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Chapter 2

Broad In Vitro Efficacy of Plant-Derived Betulinic Acid Against Cell Lines Derived from the Most Prevalent Human Cancer Types

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Abstract

Betulinic acid (BA) is a widely available plant-derived triterpene with reported activity against cancer cells of neuroectodermal origin and leukaemia's. Treatment with BA was shown to protect mice against transplanted human melanoma and led to tumor regression. In contrast, cells from healthy tissues were resistant to BA and toxic side-effects in animals were absent. These findings have raised interest in the chemotherapeutical anti-cancer potential of BA.

A comprehensive assessment of the efficacy of BA against the clinically most important cancer types is currently lacking. Therefore, we tested the in vitro sensitivity of broad cell line panels derived from lung, colorectal, breast, prostate and cervical cancer, which are the prevalent cancer types characterized with highest mortalities in woman and men. Multiple assays were used in order to allow a reliable assessment of anti-cancer efficacy of BA. After 48 hr of treatment with BA, cell viability as assessed with MTT and cell death as measured with propidium iodide exclusion showed clear differences in sensitivity between cell lines. However, in all cell lines tested colony formation was completely halted at remarkably equal BA concentrations that are likely attainable in vivo. Our results substantiate the possible application of BA as a chemotherapeutic agent for the most prevalent human cancer types.

Introduction

Betulinic acid (BA) is a plant derived pentacyclic lupine-type triterpene, which was discovered in a National Cancer Institute drug screening program of natural plant extracts, and has been recognized to possess potent pharmacological properties [1]. BA and derivatives thereof have been shown to exert anti-inflammatory [2], anti human immunodeficiency virus (HIV) [3, 4] and, most notably, anti-cancer activities. BA can be isolated from numerous botanical sources [5, 6], and its structurally related precursor, betuline, which can be readily converted into BA [7], is contained in higher quantities in widespread plant sources [8]; e.g. betuline constitutes up to 22% of the bark of the white birch tree (*Betula alba*) [9].

Initially, BA was reported to induce melanoma specific cytotoxicity [1]. In athymic mice that were challenged with human melanoma xenografts and treated after one day with BA, tumor development was strongly impeded. In addition, when treatment was initiated 41 days after tumor challenge, the established tumors regressed for more than 80% [1]. Although in this study the toxicity of BA towards tumor cell lines from non melanoma origin appeared to be limited [1], in subsequent studies it was revealed that BA induced potent cytotoxicity in various other tumor types of neuroectodermal origin next to melanoma [10-13].

Fulda and coworkers demonstrated that BA induces apoptosis in neuroblastoma, medulloblastoma and Ewing's sarcoma cell lines [10], which are the most common solid tumors in childhood. Sensitivity of neuroblastoma cell lines for BA-induced apoptosis was simultaneously observed by Schmidt et al. [11]. Primary tumor cells cultured from medulloblastoma and glioblastoma [12], glioma cell lines [13] and head and neck squamous cellular carcinoma cell lines [14] were also sensitive to BA-induced cytotoxicity. However, more recent studies disproved the selectivity of BA for neuroectodermal-derived tumors. Zuco et al. reported anti-proliferative capacity of BA in vitro in tumor cell lines originating from different tissues [15]. This study also addressed the in vivo activity of BA by showing enhanced survival times in mice grafted with a human ovarian carcinoma when treated with BA [15]. Subsequently, BA was also shown to induce apoptosis in haematological malignancies where 65% of primary pediatric acute leukaemia cells and all cell lines of this type were sensitive for BA in vitro [16].

Next to the broad specificity of BA for multiple tumor types, BA was reported to be devoid of cytotoxic effects against healthy cells. Normal human fibroblasts [15], peripheral blood lymphoblasts [15], melanocytes [17] and astrocytes [13] were shown to be resistant against BA treatment in vitro. Also, systemic in vivo toxicity was not apparent in mice treated with BA up to 500 mg/kg bodyweight [1, 15] and an earlier study did not detect BA-induced toxicity in rats as monitored with the so-called Hippocratic screening test [18].

Although the precise mechanisms contributing to BA-induced cell death have still to be unravelled in detail, several studies have provided considerable insight in BA-induced cytotoxicity. BA was shown to induce apoptosis in a p53 independent

manner [10, 13, 15, 17] by a direct effect on mitochondria [19]. In neuroectodermal cells BA induced mitochondrial membrane permeabilization [10, 20, 21] facilitating the release of cytochrome C, apoptosis-inducing factor (AIF) [19] and Smac [16]. Formation of reactive oxygen species and protein neosynthesis have been reported to be required for BA-induced cell death [10, 13, 21] and proapoptotic mitogen-activated protein kinases (MAPKs) were found to be involved [21]. Bcl-2 or Bcl-XL overexpression or treatment with bongkreikic acid, a reported stabilizer of the permeability transition pore complex, inhibited cytochrome C release and BA-induced apoptosis [10, 13, 20]. Finally, reports have also described that inhibition of topoisomerases may be involved in BA-induced cell death as an additional mechanism [22, 23].

The specific cytotoxicity induced by BA in a diversity of cancer types in conjunction with its lack of cytotoxicity for healthy cells has raised optimism that this reagent can be used as a non-toxic anti-cancer drug. The objective of the current study is to investigate the anti-tumor efficacy of BA against prevalent cancer types that are characterized by the highest mortalities [24].

BA treatment of broad cell line panels was monitored by three different assays to allow a reliable and representative assessment of BA-induced anti-cancer effects in these cancer types. We report that BA induced cytotoxic and anti-proliferative effects in cell lines derived from lung, colorectal, breast, prostate and cervix cancer, thus confirming the broad specificity of this reagent and substantiating its possible application in a non-toxic chemotherapy for these cancer types.

Material and Methods

Reagents

Betulinic acid (A.G. Scientific, San Diego, CA) was dissolved in dimethyl sulfoxide (DMSO) at 4 mg/ml and aliquots were stored prior to use at -80 C. ZVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) was obtained from Sigma.

Cell lines, healthy cells and cell culture

All cell lines were cultured in IMDM medium (Cambrex) containing 8% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Täby, Sweden) and maintained in logarithmic growth phase in 75 or 175 cm² culture flasks (Costar) prior to BA treatment.

Lung cancer cell lines H460, H322, H187, N417 were kindly provided by dr. F. Kruijt (dept. of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands), and non-small cell lung cancer cell lines GLC-2, GLC-4 and GLC-36 were kindly provided by dr. L. de Leij (dept. of Pathology and Laboratory Medicine, University of Groningen, The Netherlands). Colon cancer cell lines CO115, SW480, T84, HCT81 and LS180, lung cancer cell line MBA9812 and prostate cancer cell lines DU145 and PC3 were kindly provided by J. van Eendenburg and dr. A. Gorter (dept. Pathology, Leiden University Medical Center, The Netherlands). Breast cancer cell lines MCF7, SKBR3, MDA-231, MDL13E, BT474 and T47D were kindly provided by dr. E. Verdegaal (dept. Clinical Oncology, Leiden University Medical Center, The Netherlands). Breast cancer cell lines BT483, BT549 and ZR-75-1, and prostate cancer cell line 22Rv1 were obtained from the American Type Culture Collection. All other cell lines are from our laboratory.

Peripheral blood mononuclear cells (PBMC), cytotoxic T lymphocytes (CTL) clones and activated B cells were generated from blood obtained from healthy donors and cultured with cytokines to maintain viability as described [25].

Analysis of cell death and cell viability

Adherent cells were seeded in 6 well plates (300.000 cells/well) 24 hr prior to BA application. Suspension cells were seeded in 12 well plates (300.000 cells/well) directly before addition of BA.

Cells were treated with BA at indicated concentrations. To exclude differential effects of DMSO, for all concentrations as well as the control, DMSO was compensated to an equal concentration of 0.5%. Cells were harvested at indicated time points, resuspended in 1 ml IMDM complete medium and subsequently divided over the 3 different assays, which guaranteed equal treatment levels.

Cell death was determined by propidium iodide (PI; Molecular Probes) exclusion as previously described [26]. In short, treated cell lines were harvested and cell samples of 300 μ l were stained with PI at 1 μ g/ml for 15 min. Samples were measured by flow cytometry using a FACSCalibur system (Becton Dickinson, San Jose, CA, USA) and analysed using CellQuest software.

For quantification of apoptotic DNA fragmentation (Nicoletti assay) cells were resuspended in Nicoletti buffer containing 50 μ g/ml PI for at least 24 hr as described [27], subsequently flow cytometric measurement of PI stained nuclei was performed.

For the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability assay[28], which measures metabolic activity, 100 μ l of the treated cells were tested in triplicates in 96 well plates, subsequently 20 μ l MTT (2.5 mg/ml) was added and 2 hr later 100 μ l solubilisation buffer (75% DMSO, 5% SDS) was added. Optical density (OD) was measured after 24 hr at 560 nm (reference wavelength 655 nm) using a spectrophotometer. For data analysis, the background OD (100 μ l medium without cells, 20 μ l MTT, 100 μ l solubilisation buffer) was subtracted from each sample value. In experiments where caspase activity was blocked with the pan-caspase inhibitor zVAD.fmk, 20 μ M was applied 2 hours prior to BA-treatment and every 12 hr additional zVAD.fmk (10 μ M) was added.

Clonogenic assay

Depending on the plating efficiency of each cancer cell line, 100-1000 cells were seeded in duplicates in 6 well plates. BA was applied after 24 hr at indicated concentrations and cells were cultured without change of medium until macroscopic colonies were detected in the untreated control (usually about 6-8 days). Colonies were counted after fixation with 6% glutaraldehyde and staining with crystal violet.

Immunoblot analysis of cleaved poly-ADP ribose polymerase (PARP)

PARP western analysis was performed essentially as described before [29]. In short, cells were lysed for 20 min at 4 C in Triton X-100 buffer (1x10⁶ cells per 40 μ l) and centrifuged at high speed for 8 min. Samples of 500.000 cells were loaded per lane. Proteins were separated by 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) transfer membrane (Amersham Biosciences, Piscataway, NJ). The blot was blocked with 5% low fat milk powder (in phosphate buffered saline (PBS), 0.2% Tween-20) and probed with primary anti-PARP polyclonal antibody (1:2500, Cell Signaling) overnight. The membranes were washed in PBS with 0.1% Tween, incubated with horseradish peroxidase (HRP) conjugated anti-rabbit IgG (1:10000) as secondary antibody (Southern Biotechnology Associates, Birmingham, AL) and visualized by chemiluminescence (ECL; Amersham Biosciences). Experiments were repeated two times.

Results

BA has been reported to be active against cancer cell lines from neuroectodermal origin [10-13]. However, since additional data also suggested activity of BA against various different cancer types, we set out to investigate its cytotoxic potential in a panel of cell lines of those prevalent cancer types that are characterized with the highest mortality rates.

In the U.S., breast cancer and prostate cancer are the most commonly diagnosed cancer types for women and men respectively. However, mortality rates show that lung cancer is the most common fatal cancer in men (31%), followed by prostate cancer (10%), and colorectal cancer (10%). In women, lung (27%), breast (15%), and colorectal cancer (10%) are the leading causes of cancer death [24]. In contrast, in the developing countries cervical cancer is most frequently resulting in cancer death among woman [30, 31].

A comprehensive survey of BA-induced cell death for these cancer types has not yet been conducted. Only fragmentary and sometimes conflicting reports revealed that cell lines derived from lung cancer [15, 32, 33], colon cancer [34], prostate cancer [34] and cervical cancer [15] can be sensitive to BA-treatment. Therefore, in the current study, broad cell line panels derived from these tumor types were collected and tested for BA sensitivity in multiple assays monitoring overall cell death, metabolic activity as a measure for cell viability and clonogenic survival. First, the T cell leukemia cell line Jurkat was tested as a positive control to validate our assays and compared to healthy cell types to confirm the reported relative non-toxicity of BA against non-cancer cells under our experimental conditions.

Sensitivity to BA treatment of the Jurkat leukemia cell line and healthy cells

Because BA has been reported to act via different pathways [5], overall BA-induced cell death was our primary focus. In addition, cell metabolism and viability were assessed by the MTT dye assay. Moreover, the percentage of cells showing DNA fragmentation, which is one of the typical characteristics of apoptosis, was measured. The Jurkat T cell leukemia cell line (variant J16 [35]) was chosen as positive control because it was proven sensitive for BA treatment before [16]. Analysis of flowcytometric measurements (Fig 1A) showed that BA applied at 10 $\mu\text{g/ml}$ induced cell death that was dependent upon length of incubation, starting with approximately 30% cell death after 24 hr and leading to more than 80% cell death after 3 days of incubation (Fig 1B). This coincided with a comparable level of cells displaying DNA fragmentation at these time points (Fig 1B). Remarkably, cell metabolism, as measured in the MTT assay, was affected much more rapidly when J16 was treated with 10 $\mu\text{g/ml}$ BA. In this assay, inhibition was already observed after 6 hr. This difference between the sensitivity of J16 for BA observed in the MTT assay and the cell death assay was also observed in dose response curves. That is, a sharp decline in MTT conversion was observed already with 2.5 $\mu\text{g/ml}$ BA treatment, whereas BA concentrations of 7.5 $\mu\text{g/ml}$ and higher were

needed to induce cell death and DNA fragmentation at the same time point (48 hr) (Fig 1C). Nevertheless, concentrations of 10 $\mu\text{g/ml}$ and higher resulted in comparable toxicity when measured by MTT conversion, PI exclusion and DNA fragmentation (Fig 1C). This suggests that BA has a stronger effect on MTT conversion and affects membrane integrity (PI) and apoptosis (Nicoletti) only at higher concentrations.

To which extend the cell death induced by BA is fully caspase-dependent is still a matter of debate [5]. We therefore treated J16 with BA under conditions where caspase activity was abrogated by co-treatment with the pan-caspase inhibitor zVAD-fmk. This resulted in a complete inhibition of DNA fragmentation (Fig 1D) and (caspase-mediated) cleavage of Poly (ADP-ribose) polymerase (PARP) (Fig 1H). However, the overall induction of cell death was not reduced at all by zVAD.fmk co-treatment (Fig 1E), indicating that BA-mediated cytotoxicity ensues as efficiently when caspases are blocked. Such caspase-independent cell death has been described for other chemotherapeutics as well [36].

Because BA-induced cytotoxicity is often measured in serum free or low serum conditions in the literature[16], we compared the results of BA treatment of J16 in the presence and absence of fetal calf serum (FCS). Without FCS, J16 was more sensitive for BA (Fig 1F), which may reflect the additional effect of growth factor deprivation or may be due to the fact that BA is sequestered by serum proteins.

Finally, to ascertain that BA is non-toxic to healthy cells as was reported before [15], we tested human blood-derived PBMC, cytotoxic T lymphocyte clones and activated B cells. These cell types were highly resistant for BA-induced cytotoxic effects as measured with PI exclusion after 48 hr (Fig 1G), which is in line with the literature [15].

The primary interest of the current study is to test BA-induced anti-cancer effects irrespective from the precise mechanisms attributing to it. However, as BA may induce cell death by multiple, likely intertwined, and possibly cell type dependent mechanisms, we decided to test for multiple read-outs. In the first place, the cancer cell lines were screened for overall cell lethality by PI exclusion after 48 hr incubation with different concentrations of BA. Additionally, effects on cell survival and proliferative capacity were monitored by a clonogenic growth assay, which is time point independent. Finally, the MTT assay was included in the screening because it was found to sensitively monitor an early inhibition of cell metabolism at low BA concentrations that is missed by measuring PI exclusion alone (Fig 1B). The assays were performed in the presence of FCS because this more physiologically mimics *in vivo* circumstances than serum free conditions.

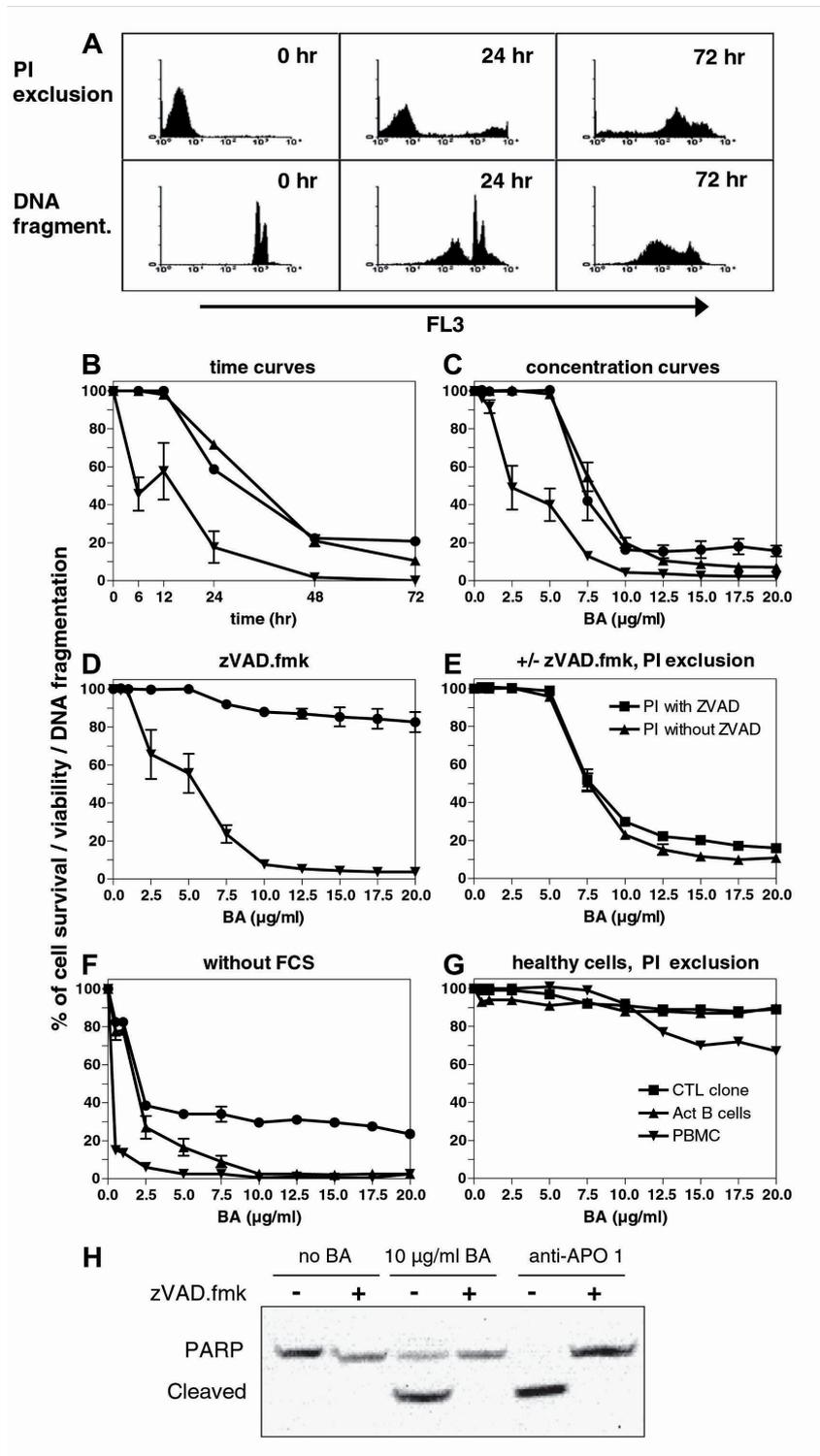


Fig 1. BA sensitivity of T cell leukemia cell line Jurkat and healthy cells

PI exclusion was used to measure cell death (▲), DNA fragmentation to measure apoptosis (●), and the MTT dye assay to measure cell viability (▼) (symbols used in panels B, C, D, G). (A) Flowcytometric FL3 histograms of Jurkat cells (J16) treated with BA (10 µg/ml) after cellular staining with PI (to measure cell death) or nuclear staining with PI (to measure DNA fragmentation). (B) Kinetics of BA-induced effects in Jurkat cell line when treated with 10 µg/ml BA. (C) Dose response curves of BA treatment measured at 48 hr. (D) Dose response curves of DNA fragmentation and MTT conversion at 48 hr in the presence of pan-caspase inhibitor zVAD.fmk. (E) Dose response curves of BA treatment in the cell death assay at 48 hr with and without pan-caspase inhibitor zVAD.fmk. (F) Dose response curves of BA treatment at 48 hr in conditions of serum free medium. (G) Dose response curves in the cell death assay of healthy cell types (PBMC, CTL clones and activated B cells) after 48 hr of BA treatment. (H) BA (10 µg/ml) induced PARP cleavage measured at 48 hr in the presence and absence of zVAD.fmk. Anti-APO1 induced PARP cleavage was included as control. Mean values and error bars (SEM) are derived from at least three experiments performed.

Sensitivity to BA treatment of lung cancer cell lines

A panel of 10 lung cancer cell lines was collected. This panel was chosen to consist primarily of cell lines derived from non-small cell lung cancer (NSCLC) because this is the most common form of lung cancer, accounting for 80% of all lung cancer cases (in the US). Non-small cell lung cancer cell lines SW1573, H460, A549, H322, GLC-2, GLC-4, GLC-36 and small cell lung cancer lines H187, N417 and MBA9812 were tested in the three assays afore mentioned (Fig 2). Overall cell death, as measured by PI exclusion, showed that 4 cell lines (A549, H187, N417, MBA9812) were efficiently killed with BA treatment at 20 µg/ml, because less than 10% of the cells were viable after 48 hr incubation (Fig 2, Table 1). At the other end of the spectrum, half maximal cell death at 48 hr was not reached for SW1573. The remaining 5 cell lines displayed intermediate BA sensitivity. The BA concentration needed for half maximal cell death (50% effective concentration; EC₅₀) at 48 hr for the lung cancer cell lines (SW1573 excluded) ranged from 6.1 to 12.3 µg/ml (Table 1). In contrast, all lines that could be tested in the clonogenic assay (8 out of 10) were halted in their growth and mostly at concentrations that were lower than the EC₅₀ derived from the PI exclusion assay (Fig 2, Table 1).

Measurement of MTT conversion at 48 hr showed a biphasic character for most cell lines, i.e. a sharp decrease was observed at low BA concentrations (up to 2.5 µg/ml), followed by a more gradual decline at higher BA concentrations. Overall, in the MTT assay, 50% reduction in MTT conversion was reached for all 10 lung cancer cell lines and ranged from 1 to 8 µg/ml (Table 1). The BA-induced effects in the MTT assay were more intense than the cytotoxic effects measured with PI exclusion for all these cell lines (Fig 2, Table 1).

Table 1
BA sensitivity of lung, colorectal, breast, prostate and cervical cancer cell lines

Cancer type	Clonogenic growth halted at ^a (µg/ml)	Cell death ^b		Viability ^c EC ₅₀ (SEM) (µg/ml)
		EC ₅₀ (µg/ml)	Max % at 20 µg/ml	
<i>Lung cancer</i>				
SW1573	10	NR ^d	47	2.2 (0.3)
H460	7.5	6.1	75	3.8 (0.4)
A549	7.5	8.3	90	2.6 (0.1)
H322	7.5	12.3	55	7.3 (0.3)
GLC-2	7.5	8.8	65	6.8 (0.1)
GLC-4	7.5	10.0	70	5.9 (0.2)
GLC-36	5	9.6	75	8.0 (0.1)
H187	NT ^d	8.7	92	2.1 (0.1)
N417	NT	6.2	85	1.0 (0.1)
MBA9812	7.5	7.6	99	2.3 (0.4)
<i>Colorectal cancer</i>				
SW1463	10	3.8	69	2.8 (0.3)
SW837	7.5	11.3	75	8.6 (0.3)
RKO	7.5	9.5	95	8.6 (0.3)
CO115	10	12.2	60	5.8 (1.7)
SW480	5	15.1	61	2.5 (0.1)
T84	10	11.3	61	11.6 (0.3)
HCT81	12.5	16.4	54	6.7 (1.7)
DLD1	10	NR	45	2.5 (0.2)
HT29	10	NR	40	5.2 (0.2)
LS180	10	11.7	62	7.7 (1.1)
<i>Breast cancer</i>				
MCF7	10	NR	22	8.3 (1.2)
SKBR3	7.5	16.2	46	5.7 (0.1)
MDA231	7.5	10.4	63	6.4 (0.1)
MDL13E	7.5	11.5	71	2.4 (0.1)
BT483	NT	12.8	67	11.6 (0.1)
BT474	NT	12.1	79	5.8 (1.2)
T47D	7.5	13.0	56	2.5 (0.1)
BT549	7.5	5.5	95	3.3 (0.1)
ZR-75-1	NT	NR	11	NR
<i>Prostate cancer</i>				
DU145	7.5	11.6	63	5.3 (0.3)
PC3	7.5	12.3	58	2.3 (0.3)
22Rv1	5	10.1	73	8.2 (0.1)
LNCaP	7.5	11.9	65	2.8 (0.5)
<i>Cervical cancer</i>				
CaSki	7.5	9.6	63	6.7 (0.2)
HeLa	7.5	14.3	60	1.8 (0.1)
SiHa	7.5	11.8	67	5.0 (0.8)

^a BA concentration at which clonogenic growth stopped was determined in duplicate with equal results.

^b Cell death as measured with PI exclusion at 48 h. Both EC₅₀ and % cell death with 20 µg/ml BA are provided.

^c Cell viability as measured in triplicate by the MTT assay at 48 h. EC₅₀ and SEM values are provided.

^d NR, not reached; NT, not tested (no colony formation in the untreated control).

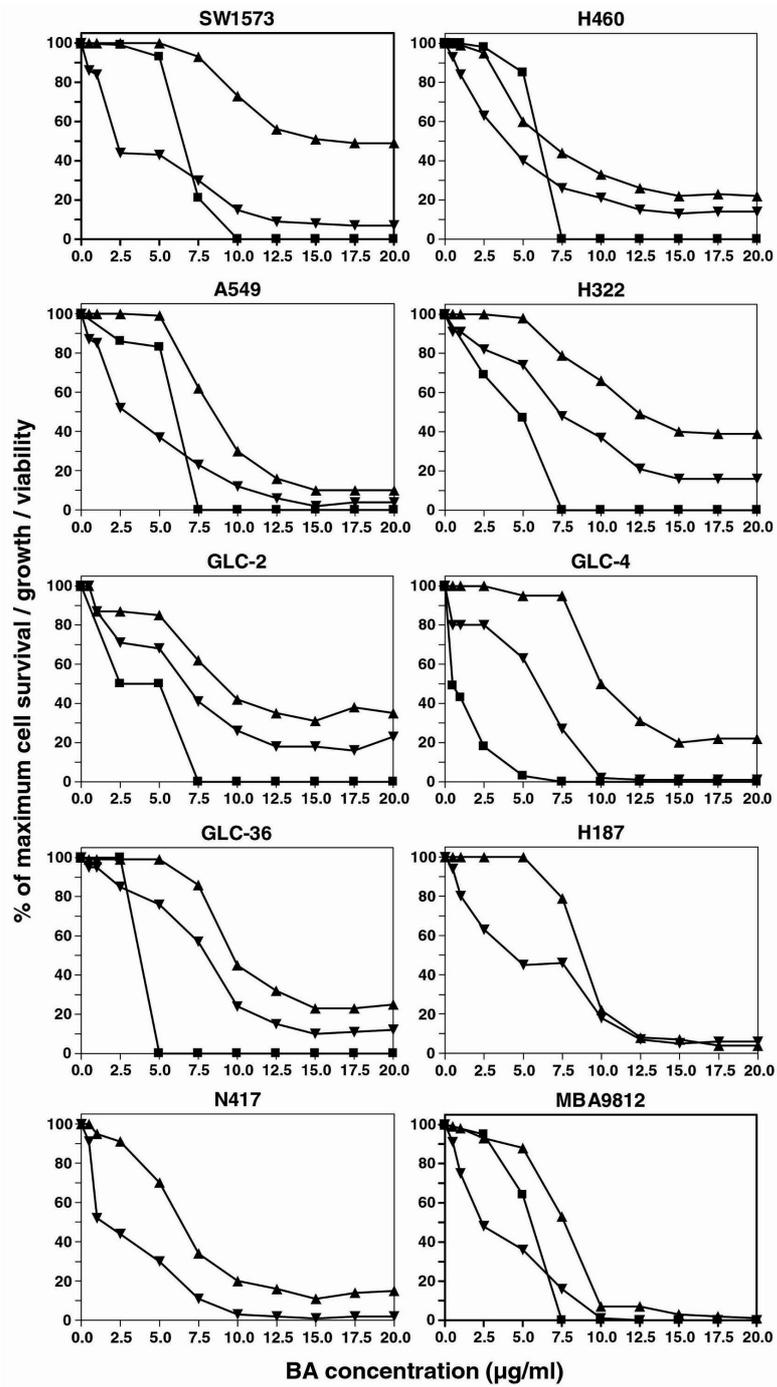


Fig 2. BA sensitivity of 10 lung cancer cell lines. Cytotoxic effects measured with PI exclusion at 48 hr (▲), cell viability measured at 48 hr with MTT conversion (▼) and clonogenic growth (■) were tested for the indicated concentrations of BA.

Sensitivity to BA treatment of colorectal cancer cell lines

A panel of 10 colorectal cell lines, consisting of rectal cancer cell lines SW1463 and SW837 and colon cancer cell lines RKO, CO115, SW480, T84, HCT81, DLD1, HT29 and LS180 was tested for BA-induced cytotoxic effects. Marked differences between the cell lines were observed for overall cell death at 48 hr as measured by PI exclusion. This ranged from sensitive cell lines, e.g. for the most sensitive cell line RKO only 5% viable cells were left after treatment with 20 µg/ml BA, to relatively insensitive cell lines, e.g. half maximal lysis (EC_{50}) was not reached for DLD1 and HT29 at the highest concentration tested (Fig 3). EC_{50} values in the cell death assay for the colorectal cell lines, excluding DLD1 and HT29, ranged from 3.8 to 16.4 µg/ml BA (Table 1).

Despite this incomplete cytotoxic effect at 48 hr, all cell lines were completely inhibited in their clonogenic growth by BA treatment. Clonogenic proliferation of 9 out of the 10 cell lines was halted at 10 µg/ml BA or below, while only HCT81 was slightly less sensitive (colony formation was inhibited at 12.5 µg/ml) (Fig 3, Table 1). Thus, long term incubation with BA resulted in a complete anti-proliferative and/or cytotoxic effect for all colon cell lines tested. Similar to the lung cancer cell lines, viability of all colon cancer cell lines in the MTT assay was affected by treatment with BA concentrations that were lower (EC_{50} range 2.5 - 11.6 µg/ml) than the BA concentrations needed for half maximal cell death at 48 hr (Fig 3, Table 1).

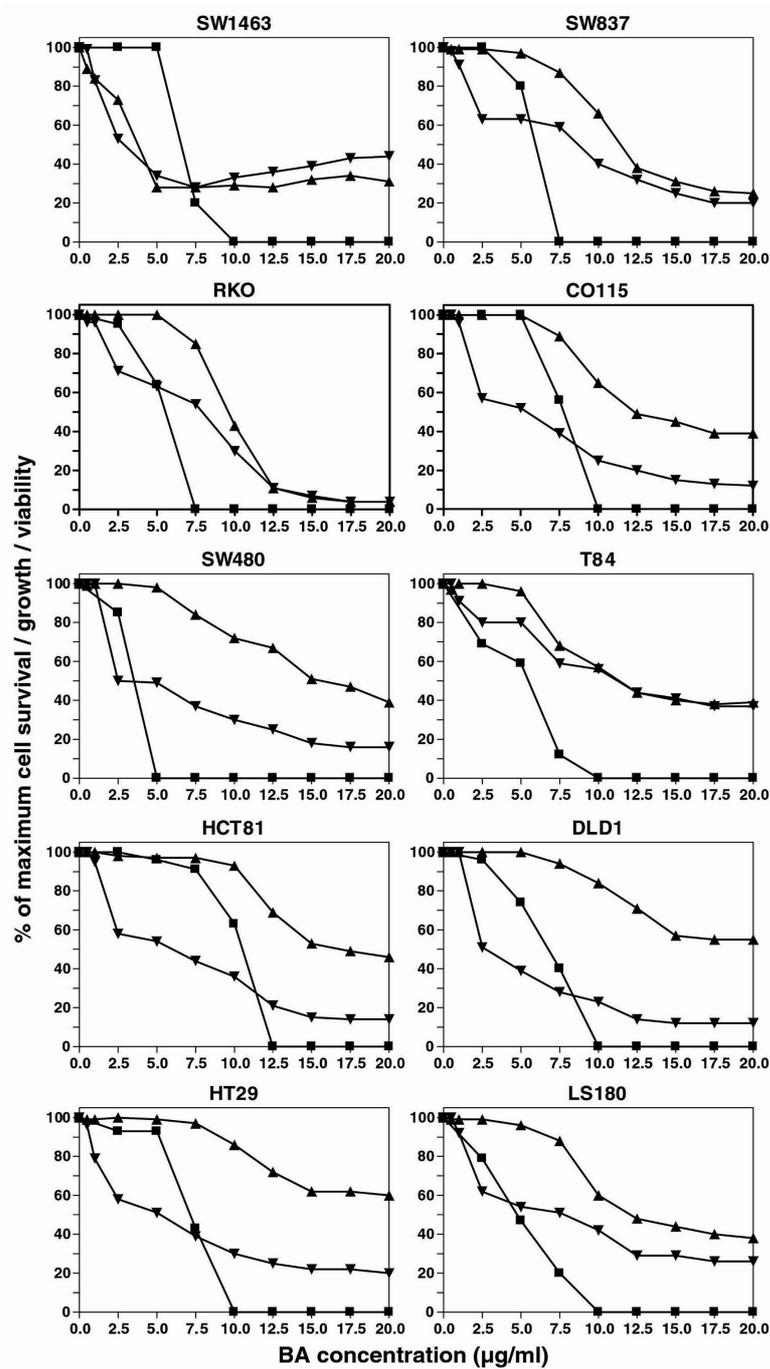


Fig 3. BA sensitivity of 10 colorectal cancer cell lines.
Cytotoxic effects measured with PI exclusion at 48 hr (▲), cell viability measured at 48 hr with MTT conversion (▼) and clonogenic growth (■) were tested for the indicated concentrations of BA.

Sensitivity to BA treatment of breast cancer cell lines

Subsequently, BA treatment of 9 breast cancer cell lines (MCF7, SKBR3, MDA231, MDL13E, BT483, BT474, T47D, BT549, ZR-75-1) was analysed.

Two of these cell lines, MCF7 and ZR-75-1, were almost resistant to BA-induced cell death at 48 hr when assayed with PI exclusion (Fig 4). Apart from MCF7 and ZR-75-1, the 7 other breast cancer cell lines reached half maximal lethality with BA concentrations ranging from 5.5 to 16.2 $\mu\text{g/ml}$ (Table 1). In some of these cell lines the highest BA concentration tested (20 $\mu\text{g/ml}$) induced significant levels of cell death, e.g. BT549 was killed for 95% after 48 hr, others were less sensitive (maximal lethalities induced by 20 $\mu\text{g/ml}$ BA varied between 95% and 44%) (Fig 4). In the clonogenic assay, the 6 breast cancer cell lines that could be tested (BT483, BT474 and ZR-75-1 failed to form colonies at all) were all halted in their clonogenic potential at 7.5 or 10 $\mu\text{g/ml}$, indicating a blockage of clonogenic growth at relatively low BA concentrations as compared to the other assays (Fig 4, Table 1). It should be noted that MCF7, which completely lacked BA-induced cell death at 48 hr, was among these cell lines. Importantly, this cell line is caspase 3 and 10 deficient [37], which indicates that caspase 3/10 deficiency does not protect MCF7 from an abrogated colony formation induced by BA treatment, which is consistent with the fact that zVAD.fmk did not protect Jurkat T cells (Fig 1E). In the MTT assay we again observed a biphasic character of BA-sensitivity in 7 out of 9 breast cancer lines. This consisted of a sharp decline in MTT conversion at low BA concentrations followed by a more gradual reduction at higher concentrations. Only BT483, lacking the initial high responsive phase, and ZR-75-1, lacking the further reduction in MTT conversion with BA treatment above 2.5 $\mu\text{g/ml}$, did not show this biphasic response pattern. Thus, ZR-75-1 was the only cell line showing high resistance against BA treatment in both the cell death assay and MTT conversion assay. The other breast cancer cell lines were highly sensitive for BA in the MTT assay and the EC_{50} values in this assay, ranging from 2.4 to 11.6 $\mu\text{g/ml}$, were considerably lower than the BA concentrations needed for half maximal cell death measured by PI exclusion.

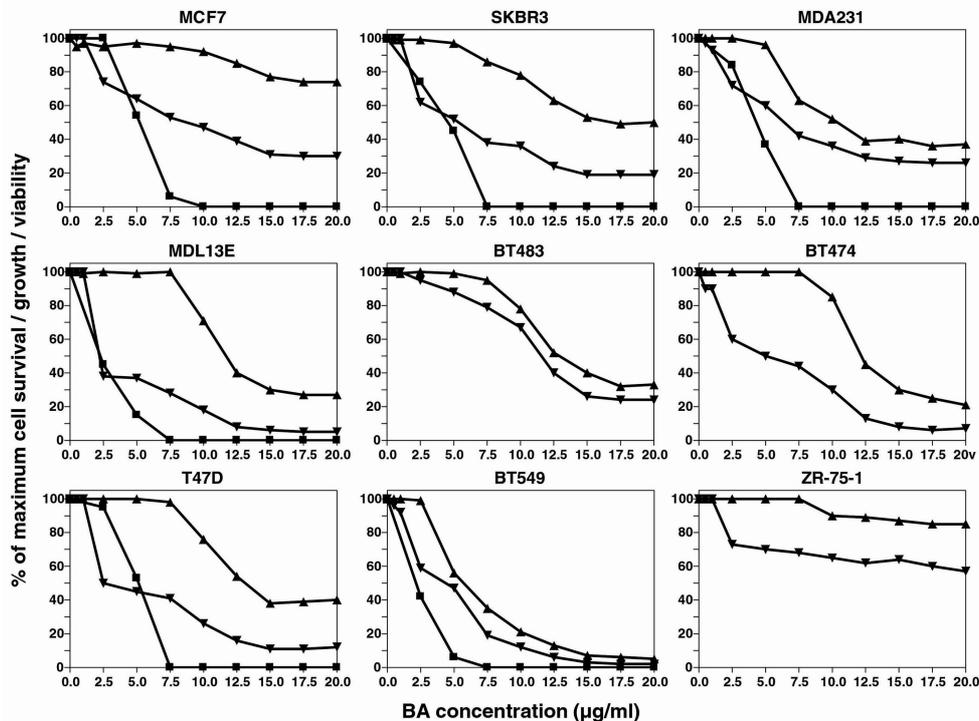


Fig 4. BA sensitivity of 9 breast cancer cell lines.

Cytotoxic effects measured with PI exclusion at 48 hr (▲), cell viability measured at 48 hr with MTT conversion (▼) and clonogenic growth (■) were tested for the indicated concentrations of BA.

Sensitivity to BA treatment of prostate and cervix cancer cell lines

Finally, we tested cell line panels derived from prostate cancer (DU145, PC3, 22Rv1, LNCaP) and from cervix carcinoma (CaSki, HeLa, SiHa). Where prostate cancer is the second cause of death from cancer in man [24], cervical cancer is leading to the highest cancer mortality in women in developing countries [30].

In general, treatment with BA of these cell lines resulted in a similar response pattern as observed in lung, colorectal and breast cancer cell lines. Maximal levels of cell death in these 7 cell lines after treatment with 20 µg/ml BA for 48 hr ranged from 73% to 58% as measured with PI exclusion (Fig 5, Table 1) and the EC₅₀ values in this assay varied between 9.6 and 14.3 µg/ml for prostate and cervix cancer cell lines together (Table 1). Clonogenic growth was halted at 7.5 µg/ml for all cell lines, except 22Rv1 which was slightly more sensitive (Fig 5). In all cell lines, a sharp decrease in the level of MTT conversion was again observed at low BA concentrations and the half maximal effect in this assay was reached at markedly lower concentrations of BA than the EC₅₀ values in the PI exclusion assay (Table 1).

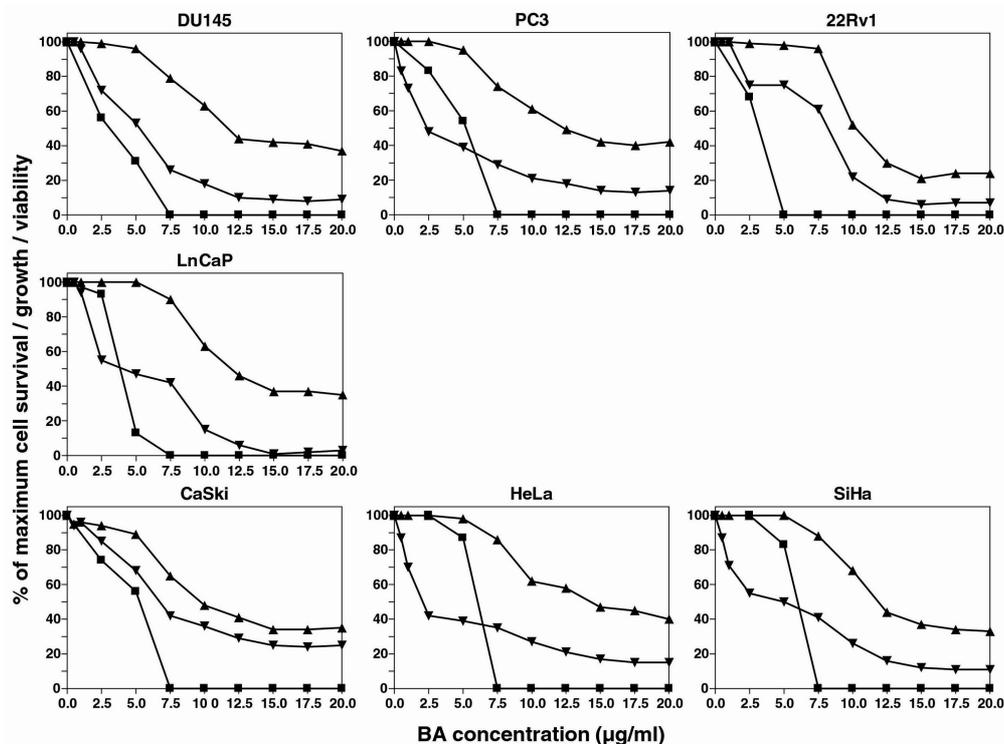


Fig 5. BA sensitivity of 4 prostate cancer cell lines and 3 cervical cancer cell lines.
 BA sensitivity of 4 prostate cancer cell lines (DU145, PC3, 22Rv1, LnCaP) and 3 cervical cancer cell lines (CaSki, HeLa, SiHa) as measured with 3 different assays. Cytotoxic effects measured with PI exclusion at 48 hr (▲), cell viability measured at 48 hr with MTT conversion (▼) and clonogenic growth (■) were tested for the indicated concentrations of BA.

Discussion

Although the efficacy of chemotherapy and other standard therapies for the majority of cancer types has been improved during the last decades, the treatment of most human malignancies is still facing high mortality rates. Moreover, toxic side-effects of the current chemotherapeutic drugs are often causing a severe reduction in the quality of life. Therefore, the development of novel potent, but non-toxic anti-cancer reagents is worth a continuous effort. Since its re-discovery in the 1990s BA has attracted considerable attention as a potential anti-neoplastic drug that may lack toxic effects towards healthy tissues.

The results of the current study show that BA treatment halted in vitro clonogenic growth of all cell lines tested - either derived from lung, colorectal, breast, prostate or cervix cancer - at remarkable uniform concentrations. Twenty seven out of 31 cell lines that were tested stopped to develop colonies at either 7.5 µg/ml or 10 µg/ml and the other 4 cell lines showed only slightly different sensitivities in this assay (Table 1). A much greater variety between the cell lines was observed in

overall cell death after 48 hr. Treatment with 20 $\mu\text{g/ml}$ BA resulted in maximum lethality at 48 hr ranging from over 90% in 5 cell lines (derived from lung, colon and breast cancer) to less than 25% in breast cancer cell lines MCF7 and ZR-75-1 (Table 1). For MCF7 this may be explained by its deficiency in caspase 3 and 10 [37], with that depriving this cell line from a main component (caspase 3) in the downstream apoptosis pathway. It should be noted, however, that co-treatment of BA together with ZVAD.fmk did not result in a significantly reduced cytotoxicity in Jurkat cells (Fig 1E), suggesting that caspase 3 deficiency is not the primary reason for the resistance of MCF7 after 48 hr. Importantly, the results of long term BA treatment in the clonogenic assay suggest that BA-induced cell death is rather delayed than absent in this cell line.

Despite the big differences in lethality within the different panels (as measured by PI uptake after 48 hr treatment with 20 $\mu\text{g/ml}$ BA) there were no major differences in BA-induced cell death between the panels (Table 1). Our observations indicate an approximate equal sensitivity for BA-induced cell death of lung cancer, colorectal cancer, breast cancer, prostate and cervix cancer.

The anti-tumor activities of BA in the cell death assay after 48 hr either expressed as EC_{50} value or as maximal lethality with 20 $\mu\text{g/ml}$ BA, did not correlate with BA sensitivities in the clonogenic assay. It is of note, for instance, that 4 out of the 5 cell lines that were not half maximally killed with 20 $\mu\text{g/ml}$ BA treatment (SW1573, DLD1, HT29 and MCF7), were not more resistant for BA in the clonogenic assay when compared to the cell lines that did reach half maximal cell death. The most remarkable cell line in this respect was MCF7, which displayed only low level of cell death after 48 hr, while colony formation was completely halted at 10 $\mu\text{g/ml}$. Therefore, BA treatment induced either strong cytostatic effects or late cytotoxic effects. The latter is more likely as we did not observe living cells after a week culture, indicating that MCF7 cells do die later on in culture.

The MTT conversion assay was included in the current study because it has frequently been used to monitor BA activity [15, 32, 34, 38-43] and because our results in the Jurkat cell line showed an early BA effect at low concentrations in this assay that was missed by monitoring cell death only (Fig 1). In the panels of cancer cell lines, we also observed a sharp drop in MTT conversion at low BA concentrations (Fig 2-5). With the exception of ZR-75-1, all cell lines reached a half maximal reduction in enzymatic MTT conversion at BA concentrations that were consistently lower than those needed for half maximal cell death as measured by PI exclusion (Table 1). For ZR-75-1, which was also refractory in the PI uptake assay, the clonogenic potential could not be assessed. Therefore, this is the only cell line for which we can not make a final judgment of its overall BA sensitivity.

We can state that it is vital to analyze multiple assays in order to determine the efficacy of BA. For instance, the moderate cell lethality observed in some cell lines at 48 hr suggested the existence of cell lines that are (partially) refractory to BA, but monitoring of colony formation revealed that BA was toxic at relatively low concentrations in all cell lines tested (Table 1). On the contrary, our results show

that the MTT assay may lead to an overestimation of the anti-cancer capacity of BA when compared to the inhibition of colony formation. Thus, the current study demonstrates the importance to monitor BA-induced effects by a combination of different assays to allow a comprehensive evaluation of the anti-cancer efficacy of BA. In this light it is important to note that some of the cell lines tested in the current study have previously been reported to be insensitive for BA. For instance, MCF7 (breast cancer) and HT29 (colon cancer) were found resistant when DNA fragmentation was monitored [10], however our approach identified these cell lines sensitive for BA in the MTT and clonogenic assays (Table 1). For MCF7, BA sensitivity in the MTT assay has also been observed by Amico et al. [38]. Similarly, BA-induced effects in LNCaP [1] and DU145 [32] (prostate cancer) were reported to be only minimal, whereas we identify these cell lines as sensitive using the different assays (Table 1). Our study does confirm the BA sensitivity of non-small cell lung cancer cell lines A549 [33] and H460 [15] and prostate cancer line PC3 [34].

In the literature only a few cancer cell lines have been tested for BA-induced effects by the clonogenic assay [17, 44, 45], and none of the cancer cell lines tested in the current study have been monitored before in this assay. Differences between our results and the literature in the assessment of BA sensitivity of some of the current cancer cell lines (HT29, LNCaP, DU145) can be attributed mainly to the inclusion of the clonogenic assay in our study. The multiple mechanisms that possibly contribute to the cytotoxic and / or cytostatic effects of BA render the clonogenic assay, which is monitoring effects after relatively long incubation times and independent of the mechanism, an especially suitable assay to evaluate the anti-cancer potency of BA.

Overall, the results presented here identify cell lines derived from lung, colorectal, breast, prostate, and cervix cancer to be sensitive for BA treatment in vitro. Therefore, our study further substantiate the notion that BA is an anti-cancer reagent with a broad specificity that is not restricted to tumors from neuroectodermal origin only [15, 16]. Although in the cell death and MTT assays clear differences between the cell lines were observed (Table 1) and in the cell death assay partially refractory cell lines were found, the BA concentration at which colony formation was halted appeared remarkably equal for all cell lines tested, irrespective of the originating cancer source. A further evaluation of the potential applicability of BA as drug for the treatment of these clinically most important cancer types now urgently awaits animal studies. The documented low toxicity of BA against human primary cells of healthy tissues in vitro [13, 15, 17], which we confirmed (Fig 1G), as well as the reported absence of toxic side-effect in mice and rats [1, 15, 18], raise hope that the therapeutic window of BA is broad enough to reach therapeutic and/or prophylactic anti-cancer effects in vivo.

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