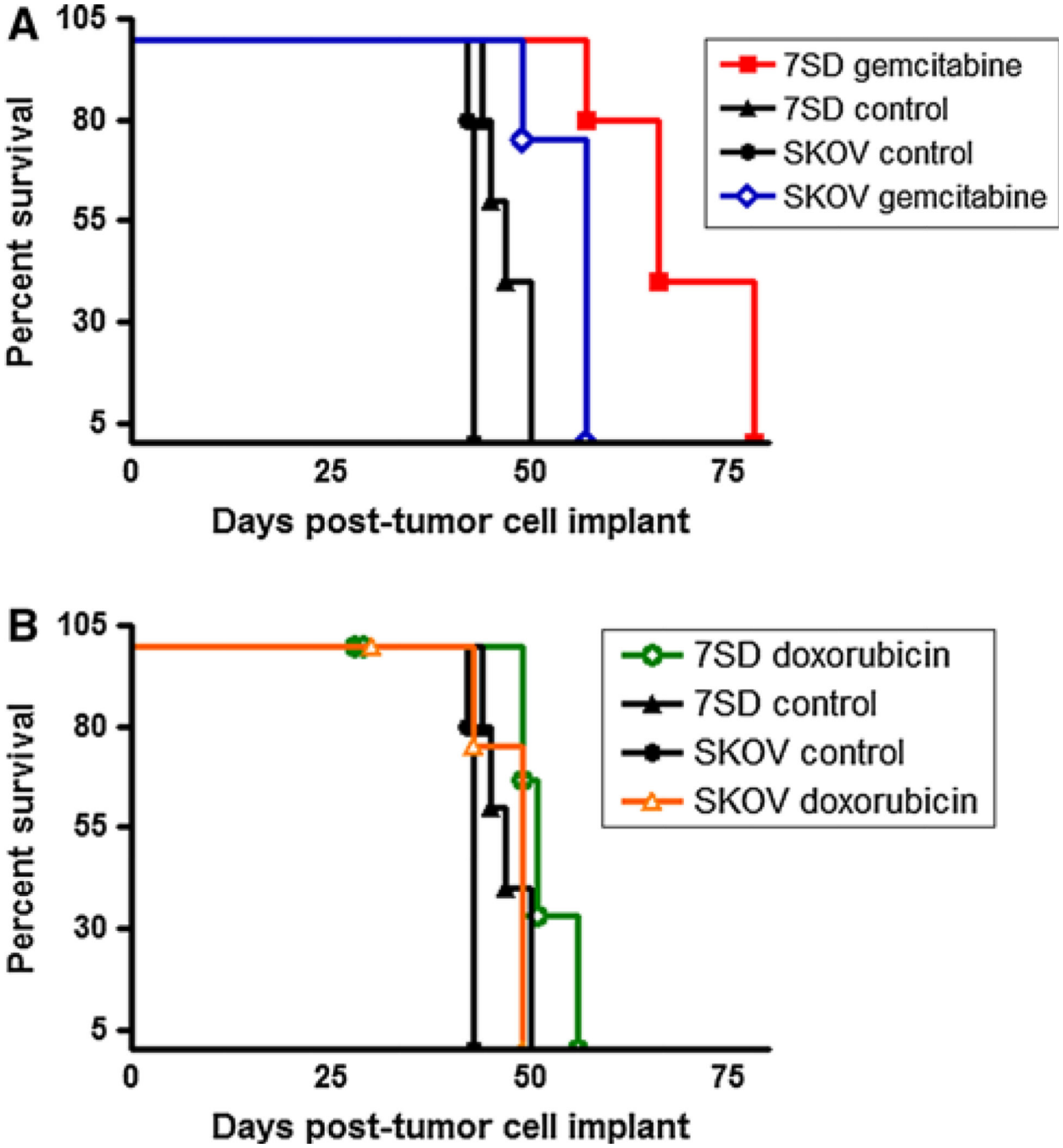

Fig. 7.

Examination of the effect of chemo-switching. **a** Gemcitabine treatment (58 mg/kg, 2× per week, ip) showed significant improvement in survival in mice implanted with either SKOV-3-13 or 7SD ($P < 0.05$; n = 5 for control and gemcitabine treatment). **b** Doxorubicin treatment (2 mg/kg, day 1 and 5; 21 day cycles) mediated a minimal but statistically significant improvement in survival in mice implanted with either SKOV-3-13 ($P < 0.05$; n = 5 for control and doxorubicin treatment). A minimal improvement in survival was noted in

mice implanted with 7SD and treated with doxorubicin (51 and 47 days for treatment and control groups), however, this did not reach statistical significance

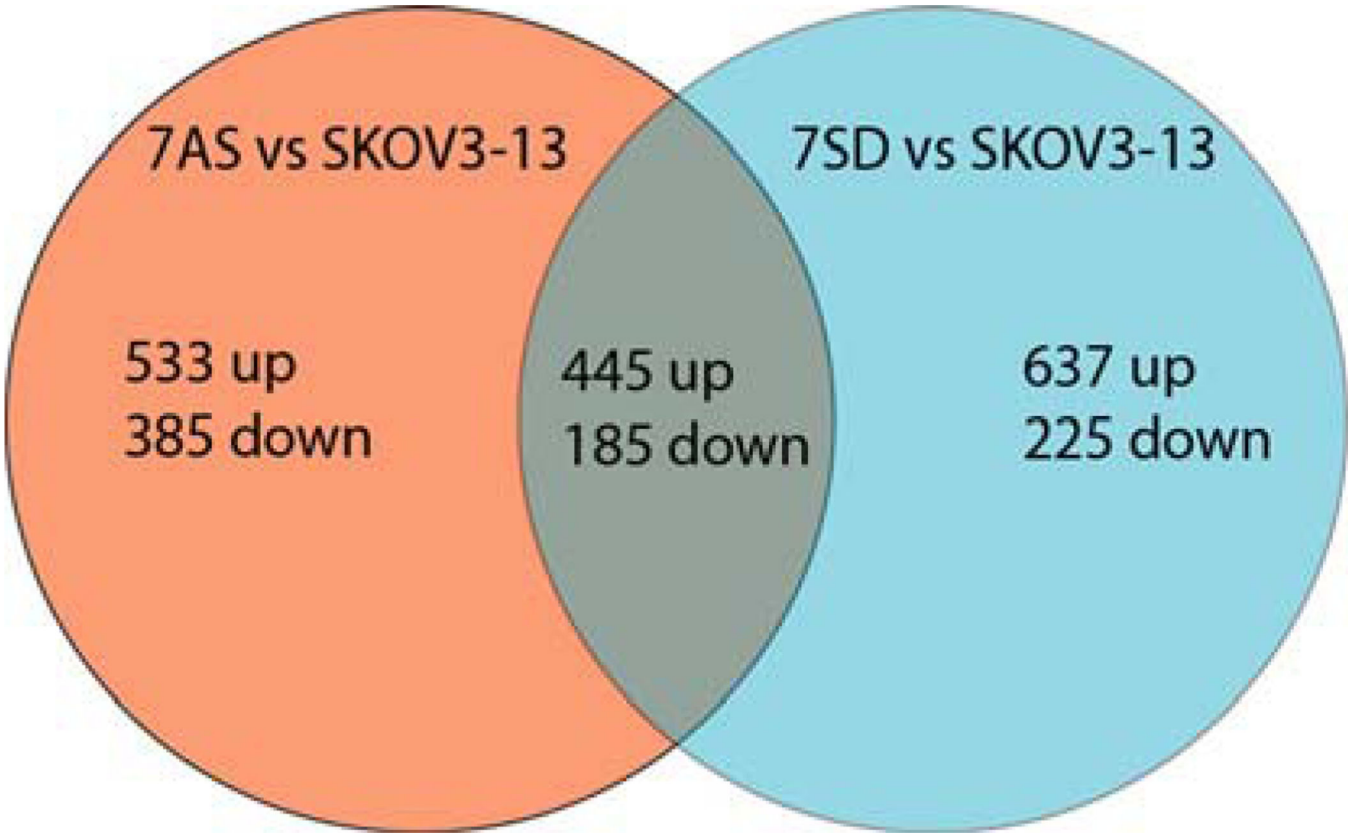

Fig. 8.

Venn diagram outlining the extent of gene expression similarities in the microarray analysis of topotecan/pazopanib resistant variants 7SD and 7AS versus the parental SKOV-3-13 cell line

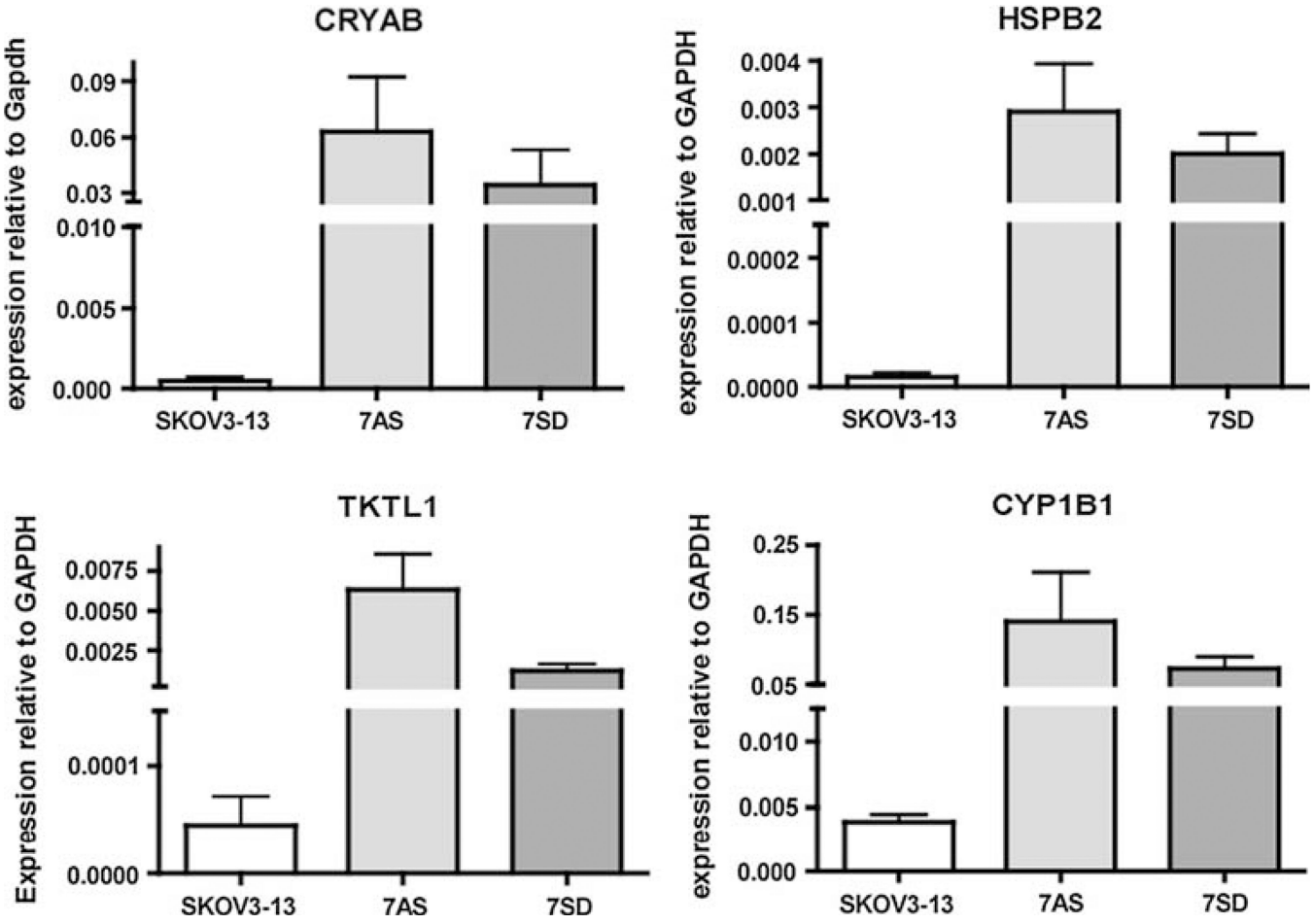

Fig. 9.

Q-RTPCR confirmation of upregulation of *CRYAB, HSPB2, TKTL1* and *CYP1B1* in the topotecan/pazopanib resistant variants 7SD and 7AS

Table 1

Molar concentration associated with IC_{50} for SN38 (CPT-11), doxorubicin and gemcitabine in the chemoresistant variants and parental cell lines

	SKOV	7SD	7AS
Doxorubicin	2.58×10^{-8}	2.45×10^{-8}	2.64×10^{-8}
Gemcitabine	1.67×10^{-9}	1.49×10^{-9}	1.11×10^{-9}
CPT11	1.43×10^{-9}	2.22×10^{-9}	2.73×10^{-9}

Table 2

Microarray studies in which high levels of expression of CRYAB, TKTL1, HSPB2 or CYP1B1 is significantly associated with poorer outcome in ovarian cancer patients

Gene	Dataset	Cancer type	Endpoint	Cohort	Contributor	Array type	Probe ID	N	Cutpoint	Minimum value	Corrected value
TKTL1	GSE14764	Ovarian cancer	Overall survival	TOC	Denkert	HG-U133A	216370_s_at	80	0.56	0.000197	0.006343
HSPB2	GSE26712	Ovarian cancer	Disease free survival	MSKCC (1990–2003)	Bonome	HG-U133_Plus_2	205824_at	185	0.80	0.001782	0.030984
HSPB2	DUKE-OC	Ovarian cancer	Overall survival	Duke	Bild	HG-U133A	205824_at	133	0.71	0	0
HSPB2	GSE9891	Ovarian cancer	Overall survival	AOCS, RBH, WH, NKI-AVL (1992–2006)	Tothill	HG-U133_Plus_2	205824_at	278	0.73	0.001263	0.041191
CRYAB	GSE9891	Ovarian cancer	Overall survival	AOCS, RBH, WH, NKI-AVL (1992–2006)	Tothill	HG-U133_Plus_2	209283_at	278	0.65	0.000346	0.01033
CRYAB	DUKE-OC	Ovarian cancer	Overall survival	Duke	Bild	HG-U133A	209283_at	133	0.76	0.000052	0.001972
CRYAB	GSE8841	Ovarian cancer	Overall survival	Milan (1992–2003)	Marchini	G4100A	5559	81	0.85	0.000088	0.003139
CRYAB	GSE8841	Ovarian cancer	Overall survival	Milan (1992–2003)	Marchini	G4100A	1818	81	0.78	0.000222	0.007027
CRYAB	GSE17260	Ovarian cancer	Progression free survival	Niigata (1997–2008)	Yoshihara	G4112A	A_24_P206776	110	0.89	0.000642	0.017534
CYP1B1	DUKE-OC	Ovarian cancer	Overall survival	Duke	Bild	HG-U133A	202436_s_at	133	0.86	0.001277	0.031267
CYP1B1	DUKE-OC	Ovarian cancer	Overall survival	Duke	Bild	HG-U133A	202437_s_at	133	0.83	0.001997	0.045241