

# Cribrostatin 6 induces death in cancer cells through a reactive oxygen species (ROS)-mediated mechanism

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Received: 23 October 2009 / Accepted: 13 January 2010 / Published online: 20 February 2010  
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**Summary** Cribrostatin 6 is a quinone-containing natural product that induces the death of cancer cell lines in culture, and its mechanism of action and scope of activity are unknown. Here we show that cribrostatin 6 has broad anticancer activity, potentially inducing apoptotic cell death that is not preceded by any defined cell cycle arrest. Consistent with this data, we find that cribrostatin 6 treated cells have large amounts of reactive oxygen species (ROS) and, based on transcript profiling experiments and other data, this ROS generation is likely the primary mechanism by which cribrostatin 6 induces apoptosis. Given the success of certain ROS producers as anticancer agents, cribrostatin 6 has potential as a novel chemotherapeutic agent.

**Keywords** Cribrostatin 6 · ROS · Quiescent 3T3 · Quinone · HMOX1 · Apoptosis · Cancer stem cells · Transcript profile · Quiescent cells · Drug-resistant cell lines

## Introduction

Certain quinone-containing small molecules have utility as anticancer agents, as demonstrated by FDA-approved drugs such as mitoxantrone, mitomycin C (MMC), and doxorubicin (Fig. 1a). Most investigations into the mode of action for anticancer quinones focus on some combination of DNA intercalation, topoisomerase inhibition, DNA alkylation, and reduction/oxidation-cycling properties. Indeed, several quinones appear to induce death via topoisomerase inhibition [1], others through covalent alkylation of DNA (sometimes preceded by bioreductive activation) [2–4], and others generate cellular reactive oxygen species (ROS), leading to cell death [5–7].

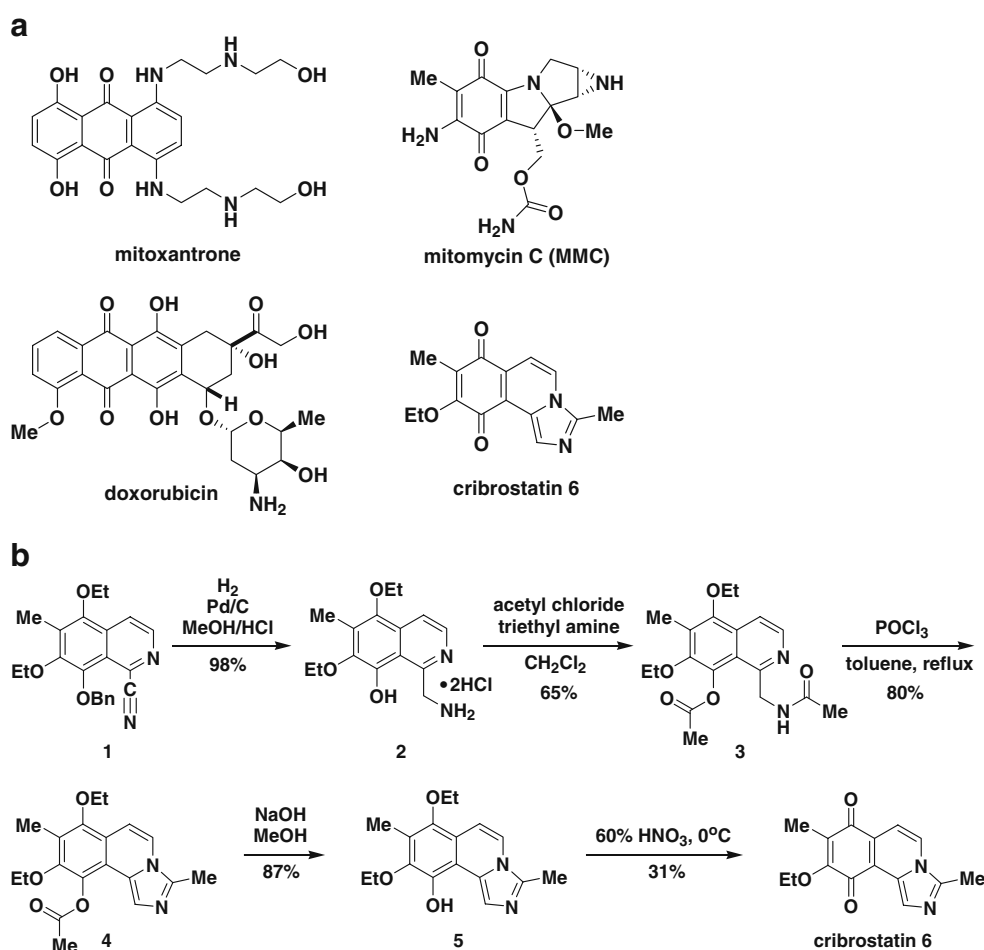
In many cases, the data suggests more than one of the above mechanisms of death being operational in cells treated with anticancer quinones. For example, doxorubicin both inhibits topoisomerase II and generates ROS through a 1-electron reduction pathway [1, 8]. There is also evidence that doxorubicin forms covalent adducts with DNA in cell culture as well as in carcinomas of human patients [9, 10]. MMC can be activated to a cytotoxic species through both 1- and 2-electron reduction pathways to give the MMC semi-quinone and hydroquinone, respectively [11].

The cribrostatins are a family of cytotoxic isoquinoline quinones isolated from the sea sponge *Cribochalina* [12–14], or *Petrosia* [15]. Members of this family have anti-neoplastic activity, with IC<sub>50</sub> values in the range of 0.045–25 μM against the P-388 murine leukemia cell line, as well as anti-bacterial and anti-fungal properties [12–14, 16, 17]. Cribrostatin 6 (Fig. 1a), a natural product isolated in 2003, was reported to induce death in P-388 cells (GI<sub>50</sub> value of 1.1 μM) as well as many different Gram-positive bacteria, including some that are multi-drug resistant [13]. However, the mechanism by which cribrostatin 6 kills cancer cells and bacterial cells was unknown. With the goal of defining the mode of cell death induced by this compound, cribrostatin 6 was synthesized and evaluated in a battery of biological assays. These experiments indicate that cribrostatin 6 has broad anticancer activity (including against both quiescent

**Electronic supplementary material** The online version of this article (doi:10.1007/s10637-010-9390-x) contains supplementary material, which is available to authorized users.

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**Fig. 1 a** Quinone-containing anticancer agents. **b** Synthesis of cribrastatin 6



and rapidly-dividing cells), does not appreciably inhibit topoisomerases, and appears to induce cell death through the generation of reactive oxygen species.

## Results

### Synthesis of cribrastatin 6

Cribrastatin has been the subject of three different total syntheses, by Nakahara and Kubo in 2004 [16, 18, 19], Markey and Kelly in 2008 [20], and most recently by Kneuppel and Martin [21]. To obtain cribrastatin 6 for our mechanistic experiments, the compound was synthesized via a route following the Nakahara route to compound **1** [18], and then converted to cribrastatin 6 through a slightly different series of transformations (Fig. 1b). Specifically, compound **1** was hydrogenated to provide **2** in a 98% yield. Treatment of **2** with acetyl chloride and triethylamine gave amide **3**, which was readily converted to tricycle **4** with phosphorous oxychloride. Compound **4** was saponified to afford phenol **5**, which was then oxidized to provide

milligram quantities of cribrastatin 6; spectral data of synthetic cribrastatin 6 matched that previously reported [13].

Cribrastatin 6 induces death in multiple cancer cell lines

Previously, the cribrastatins were identified as potent anticancer agents in the P-388 mouse leukemia cell line [12–14]. Thus, a further characterization of the potency of cribrastatin 6 was first obtained through evaluation of its toxicity in a variety of cancer cell lines including HeLa (human cervical cancer), MCF-7 (human breast cancer), SK-MEL-5 (human melanoma), U-937 (human lymphoma) and HL-60 (human leukemia). To determine the IC<sub>50</sub> values for cribrastatin 6, cells were incubated with compound over a range of concentrations (0–100 μM). After 24 h, the cellular toxicity was determined by either a sulforhodamine B (SRB) assay [22] for adherent cell lines (HeLa, MCF-7, and SK-MEL-5) or an MTS assay for suspension cell lines (U-937 and HL-60), and logistical dose-response curves were used to calculate IC<sub>50</sub> values. In general, cribrastatin 6 exhibited reasonably potent (≤10 μM) toxicity across cell lines from

various origins, with U-937 cells being the most susceptible of the cell lines tested,  $IC_{50}=0.6 \mu\text{M}$  (Table 1). The potency of cribrastatin 6 was also tested in HL-60 VCR, a HL-60 cell line that is resistant to vincristine and colchicine by virtue of enhanced expression of P-glycoprotein (Pgp) [23]. Pgp actively pumps many compounds out of the cancer cell, thus enabling the cell to resist the effect of many cytotoxins [23]. The  $IC_{50}$  value for cribrastatin 6 in the HL-60 VCR cell line was determined to be 4.8-fold higher than wild type HL-60; however, as shown in Table 1, the HL-60 VCR cell line was extremely (>200-fold) resistant to colchicine. Overall, cribrastatin 6 induces cell death across a variety of cancer cell types.

#### Cribrastatin 6 inhibits the growth of quiescent 3T3 fibroblasts

Chemotherapeutics typically target rapidly dividing cells, and these compounds are frequently inactive against quiescent cells, cells that are resting outside of the cell cycle [24]. Due to this disparity in toxicity, quiescent cancer cells may be responsible for some tumor resistance to common therapeutics [24–28], and in some cases it might be useful to have compounds that kill quiescent cells. BALB/3T3 cells were utilized to evaluate cribrastatin 6 versus isogenic quiescent and rapidly dividing cells. These 3T3 cells rapidly divide until the cells reach contact inhibition, at which point they become quiescent [29, 30]. Thus, both low density and confluent 3T3 cells were treated with cribrastatin 6 to assess its activity against actively dividing and quiescent cell types. As a comparison, several other chemotherapeutic agents (representing a variety of anticancer mechanisms) were also evaluated. For these experiments cells were incubated with compound for 72 h, at which point cytotoxicity was determined by the sulforhodamine B assay and  $IC_{50}$  values were calculated from logistical dose-response curves.

All anticancer compounds tested inhibited growth and caused death in rapidly dividing 3T3 cells with nanomolar to low micromolar  $IC_{50}$  values (Table 2). However, when quiescent 3T3 cells were treated with the same compounds for 72 h, paclitaxel, cisplatin, and etoposide induced no cell

death or growth inhibition ( $IC_{50}>100 \mu\text{M}$ ). Doxorubicin and MMC retained cytotoxic activity against the quiescent 3T3 cells, although their potencies were reduced 60- and 16-fold, respectively, relative to the non-contact inhibited cells. In contrast, cribrastatin 6 had only a minimally reduced (~2.6-fold) potency against the quiescent 3T3 cells. Thus, cribrastatin 6 is able to kill 3T3 cells that are not actively dividing.

#### Cribrastatin 6 induces apoptotic death in cancer cells

To determine the mode of cell death induced by cribrastatin 6, annexin V/propidium iodide (PI) co-staining assay was utilized, enabling discrimination between apoptotic and necrotic cell death. U-937 cells were treated with cribrastatin 6 (10  $\mu\text{M}$ ) for 12 and 16 h, then double stained with FITC-conjugated annexin V and PI and analyzed by flow cytometry (Fig. 2a). After 12 h of cribrastatin 6 treatment a small population of cells (~9%) had either died or was in late stage apoptosis (Annexin V positive, PI positive), and ~24% of cells were in early stage apoptosis (Annexin V positive, PI negative). After a 16 h exposure to cribrastatin 6, ~56% of cells were in the dead/late apoptotic quadrant, ~36% of cells were in early stage apoptosis, and only ~8% of the cells were viable. Thus a significant population of cribrastatin 6 treated cells progress through the annexin V positive/PI-negative quadrant, strongly suggesting that these cells are dying through apoptosis.

#### Cribrastatin 6 minimally displaces ethidium bromide from DNA

To test if cribrastatin 6 interacts with DNA in a manner similar to known DNA binders doxorubicin and 9-aminoacridine, an in vitro ethidium displacement assay was performed based on the known increase in ethidium bromide fluorescence upon intercalation into DNA [31]. As shown in Fig. 2b, addition of cribrastatin 6 to DNA induces only a small change (~20%) in ethidium bromide fluorescence; in contrast, doxorubicin and 9-aminoacridine show significantly more ethidium bromide displacement. Thus, cribrastatin 6 does not appear to interact with DNA to the same degree as 9-aminoacridine or doxorubicin.

**Table 1**  $IC_{50}$  values for cribrastatin 6 against various human cancer cell lines, and the effect of colchicine versus HL-60 and HL-60 VCR. Cells were treated with compound for 24 h then analyzed as described in the text. Error is standard error of the mean,  $n=3$

Cell line	Cribrastatin 6 $IC_{50}$ ( $\mu\text{M}$ )	Colchicine $IC_{50}$ ( $\mu\text{M}$ )
HeLa (cervical)	10±0.7	–
MCF-7 (breast)	8±2	–
SK-MEL-5 (melanoma)	9±3	–
U-937 (lymphoma)	0.6±0.2	–
HL-60 (leukemia)	6±5	0.5±0.1
HL-60 VCR (multi-drug resistant leukemia)	29±5	>100

**Table 2** Assessment of cell death induction in quiescent and actively dividing 3T3 fibroblasts. For quiescent experiments, 3T3 cells were grown in media until contact inhibited. Both quiescent and rapidly

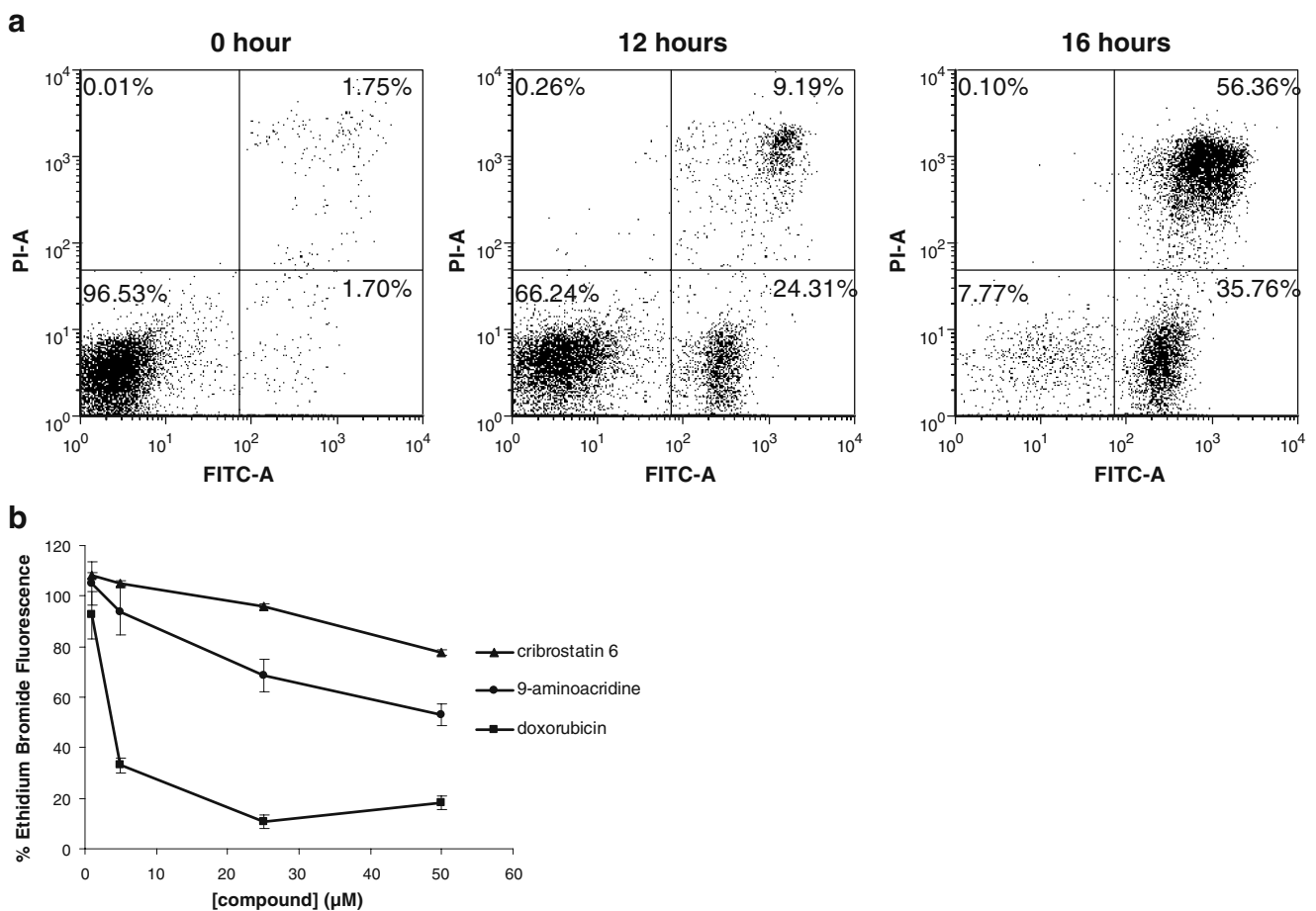
dividing cells were treated with compound for 72 h followed by analysis of cell growth with the sulforhodamine B assay. Error is standard error of the mean,  $n=3$

Compound	IC <sub>50</sub> vs. quiescent 3T3 (μM)	IC <sub>50</sub> vs. dividing 3T3 (μM)	Fold difference
cisplatin	>100	3±0.8	>33
cribrostatin 6	13±2	5±2	2.6
doxorubicin	0.6±0.03	0.01±0.003	60
etoposide	>100	0.05±0.003	>2000
MMC	16±2	1±0.4	16
paclitaxel	>100	0.02±0.005	5000

Cribostatin 6 is not a potent inhibitor of Topoisomerase I or II

Inhibition of topoisomerase enzymes is a known mechanism of approved cancer drugs doxorubicin, camptothecin, mitoxantrone, and etoposide [32–34]; many topoisomerase

inhibitors contain quinones [35]. To determine if cribrostatin 6 inhibits these enzymes, *in vitro* inhibition assays with purified topoisomerases were performed. To assess the inhibition of topoisomerase I, purified topoisomerase I and supercoiled pBR322 plasmid DNA were incubated together for 30 min at 37°C. Topoisomerase I catalyzes the

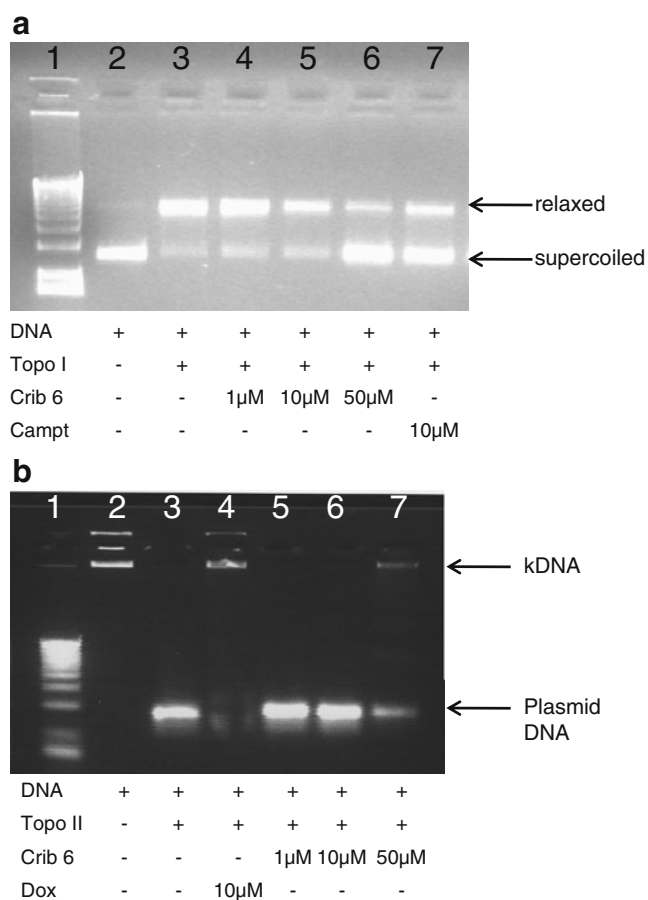


**Fig. 2 a** Cribrostatin 6 induces apoptosis in U-937 cells. Cells were treated with 10 μM cribrostatin 6 for the indicated time, then stained with PI and annexin V-FITC and analyzed by flow cytometry. Population movement through the lower right-hand quadrant indicates

apoptotic death. Data shown is representative data of 3 experiments. **b** Interaction of known intercalators and cribrostatin 6 with DNA, as monitored by an ethidium bromide displacement assay. Error bars are standard error of the mean,  $n=3$

conversion of supercoiled plasmid DNA to relaxed circular plasmid DNA, and the supercoiled and relaxed forms of DNA can be resolved and readily visualized by agarose gel electrophoresis [33, 36–38]. As shown by the data in Fig. 3a, cribrastatin 6 inhibits the topoisomerase I-induced relaxation of supercoiled pBR322 only at a concentration of 50  $\mu$ M. In contrast, camptothecin, a known topoisomerase I inhibitor, inhibits the relaxation of pBR322 at a concentration of 10  $\mu$ M. Cribrastatin 6 does not appear to be a strong inhibitor of topoisomerase I *in vitro*.

The inhibition of topoisomerase II can be similarly investigated using a gel electrophoresis DNA shift assay. In this assay, purified topoisomerase II was incubated with catenated plasmid DNA (kDNA). Topoisomerase II decatenates the kDNA into single plasmids, and agarose gel electrophoresis resolves these two forms of DNA [39–41].



**Fig. 3** **a** Camptothecin is more potent than cribrastatin 6 in an *in vitro* topoisomerase I inhibition assay. Topoisomerase I and pBR322 supercoiled plasmid DNA were incubated in the presence of vehicle or compound for 30 min and then analyzed by gel electrophoresis. Lane 1 contains 1  $\mu$ g DNA ladder. **b** Doxorubicin is more potent than cribrastatin 6 in an *in vitro* topoisomerase II inhibition assay. Topoisomerase II and kDNA were incubated for 30 min in the presence of vehicle or compounds. Lane 1 contains 1  $\mu$ g DNA ladder

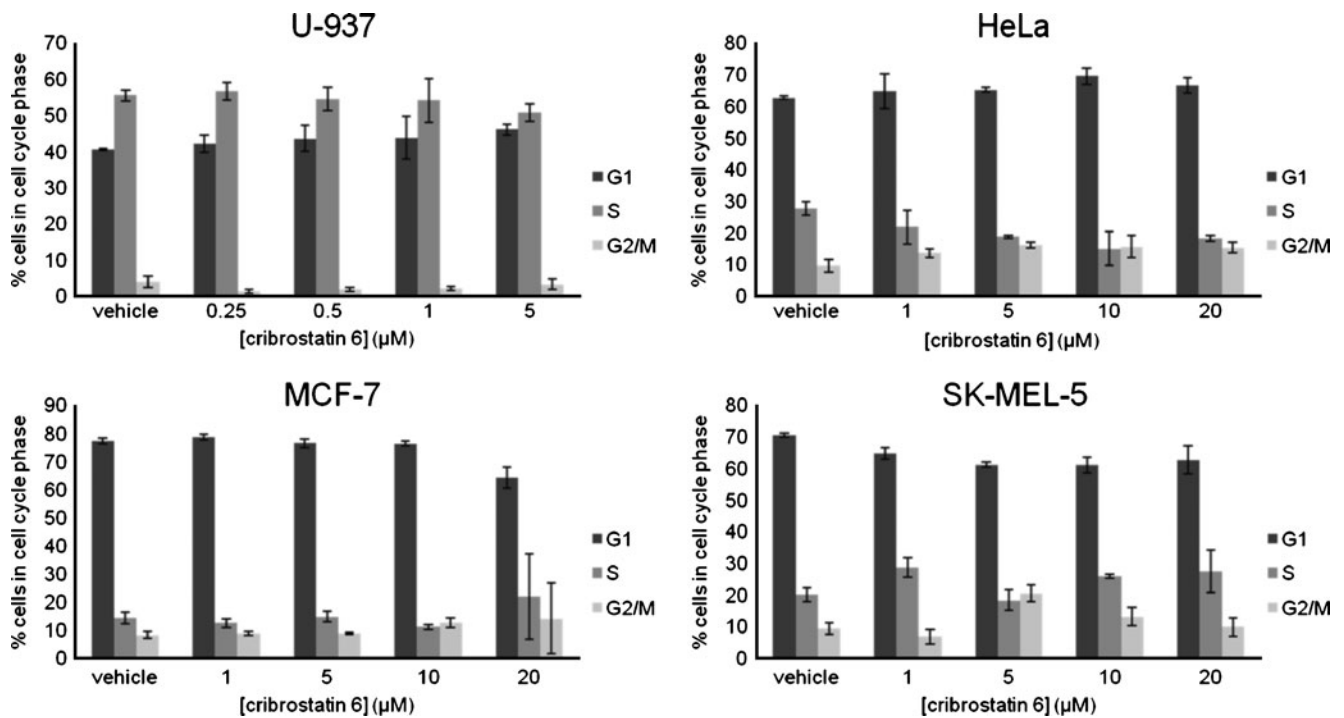
As shown in Fig. 3b, a 10  $\mu$ M concentration of doxorubicin completely inhibited decatenation by topoisomerase II. In contrast, only at 50  $\mu$ M did cribrastatin 6 inhibit decatenation of kDNA by topoisomerase II, indicating that cribrastatin 6 is not a strong inhibitor of topoisomerase II *in vitro*. In total, the high cribrastatin 6 concentrations needed to inhibit the topoisomerase enzymes *in vitro* suggest that topoisomerase inhibition is not a primary mechanism of cell death induced by this compound.

Cribrastatin 6 induced death of cancer cells is not preceded by cell cycle arrest

Prior to death, cells treated with many cytotoxins arrest in a certain phase of the cell cycle [42, 43]; the phase of arrest can be a clue to the mechanism of action of a compound. To determine if cribrastatin 6 arrests cells in a certain phase of the cell cycle, four different cell lines were treated with increasing concentrations of cribrastatin 6 for 16 h. Cells were then fixed and stained for the amount of DNA present using propidium iodide (PI), and analyzed by flow cytometry. Treatment of U-937, SK-MEL-5, MCF-7, and HeLa cells with cribrastatin 6 caused no significant changes in the levels of cells in each phase of the cell cycle within 16 h relative to controls (Fig. 4). This data indicates that cribrastatin 6 does not induce cell cycle arrest in these cell lines. The inability of cribrastatin 6 to induce cell cycle arrest corroborates the data suggesting that topoisomerase inhibition may not be the primary mechanism of action for cribrastatin 6, as small molecules topoisomerase inhibitors are known to arrest cells in distinct phases of the cell cycle. For example, camptothecin arrests cells in S and G2 phase due to DNA replication fork damage [44, 45]. Similarly, doxorubicin causes G2/M phase arrest through its inhibition of topoisomerase II [46]. Although most cytotoxins induce some form of cell cycle arrest, there are a handful of compounds known to effect cell death in a cell cycle independent manner [47–49].

Cribrastatin 6 induces ROS production in cancer cells

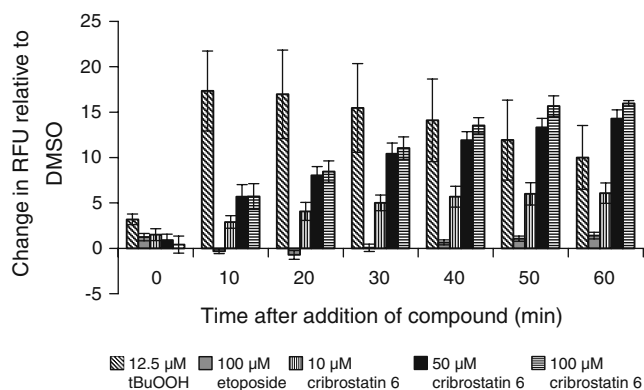
Reactive oxygen species (ROS) can be a cause or an effect of cancer cell death [50]. Many compounds known to have anticancer properties produce ROS such as elesclomol [51], CDDO [52], and doxorubicin [53]. To determine if ROS production is an early effect of cribrastatin 6 treatment, and therefore more likely a cause of death of treated cells, U-937 cells were treated with the compound and the levels of ROS produced was monitored with dichlorofluorescein diacetate (DCF), a non-fluorescent dye that reacts with peroxide to produce fluorescent dichlorofluorescein. The level of intracellular fluorescence from this dye in live cells was quantified by flow cytometry. As a positive control for



**Fig. 4** Cribrastatin 6 does not cause cell cycle arrest prior to death. Cells were treated with varying concentrations of cribrastatin 6 for 16 h after which time the cells were trypsinized and fixed/

permeabilized. No significant change in the distribution of cells in growth phases was observed. Error bars represent standard error of the mean,  $n \geq 3$

these experiments, *t*-butyl peroxide was used, as it is known to create peroxide in cells [54]. As shown in Fig. 5, cells treated with cribrastatin 6 produced significant concentrations of peroxide in an hour, as indicated by DCF staining. Compounds that do not induce ROS through their primary mechanism, such as etoposide, show no response in this assay (Fig. 5). The rapid formation of ROS suggests that it is directly produced by cribrastatin 6 and is not a byproduct of cell death.



**Fig. 5** Cribrastatin 6 produces peroxides in 10 min as observed with the dye DCF. *t*-Butyl peroxide is known to produce peroxides in cells, whereas etoposide does not. Error bars represent standard error,  $n = 3$

Cribrastatin 6 causes an increase in the HMOX1 transcript, as indicated by global transcript profiling

The analysis of global transcriptional changes in response to compound treatment can be used to identify cellular pathways affected by a small molecule, and in some cases can suggest a macromolecular target [55–57]. Whole genome transcriptional profiling of human lymphoma U-937 cells treated with cribrastatin 6 (15  $\mu$ M) for 6 h was performed using the Illumina humanHT-12 array. This concentration and time point were selected so that the data gathered could be compared to the Connectivity Map, a database of small molecule transcript profiling experiments [57].

Analysis of the data from this experiment indicates that cribrastatin 6 treated cells had elevated levels of several antioxidant transcripts, including heme oxygenase 1 (HMOX1) (28.7 fold change) and various ferritins. These ferritins are under the transcriptional control of NRF2, a transcription factor that is activated under oxidative stress [58, 59]. The transcripts that were elevated and reduced (top 20 for both) in response to cribrastatin 6 treatment of U-937 cells are shown in Table 3. Other oxidative stress associated transcripts affected included the upregulation of sulfiredoxin 1 (SRXN1), oxidative stress induced growth inhibitor (OKL38) and the glutamate-cysteine ligase modifier subunit

**Table 3** Top 20 up- and down-regulated genome-wide transcripts in human lymphoma U-937 cells treated with cribrastatin 6 (15  $\mu$ M) for 6 h. The gene symbol identification, protein associated with that gene,function of the protein, *p*-values corrected for multiple hypothesis testing using the False Discovery Rate method and fold changes in transcript versus DMSO control are shown in the table

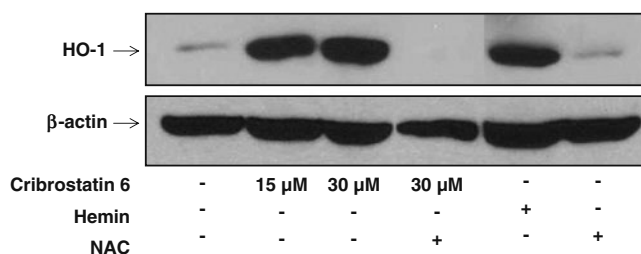
Symbol	Protein	Function	<i>p</i> value	Fold change
HMOX1	heme oxygenase 1	antioxidant	0	28.7
IL8	interleukin 8	inflammation	0	4.7
IL8	interleukin 8	inflammation	0	4.1
SRXN1	sulfiredoxin 1 homolog	oxidative stress	0	3.5
OKL38	oxidative stress induced growth inhibitor 1	oxidative stress	0	3.5
TNF	tumor necrosis factor	death ligand	0	3.3
FTH1	ferritin, heavy polypeptide-like 1	intracellular iron storage	0	3.3
FTHL12	ferritin, heavy polypeptide-like 12	intracellular iron storage	0	2.8
FTHL8	ferritin, heavy polypeptide-like 8	intracellular iron storage	0.0000001	2.8
SQSTM1	sequestosome 1	regulation of NF-kB)	0.0000003	2.6
GCLM	glutamate—cysteine ligase modifier subunit	glutathione synthesis	0	2.6
SLC7A11	solute carrier family 7 member 7	cationic amino acid transporter	0	2.6
FTHL11	ferritin, heavy polypeptide-like 11	intracellular iron storage	0.0000006	2.5
MAFB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B	represses ETS1-mediated transcription	0	2.5
RIT1	Ras-like without CAAX 1	activation of EPHB2 and MAPK14 signaling	0	2.5
TM4SF1	transmembrane 4 L six family member 1	cell surface antigen expressed in carcinomas	0.0000001	2.4
FTHL3	ferritin, heavy polypeptide-like 3	intracellular iron storage	0.0000036	2.3
FTHL2	ferritin, heavy polypeptide-like 2	intracellular iron storage	0.0000235	2.3
FTHL11	ferritin, heavy polypeptide-like 11	intracellular iron storage	0.0001174	2.3
CDKN1A	cyclin-dependent kinase inhibitor 1A	p53-dependent G1 phase arrest	0	2.3
FAM46C	hypothetical protein LOC54855		0.0000003	−2.0
LOC201164	phosphatidylcholine-hydrolyzing phospholipase D6		0.0000225	−1.8
TFRC	transferrin receptor	cellular iron uptake	0.0011374	−1.8
VCX	variably charged protein X-B1		0.0000998	−1.7
LOC649856			0.001137	−1.7
PTPLAD1	protein tyrosine phosphatase-like A domain containing 1	Rac1-signaling	0.0000882	−1.6
VCX-C	variably charged protein X-C		0.0001519	−1.6
FAM46A	hypothetical protein LOC55603		0.0002401	−1.6
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphate 4	fructose 2,6-biphosphate synthesis	0.0479886	−1.6
VCX3A	variably charged protein X-3A		0.0002669	−1.5
ELOVL3	cold-inducible glycoprotein of 30 kDa	neutral lipid formation	0.0012824	−1.5
NFE2	leucine zipper protein NF-E2	hemoglobin production	0.0243916	−1.5
CD47	CD47 molecule	membrane transport/signal transduction	0.0046719	−1.5
MYB	c-myb protein	transcriptional activator	0.0000765	−1.5
SYNC1	syncollin, intermediate filament 1		0.0033578	−1.5
ZNF395	zinc finger protein 395	papilloma virus genes transcription	0.0040402	−1.5
DHRS9	3-alpha hydroxysteroid dehydrogenase	retinoic acid biosynthesis	0.0031682	−1.5
GJB2	gap junction protein beta 2	cell-to-cell ion/small molecules transfer	0.0081905	−1.5
GFI1	growth factor independent 1 transcription repressor	S-phase gene transcription	0.0006575	−1.4
PDYN	proenkephalin B		0.023222	−1.4

(GCLM) involved in glutathione synthesis. The down regulation of the transferrin receptor (TFRC) involved in iron uptake and the leucine zipper protein NF-E2 involved in hemoglobin production was also observed (Table 3). The statistically most significant canonical pathways affected by cribrastatin 6 were the NRF2 oxidative stress mediated pathway ( $p$  value  $9.51 \times 10^{-11}$ ), followed by the TREM1 ( $p$  value  $3.06 \times 10^{-7}$ ) and the glucocorticoid receptor signaling pathways ( $p$  value  $1.7 \times 10^{-5}$ ), which modulate inflammatory responses (see Supporting Information for canonical pathway diagrams).

Heme oxygenase 1 (HO-1), the product of the *HMOX1* gene, is a 32 kDa heat-shock protein that prevents cell death by converting heme to the powerful antioxidant biliverdin [60]. Biliverdin production from heme produces carbon monoxide, a potential neurotransmitter, and free iron, which can serve as an oxidative stress signal [58]. Biliverdin is also rapidly degraded into bilirubin, another potent antioxidant [58]. Ferritins, which were also upregulated in cribrastatin 6 treated cells, are responsible for the storage of free iron in a soluble and non-toxic form in the cell.

In order to confirm that treatment of cells with cribrastatin 6 elevates HO-1 at the protein level, a Western blot for HO-1 was performed on cells treated with cribrastatin 6 for 6 h. Hemin, a compound that induces the transcription of NRF2 and HO-1 [61], was used as a positive control for elevation of HO-1 protein. As shown in Fig. 6, cribrastatin 6 treatment (at 15 and 30  $\mu$ M) elevated HO-1 protein levels in U-937 cells. This elevation could be prevented by co-treatment of cells with the antioxidant *N*-acetyl cysteine (NAC), strongly suggesting that HO-1 upregulation by cribrastatin 6 is ROS-mediated.

The gene expression signature of cribrastatin 6-treated U-937 cells was compared to analogous signatures for over 1300 biologically active small molecules; the macromolecular target is known for most of these compounds. Thus, use of this Connectivity Map database can allow the identification of the mechanism of action of bioactive



**Fig. 6** Western blot for HO-1. Treatment of U-937 human lymphoma cells with cribrastatin 6 induces heme oxygenase 1 (HO-1) protein expression that can be prevented by the antioxidant NAC. Hemin is a known up-regulator of HO-1 protein levels. Cells were treated with hemin at 10  $\mu$ M and NAC at 10 mM

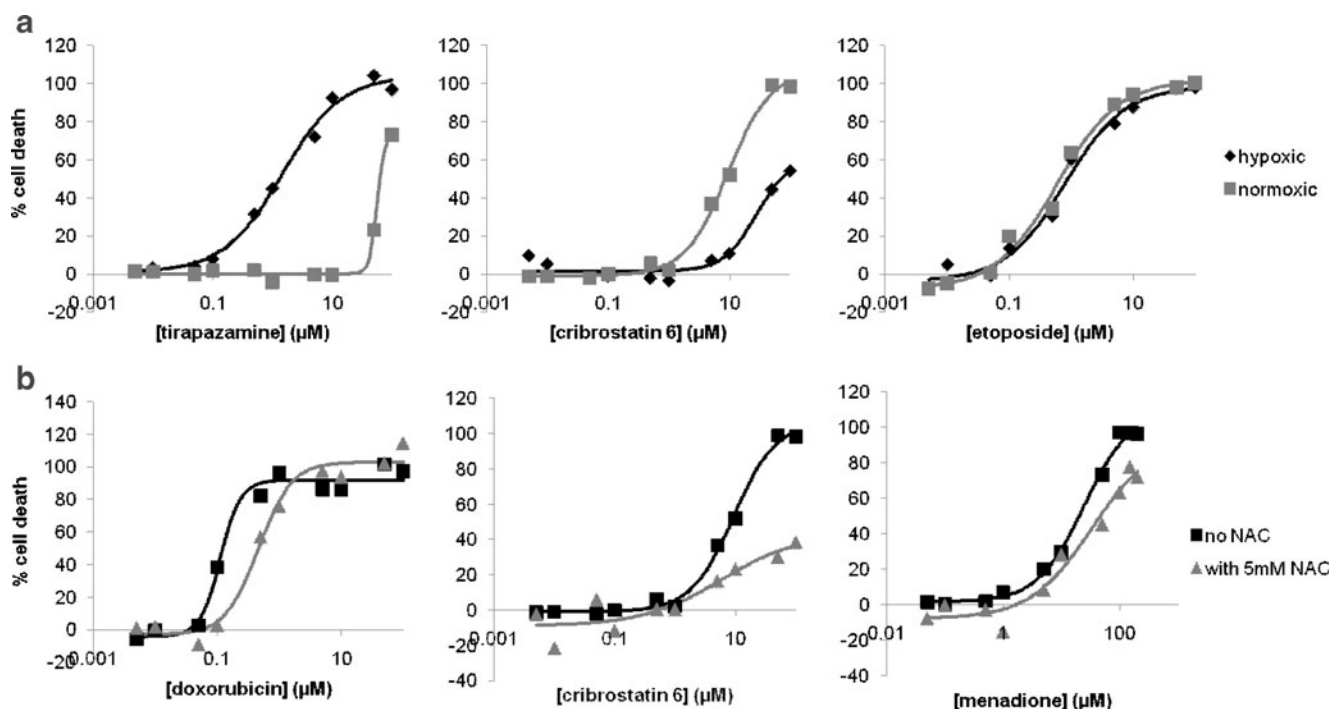
molecules by comparison to the gene expression signature of compounds with known modes of action [56]. In the case of cribrastatin 6, the connectivity map did not yield strong matches (best score  $<0.6$ ) to the compounds in the database. Importantly, this database contains several quinones and multiple topoisomerase inhibitors, including doxorubicin, daunorubicin, mitoxantrone, etoposide, camptothecin, and irinotecan.

#### Hypoxic environment protects cancer cells from cribrastatin 6

Hypoxic environments can affect the cellular activity of a compound. For example, if a compound causes cell death by stimulating superoxide production from molecular oxygen, then the potency of the compound should decrease in hypoxia. To investigate if cribrastatin 6 displays differential toxicity in hypoxic and normoxic cells, HeLa cells were treated with cribrastatin 6 and incubated in a hypoxic (1%  $O_2$ ) or normoxic (ambient  $O_2$  concentration,  $\sim 20\%$ ) incubator in 96 wells plates. After 48 h, the plates were analyzed using SRB staining to determine  $IC_{50}$  values. The toxicities of cribrastatin 6 in each environment were then compared. Tirapazamine was used as a hypoxia active control [62, 63], and etoposide was used as a non-hypoxic sensitive control. As shown in Fig. 7a and consistent with literature reports, tirapazamine is significantly more potent in hypoxia (by over 35-fold) and the potency of etoposide is not altered in the hypoxic environment. In contrast, cribrastatin 6 is less potent, by more than 10-fold, on cells grown in hypoxia. This reduction in toxicity suggests that cribrastatin 6 produces toxic superoxide through redox cycling with  $O_2$ .

#### *N*-acetyl cysteine protects cells against cribrastatin 6

ROS can be indirectly sensed through use of *N*-acetyl-L-cysteine (NAC). NAC is a precursor to glutathione, a major ROS scavenger, and NAC is a radical scavenger in its own right. If cells in culture are co-treated with NAC and a ROS producing compound, levels of ROS (as detected by a dye like DCF) and transcription upregulation are generally reduced [64]. In addition, NAC can rescue cells in culture from ROS-mediated cell death including death caused by mitomycin C [65], doxorubicin [53], and menadione [50, 51, 66–68]. To determine if NAC can rescue cells from cribrastatin 6 treatment, HeLa cells were pretreated with 5 mM NAC for 1 h prior to treatment with cribrastatin or control compounds in 96 well plates. Cell death was determined by SRB staining after 48 h incubation with compounds. As shown in Fig. 7b, NAC treatment has a modest effect on the toxicity of doxorubicin and menadione. However, cribrastatin 6 induced cell death is dramatically reduced in the presence of NAC (Fig. 7b). This data



**Fig. 7** NAC and hypoxia protect cancer cells from cribrostatin 6 induced cell death. **a** Tirapazamine is more potent in hypoxia, etoposide is equipotent in hypoxia and normoxia, whereas cribrostatin 6 is less potent to cells grown in hypoxia. HeLa cells were preincubated in hypoxic (1% O<sub>2</sub>) or normoxic environments then treated with indicated concentrations of compounds. Cell death was assessed after 48 h by SRB assay. Graphs are representative of three

different experiments. **b** NAC treatment has a modest effect on doxorubicin and menadione toxicity, but a significant effect on the ability of cribrostatin 6 to induce cell death. HeLa cells were preincubated with NAC (5 mM), and then treated with compound at the given concentrations. Cell death was assessed 48 h later by SRB assay. Graphs are representative of three different experiments

is consistent with the Western blot in Fig. 6, and further implicates ROS in the mode of cell death induced by cribrostatin 6.

## Discussion

Cytotoxic quinones induce death through a spectrum of mechanisms, including topoisomerase inhibition and direct DNA damage. The data described herein suggest that the mode of action of cribrostatin 6 does not map onto any of these known cytotoxins. Cribrostatin 6 treated cancer cells die through apoptosis, as detected by positive annexin V/PI staining. In vitro, cribrostatin 6 has only weak intercalative and topoisomerase inhibitory properties, suggesting that DNA binding and topoisomerase inhibition are not primary modes of cell death induction. Indeed, these observations are consistent with data from cell cycle arrest experiments: cribrostatin 6 does not induce cell cycle arrest, unlike quinones such as doxorubicin [69], menadione [70], or mitomycin C [71]. In addition, unlike most cytotoxins cribrostatin 6 is able to induce death in both actively dividing and

quiescent cells, suggesting a mechanism of cell death not related to, or dependent on, the cell cycle.

The weight of the available evidence suggests that cribrostatin 6 induces cell death through a ROS-mediated mechanism. As measured with DCF, cribrostatin 6 induces cellular ROS within minutes of treatment. NAC treatment protects cells from cribrostatin 6 induced cell death, as does growth under hypoxic conditions. Consistent with this data, global transcript profiling shows that *HMOX1*, an antioxidant gene, is the most elevated transcript in cribrostatin 6 treated cells, with a >28-fold upregulation. Western blots confirm that the HO-1 protein is elevated upon cribrostatin 6 treatment, and that this effect is abrogated upon treatment with the ROS scavenger NAC. Finally, cribrostatin 6 induces death in growing cells without cell cycle arrest, and it is able to kill cells that are not actively cycling; both of these features are consistent with direct ROS generation. It is likely that a one-electron bioreduction converts cribrostatin 6 to its semiquinone, which upon oxidation with O<sub>2</sub> produces superoxide leading to cell death.

Increasing cellular ROS levels is a possible strategy to target cancer cells [72]. According to this theory, cancer

cells have higher levels of oxidative stress than normal cells, therefore, an external source of ROS can bring the cancer cell over its oxidative damage buffering capacity and cause cancer cell death. Normal cells will be insulted similarly with the external ROS source; however, their lower endogenous level of ROS enables the normal cells to resist a certain amount of oxidative damage. One potential application of ROS-generating compounds could be their use in combination therapies, where standard cytotoxins kill the rapidly dividing portion of a tumor and the ROS-generating compound kills the resistant cancer stem cells [25, 28].

An experimental therapeutic that appears to operate via a ROS-producing mechanism is elesclomol. Elesclomol is an anticancer compound that induces the upregulation of HSP70, increases the DCF signal, and its cell death induction is mitigated by the addition of NAC, all traits consistent with ROS production [51]. During Phase I trials, a combination of elesclomol and paclitaxel was tolerated by a group of patients with refractory solid tumors [73]. The compound continued to Phase II trials where progression-free survival was seen to double in metastatic melanoma patients treated with elesclomol and paclitaxel in combination (3.7 months) rather than paclitaxel alone (1.8 months) [74]. Despite these seemingly positive results, Phase III trials were halted in 2009 due to increased mortality in cancer patients treated in combination with paclitaxel and elesclomol [75]. The reason for the increase in mortality has not been determined. It is worth noting that although cribrostatin 6 and elesclomol both appear to induce cell death through oxidative stress, the transcript profile of elesclomol-treated cells shows a different pattern of oxidative death responsive genes than cribrostatin 6 [51].

In conclusion, cribrostatin 6 appears to induce death in cancer cells through a ROS-mediated mechanism. When compared to similar quinone drugs, cribrostatin 6 has certain attractive features, including its ability to induce death in non-dividing cells and in cells that are resistant to standard anticancer agents. Although more animal studies need to be completed, *in vivo* studies have shown that mice can tolerate cribrostatin 6 injected *i.p.* at 1 mg/kg twice daily for 5 days [17]. Further investigations with cribrostatin 6 will help to shed light on the potential of ROS-generating compounds as anticancer agents.

**Acknowledgements** We would like to thank Justin Lamb (Broad Institute) for advice regarding use of the Connectivity Map database, the Keck facility at the University of Illinois for performing the microarray experiment and data processing, Russel J. Mumper (U. Kentucky) for the kind gift of the HL-60 VCR cell line, Joseph S. Bair (UIUC) for synthesis of tirapazamine, and Dr. Benjamin J. Leslie (UIUC) for preliminary HL-60 VCR assays. Funding was provided by the University of Illinois.

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