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ORIGINAL ARTICLE

Conditional drug screening shows that mitotic inhibitors induce AKT/PKB-insensitive apoptosis

Maria Berndtsson · Emma Hernlund · Maria C. Shoshan · Stig Linder

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Abstract The phosphatidylinositol 3-kinase (PI3K)/AKT pathway is frequently upregulated in human cancer. Activation of this pathway has been reported to be associated with resistance to various chemotherapeutical agents. We here used a chemical biology/chemical informatic approach to identify apoptotic mechanisms that are insensitive to activation of the PI3K/AKT pathway. The National Cancer Institute (NCI) Mechanistic Set drug library was screened for agents that induce apoptosis in colon carcinoma cells expressing a constitutively active form of AKT1. The cytotoxicity screening data available as self-organized maps at the Developmental Therapeutics Program (DTP) of the NCI was then used to classify the identified compounds according to mechanism of action. The results showed that drugs that interfere with the mitotic process induce apoptosis which is comparatively insensitive to constitutive AKT1 activity. The conditional screening approach described here is expected to be useful for identifying relationships between the state of activation of signaling pathways and sensitivity to anticancer agents.

Keywords Chemical biology · Apoptosis · AKT · Microtubuli-interacting agents

Introduction

The phosphatidylinositol 3-kinase (PI3K)/AKT pathway is frequently upregulated or activated in human cancer. The class I subgroup of PI3Ks is thought to exclusively phosphorylate PtdIns(4,5)P2 to generate PtdIns(3,4,5)P3 (PIP3) in vivo [41]. PIP3 induces the membrane localization and activation of the proto-oncogenic kinase AKT/protein kinase B (PKB) [39]. While AKT mutations are less common, PI3K is often amplified in cancer [39]. Oncogenic signaling via activated tyrosine kinase receptors and mutated Ras will also lead to high constitutive activation of PI3K and hence also of AKT [14,34]. Furthermore, the counteractive tumor suppressor PIP3 phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) is frequently mutationally inactivated in many types of cancer [26]. Downstream effector pathways of AKT include the anti-apoptotic protein Bad, inhibition of Mdm2, p27Kip, and activation of mammalian target of rapamycin, mTOR [11]. mTOR regulates both cell growth and cell cycle progression through its ability to integrate signals from nutrient and growth factor stimuli.

In addition to contributing to tumorigenesis, the PI3K/AKT pathway is strongly implicated in chemoresistance. A number of investigators have reported that AKT is a key factor in conferring cellular resistance to various chemotherapeutical agents in vitro, including cisplatin, oxaliplatin, and etoposide [1,2,4,6,8,23,25,29,36]. Furthermore, AKT promotes cell survival after exposure to such different death stimuli as UV irradiation, growth factor withdrawal, TGF-ß, anti-Fas antibody, glutamate, and bile acids [9,10,12,16,21,22].

Cellular responses to anticancer drugs are complex and may include apoptosis, necrosis, premature senescence, and mitotic catastrophe [7,35]. In order to examine the role of AKT specifically in apoptosis induced by chemotherapeutic drugs and to identify drugs which induce apoptosis independently of AKT1 activity, we have here screened a chemical library on an isogenic pair of HCT116 cell lines, one of which expresses wild-type AKT1 which by myristylation is made constitutively membrane-bound and active.

M. Berndtsson · E. Hernlund · M. C. Shoshan · S. Linder (✉)
Cancer Center Karolinska,
Department of Oncology and Pathology,
Karolinska Institute and Hospital, Cancer Center Karolinska,
R8:00, S-171 76 Stockholm, Sweden
e-mail: Stig.Linder@ki.se

Materials and methods

Materials

The mechanistic set was obtained from the Developmental Therapeutics Program of the US National Cancer Institute (http://www.dtp.nci.nih.gov). AKT antibody was from Cell Signaling (Beverly, MA), β-tubulin antibody from Sigma–Aldrich (St Louis, MO), and antibody to phosphorylated GSK3β was a gift from Katja Pokrovskaja (Cancer Center, Karolinska).

Cell culture

HCT116 colon carcinoma cells stably expressing constitutively active myristoylated AKT (myr-AKT), and parental cells were generously provided by Dr Nissim Hay (University of Illinois at Chicago, IL). Cells were maintained in McCoy's 5A modified medium supplemented with 10% fetal calf serum, L-glutamate, penicillin, and streptomycin (and 10 μg/ml puromycin for AKT-transfected cells) at 37 in 5% CO_2. Cell survival was examined using the sulphorhodamine (SRB) assay as described [17].

Screening and assessment of apoptosis

HCT116 cells were seeded in 96-well microtiter plates at 10,000 cells per well in 200 μl medium and incubated overnight. Drugs were then added to a final concentration of 2.5 or 5 μM from stocks in DMSO (to a final concentration of 0.5%). Control wells received vehicle only. After 24 h of incubation, NP-40 was added directly to the tissue culture medium to a final concentration of 0.1% followed by mixing on a rotatory shaker for 5 min. A 25-μl aliquot of the content of each well was assayed for caspase-cleaved CK18 using the M30-CytoDeath® ELISA assay (a modification of the M30-Apoptosense® ELISA intended for in vitro use; PEVIVA AB, Bromma, Sweden) [19]. Formation of the cleaved product depends on caspase activity and is inhibited by pan-caspase inhibitors such as zVAD-fmk.

siRNA transfection

Cells were seeded in the presence of transfection reagents (25 nM siRNA, HiPerfect Transfection reagent; Dharmacon) and incubated for 48 h. Cells were then either drug

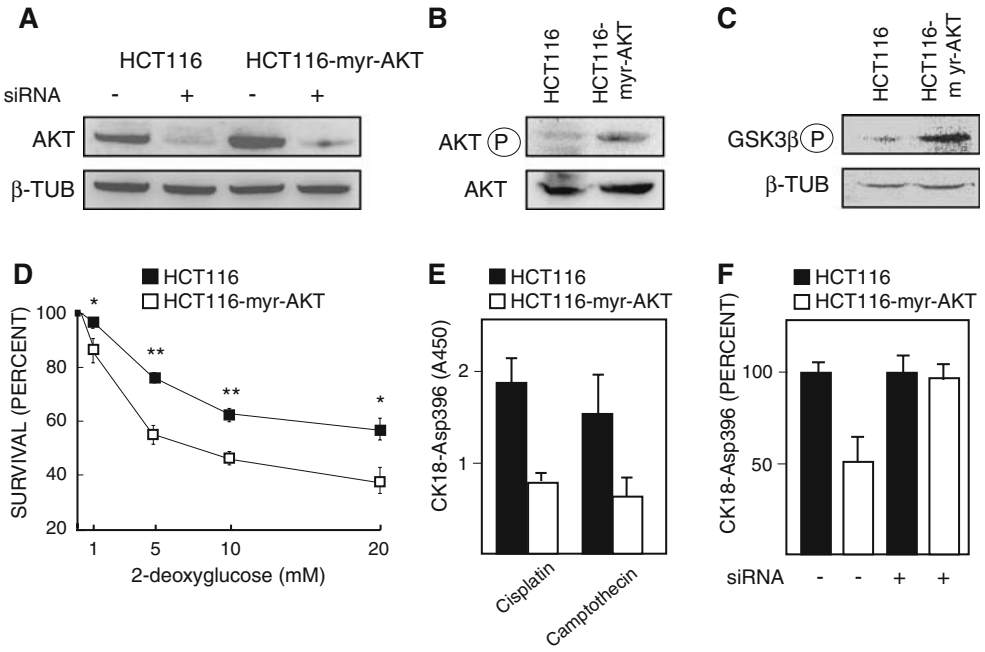

Fig. 1 Phenotype of HCT116-myr-AKT cells. **a** AKT expression in HCT116 and HCT116-myr-AKT cells. Cell extracts were analyzed for AKT expression by western blotting using an antibody to human AKT. Cells were transfected with AKT siRNA where indicated; **b** increased phosphorylation of AKT at T473 in HCT116-myr-AKT cells. AKT phosphorylation was examined by western blotting using an antibody to the phosphorylated form of the protein. The same blot was probed with an antibody to total AKT. **c** Increased phosphorylation of GSK3β in HCT116-myr-AKT cells. GSK3β phosphorylation was examined by western blotting using an antibody to the phosphorylated form of the protein. The same blot was probed with an antibody to β-tubulin. **d** Increased sensitivity of HCT116-myr-AKT cells to 2-deoxyglucose. Cells were treated with the indicated concentrations of 2-deoxyglucose and survival was examined using the SRB assay; $*p<0.05$; $**p<0.005$. **e** Apoptosis induction by cisplatin and camptothecin in HCT116-myr-AKT cells. Cells were treated for 24 h, and apoptosis was quantified using the M30 CytoDeath® ELISA. **f** Effect of AKT siRNA on apoptosis induced by one of the agents in the set (*NSC* NSC632841). Cells were transfected with siRNA as indicated and treated with the drug for 24 h. Caspase-cleaved CK18 was determined using the M30 CytoDeath® ELISA

treated for apoptosis assessment or lysed for Western blotting. Cell extract proteins were resolved by NuPage Bis-Tris gels (Invitrogen) and transferred onto a polyvinylidene difluoride membrane. Membranes were probed with antibodies to AKT (dilution 1: 2,000), AKT T473 (dilution 1:1,000), or β-tubulin (dilution 1:1,000).

SOM clustering

The web-based tool available at http://spheroid.ncifcrf.gov/spheroid/HTMLNscNumberProjection.cfm was used to map the apoptotic compounds onto the self organizing maps (SOMs). This tool has been developed by the Covell group at the National Cancer Institute at Frederick and utilizes biological data (tumor cell growth inhibition (GI_{50})) to generate SOMs [32]. The SOMs represent a neural network-based algorithm [24] able to project high-dimensional data into lower dimensional space. For compounds that generated more than one map position, the strongest association was used.

Results and discussion

Characterization of an HCT116 cell line expressing myristylated AKT/PKB We here used an HCT116 colon carcinoma cell line engineered to express a myristylated form of AKT that is constitutively membrane-bound and active (HCT116-myr-AKT, generously provided by Dr. Nissim Hay). Western blot analysis confirmed higher levels of AKT in HCT116-myr-AKT cells relative to parental cells (Fig. 1a) and increased phosphorylation of AKT at S473 (Fig. 1b). Furthermore, increased phosphorylation of the AKT target GSK3β was observed in HCT116-myr-AKT cells (Fig. 1c). In accordance with a requirement for glycolysis in the survival functions of AKT [13], HCT116-myr-AKT cells showed an increased sensitivity to the glycolysis inhibitor 2-deoxyglucose (1–20 mM), as assessed in a survival assay over 48 h (Fig. 1d). Furthermore, HCT116-myr-AKT cells were less sensitive to apoptosis induced by cisplatin and camptothecin (Fig. 1e).

In order to confirm the role of myr-AKT expression for the apoptotic response, we transfected HCT116-myr-AKT and control cells with AKT siRNA. The transfection resulted in significantly decreased AKT expression in both cell lines (Fig. 1a). Transfected cells were then treated with one of the apoptotic compounds from the NCI Mechanistic Set (4-piperidinone, 1-(1-oxo-2-propenyl)-3,5-bis(phenylmethylene)), As shown in Fig. 1f, untransfected control and myr-AKT cells showed different responses to NSC632841, whereas the apoptotic response was similar in both cell lines when transfected with AKT siRNA. We conclude from these experiments that HCT116-myr-AKT cells show the expected phenotype with regard to signaling and drug sensitivity.

Screening for compounds that induce myr-AKT-insensitive apoptosis In order to identify agents that are effective in inducing apoptosis of HCT116-myr-AKT cells, we exposed the pair of cell lines to the NCI Mechanistic drug set at 2.5 or 5 μM. This drug collection contains 827 compounds selected from approximately 40,000 compounds on the basis on different mechanisms of action with regard to cell growth inhibition of the NCI60 tumor cell line panel. We initially screened the entire drug set for compounds effective in inducing apoptosis of HCT116 then used a selection of apoptosis-inducing compounds on the cell pair. Apoptosis was measured by the M30 CytoDeath® ELISA, an assay which is specific for a caspase-cleaved product of cytokeratin-18 formed in apoptotic cells [19]. The apoptosis product accumulates in cell cultures and was measured at a single time point (24 h). The signals from untreated myr-AKT and control cells were set to 1, respectively. For each drug, the induced levels of apoptosis in each cell line were then plotted against each other (Fig. 2). Drugs that induced less than twofold the background apoptosis in parental cells are excluded from the figure. Most of the drugs shown generated a sufficient signal at a concentration of 2.5 μM. It is clear

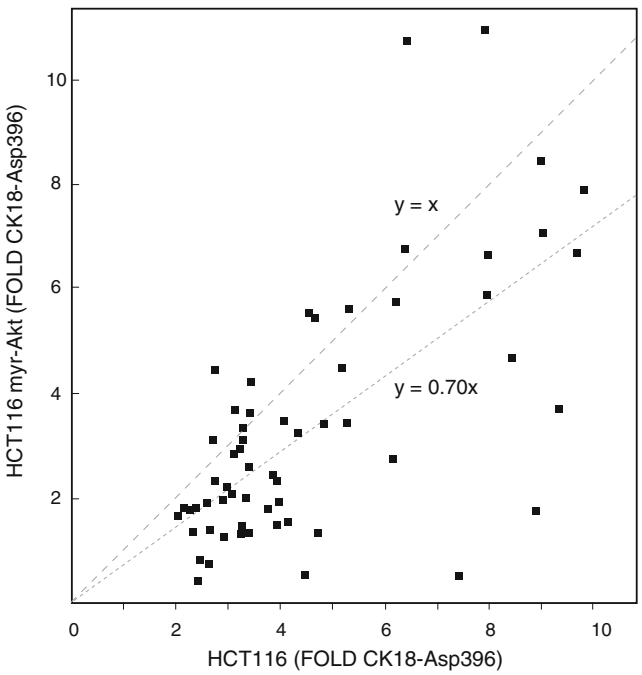

Fig. 2 Apoptotic responses of myr-AKT and control HCT1116 cells treated with 2.5 μM of NCI Mechanistic Set agents. With a minority of agents, 5 μM was required. Caspase-cleaved CK18 was measured in extracts and medium after 24 h of treatment using the M30 CytoDeath® ELISA. Only agents inducing caspase-cleaved CK18 of greater than twofold control are included in the figure. The best-fit line of the dataset is shown

from the result (Fig. 2) that most compounds induced stronger apoptotic responses in control cells compared to myr-AKT cells (the slope of the best-fit curve is 0.70). However, and importantly, a number of compounds induced similar levels of apoptosis regardless of cellular AKT status.

Drugs whose ratios of apoptosis induction in HCT116-myr-AKT to control HCT116 cells were ≥0.9 were classified as AKT-insensitive. There were 17 such drugs (Table 1). Fifteen compounds were found to induce an apoptotic response in HCT116-myr-AKT cells that was <50% of control cells and were classified as AKT-sensitive (Table 2).

Characteristics of compounds inducing similar levels of apoptosis in myr-AKT and control HCT116 cells Based on responses in the NCI60 cancer cell line panel, the profiles of cell growth inhibition of the drugs tested by the NCI Developmental Therapeutics Program have been assembled into SOMs, where the position of a particular drug reflects its main target process or mechanism of action [32]. Most of the agents currently in clinical use map to the M and S areas of these maps (M = mitosis, S = nucleic acid synthesis; Fig. 3a). We used a web-based tool to determine where the apoptotic compounds map on these SOMs (see "Material and methods"). Approximately half (34/62) of the compounds identified by screening as inducing apoptosis in HCT116 cells (shown in Fig. 2) mapped in the M and S areas and 15 mapped to the Q region which is associated with metabolic stress.

AKT-sensitive and AKT-insensitive compounds showed different distributions on the maps (Fig. 3c, d). AKT-sensitive drugs mapped in the S region (6/15), in the Q region (6/15), but not in the M region (0/15). In contrast, 11/17 AKT-insensitive drugs mapped in the M-region and only 2/17 in the S region. The difference in distribution of AKT-sensitive and AKT-insensitive drugs to the M area was statistically highly significant (Fisher exact test: $p=0.00011$).

As expected, the group of AKT-insensitive drugs mapping to the M region included typical microtubuli-targeting drugs such as colchicine, vinblastine, and podophyllotoxin. In addition, drugs such as actinomycin-D, tetrocarcin, and thaspine were found in this group. Whereas actinomycin-D has been observed to inhibit tubulin polymerization in vitro [33], such activities have not been reported for tetrocarcin A or thaspine. Tetrocarcin A has been reported to inhibit Bcl-2 function [28], while the mechanism of thaspine cytotoxicity is not well characterized.

Table 1 myr-AKT-insensitive drugs

NSC number	Name	SOM Region(s)	Cancer active[a]
757	Colchicine	M	Leukemia Carcinoma Sarcoma
18268	Dactinomycin	M	Leukemia Carcinoma
24819	Peltatin	M	Leukemia Carcinoma
24818	Podophyllotoxin	M	Leukemia Sarcoma
33410	Colchicine, N-Benzoyl-deacetyl	M	Leukemia Melanoma
49842	Vinblastine	M	Leukemia Carcinoma
76022	Thaspine	M	Sarcoma
83265	Tritylcysteine	N	Leukemia Carcinoma
85236	Helenalin	Q	Leukemia
219734		M	Leukemia Carcinoma
333856	Tetrocarcin	M	Melanoma
345647	Chaetochromin	M	Carcinoma
639828		M	nt
647889		P	nt
651079		S	nt
687850		Q	nt
705701	Alsterpaullone	S	nt

Drugs inducing similar levels of apoptosis in parental and myr-AKT expressing HCT116 cells

[a] Drugs that induce increased survival and/or decreases in tumor mass according to testing performed at the National Cancer Institute (http://www.dtp.nih.nci.gov) were classified as cancer active

nt not tested

Table 2 myr-AKT-sensitive drugs

NSC number	Name	SOM region(s)
285116	Siomycin	P
72961	8-Adenosine	V
47147	Prodigiosin	S
152731		S
123111	Mitomycin derivative	S
162907	Ellipticine, 6-(5-hexen-1-yl-)	R
335142		S
651084		Q
687849		Q
638645		Q
636676		Q
173904	Carbamic acid, ester	Q
629971	9-Amino-20-camptothecin	S
697923		Q
146604	5-Fluorouridine	S

Drugs that induced more apoptosis in parental than in myr-AKT expressing HCT116 (ratio>2)

Of the 17 AKT-insensitive drugs, 12 have been tested for anti-tumor activity by the Developmental Therapeutics Program (http://dtp.nci.nih.gov). All 12 were found to have antitumor activity in animal models (Table 1). Three of the active compounds are well-known cytostatics, podophyllotoxin, vinblastine, and actinomycin-D. Of the remaining, NSC219734 is a DNA intercalating diacridine [15]. NSC526417 is dithiocarbamate, an agent found to have activity on leukemias and carcinomas. Finally, thaspine (NSC76022) is an alkaloid present in the cortex of the tree *Croton lechleri* which grows in the upper Amazon region of Peru, Ecuador, and Colombia. A red latex, Dragon's blood (Sangre de drago), is extracted from the cortex of the tree and is extensively used by different tribes of the Amazonian basin for medicinal purposes. Thaspine was previously reported to be cytotoxic [20,40] and to have antitumor activity [40] and may be an interesting anticancer drug. Helenalin (NSC85236) is often used in vitro as an NFκB inhibitor, but apoptosis induction by helenalin in the myr-AKT cells is in line with its reported inhibitory effect on AKT [3] and its SOM location in the Q region. We have reported that helenalin induces apoptosis via CaMKII, ASK1, and JNK [30].

Based on the present apoptosis screening and further analysis using SOMs, we conclude that expression of constitutively active AKT mainly affected apoptosis induced by DNA-damaging drugs, whereas AKT-insensitive apoptosis was associated mainly with drugs that interfere with the process of mitosis. This conclusion is supported by the report that expression of constitutively active AKT1 in A549 human non-small cell lung carcinoma cells resulted in increased survival in response to mitoxantrone and cisplatin but not to microtubuli-interacting agents such as paclitaxel [36]. That apoptosis induced by mitotic inhibitors is insensitive to AKT overexpression is not altogether unexpected since apoptosis induced by mitotic inhibitors is likely to be secondary to mitotic catastrophe and not a primary signaling event [27]. In contrast to these results, other authors have reported that myr-AKT confers resistance to microtubuli-interacting agents [38]. We suggest that this different result is due to the use of non-transformed IL-3 dependent hematopoietic cells rather than transformed epithelial cells. These cell types likely differ in signaling pathways that regulate survival energy metabolism and microtubule functions. Furthermore, due to

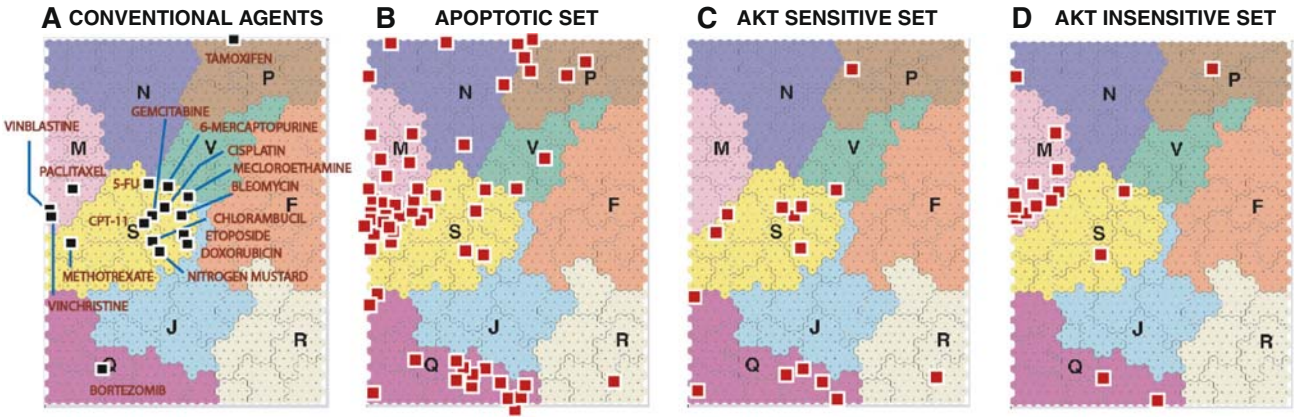

Fig. 3 Exploration of mechanisms of action of different sets of drugs using self-organizing maps (3D Mind resource; http://spheroid.ncifcrf.gov/spheroid). SOM clustering of the NCI60 GI_{50} data segregates compounds into nine major response categories: mitosis (*M*); nucleic acid metabolism (*S*); metabolic stress and cell survival (*Q*); membrane function (*N*); kinases/phosphatases and oxidative stress (*P*); and four unexplored regions, *R*, *F*, *J*, and *V* [18]. **a** Examples of drugs are in clinical use (mostly mapping in the *M* and *S* areas); **b** Mapping of 62 drugs in the NCI mechanistic set which induce strong apoptosis of HCT116 cells; **c** mapping of AKT-sensitive drugs (note the preferential location to the *S* and *Q* regions); **d** Mapping of AKT-insensitive drugs (note the preferential location to the *M* region)

the documented roles of AKT in anti-apoptotic pathways [31], we have focused exclusively on acute apoptosis, whereas the other report is based on survival seen as levels of propidium iodide exclusion after 72-h treatment [38]. Over this time interval, microtubuli-interacting agents are expected to induce mitotic arrest followed by secondary apoptosis, and it is possible that this cell death is sensitive to blocking by constitutively active AKT.

Cell-based assays have the advantage of reasonable experimental through-put while preserving disease-relevant molecular-pathway interactions [5]. This is particularly important in the field of oncology due to the complex mechanisms of action of cytotoxic drugs [37]. The conditional cell-based phenotypic screening approach used here promises be effective in defining drug mechanisms that are insensitive to overexpression of various oncogenes, loss of tumor suppressors, or other phenotypic traits. Such studies are important in order to define the proper use of cancer drugs in clinical oncology.

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