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N′-Formyl-2-(5-nitrothiophen-2-yl)benzothiazole-6-carbohydrazide as a potential anti-tumour agent for prostate cancer in experimental studies

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Keywords
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Abstract

Objectives Benzothiazoles (BZTs) represent organic compounds with different biological actions. In this study we aimed to investigate ten newly synthesized BZT derivatives as potential anti-tumour agents against prostate cancer *in vitro* and *in vivo*.

Methods The cytotoxic effect of these compounds was screened on the human prostate cancer cell lines PC-3 and LNCaP. The most effective compound, *N*′-formyl-2-(5-nitrothiophen-2-yl)benzothiazole-6-carbohydrazide, was further characterized regarding its dose- and time-dependent effects on cell viability and proliferation (XTT test) as well as on adhesion and spreading (real-time cell analyzer xCelligence), migration (scratch-wound repair assay) and invasion (Boyden chamber) of the cells. This BZT derivative was also tested as an inhibitor of angiogenesis (chicken chorioallantoic membrane assay), clonogenic activity (soft agar) and matrix metalloproteinase 9 (gelatin zymography).

Key findings *N*′-Formyl-2-(5-nitrothiophen-2-yl)benzothiazole-6-carbohydrazide significantly inhibited all tested properties of the prostate cancer cell lines and showed low toxic *in vitro* and *in vivo* effects. The *in vitro* anti-tumour activity of this compound was confirmed by the *in vivo* effects on PC-3 xenografts in nude mice. Tumour growth was decreased in treated compared with untreated mice.

Conclusions These results suggest the potential capacity of BZTs and in particular *N*′-formyl-2-(5-nitrothiophen-2-yl)benzothiazole-6-carbohydrazide as anti-tumour agents for the treatment of prostate cancer.

Introduction

Prostate cancer (PCa) continues to be the most common cancer in men with a predicted incidence of 241 470 for 2012 in the USA.[1] Despite progress in the early diagnosis and curative treatment options for PCa, 4–12% of patients already suffer from metastases at the time of diagnosis.[1] Approximately 30% of patients experience a relapse within 10 years following radical prostatectomy.[2] These patients require hormonal treatment and later, in the hormone refractory stage, chemotherapy. However, the limited efficacy and significant side effects of the available chemotherapeutic agents demonstrate a need for the development of novel antineoplastic agents for the treatment of advanced PCa.[3]

Benzothiazole (BZT) compounds and their active cytochrome P450 (CYP) 1A1 metabolites are chemicals with diverse pharmacological uses such as analgesics, anticonvulsants, antimicrobials and antineoplastics.[4,5] Several studies have demonstrated the potent anti-tumour effects of different BZT compounds, whereby various mechanisms targeting the basic properties of adhesion, proliferation,

migration and invasion of cancer cells have been described (reviewed by Ahmed *et al*.[6]). CYP1A1, a CYP isoform, modifies BZTs to active and inactive metabolites and essentially influences the mode of action of several BZT derivatives.[7] Recently, a suppressed secretion of several matrix metalloproteinases (MMPs) by a BZT analogue was shown.[8] This effect is of special interest since the upregulation of MMPs, among them particularly MMP-2 and MMP-9, is correlated with the degree of malignancy and tumour progression.[9] Thus, MMP-9 could be considered a potential target for the development of new anti-cancer compounds.

The antineoplastic activity of certain BZTs prompted us to synthesize novel BZT derivatives as potential agents against PCa. The aim of this study was to evaluate the *in vitro* and *in vivo* effects of these new compounds with regard to their characteristics as potential inhibitors of the essential steps of tumour development, such as cell viability, growth, adhesion, migration, invasion and neoangiogenesis, and of MMPs.

Materials and Methods

Synthesis of benzothiazole derivatives

The synthesis of the BZT compounds followed the general scheme shown in Figure 1. Ten different BZT derivatives were synthesized and used in this study. Detailed data on the synthesis procedures, including the physico-chemical characteristics of these compounds, are provided as supplementary information (Appendix S1 that accompanies the online version of the article).

Cell lines

Human prostate tumour cell lines (PC-3, LNCaP) and non-tumour epithelial cell lines (HK-2, proximal tubule epithelial cells; ARPE-19, retinal pigment epithelium cells) were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and American Type Culture Collection (Manassas, VA, USA). For cell growth, RPMI medium supplemented with 10% fetal bovine serum (FBS), streptomycin (50 μg/ml) and penicillin (50 U/ml) was used in a humidified atmosphere of 95% air and 5% CO_2 at 37°C (Gibco-InVitrogen, Karlsruhe, Germany and PAA Laboratories, Pasching, Austria).

Measurement of cell viability and cell proliferation

A 96-well microtitre plate (tissue culture grade) with 0.1 ml of RPMI medium per well was seeded with 5×10^3 PC-3 or 1.2×10^4 LNCaP cells. After 24 h at 37°C, the cells were exposed to the BZT compounds (5–100 μg/ml for 72 h), and the dose-dependent outcome on survival was evaluated compared with the vehicle control.[10] The compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with cell culture medium so that a final concentration of the DMSO solvent in the culture medium was always lower than 0.2%, a dose which was not cytotoxic and does not interfere with the colorimetric XTT test (Roche Diagnostics, Mannheim, Germany).

A 50% reduction in cell viability for the tested compounds compared with vehicle-treated cells was defined as the cytotoxic IC50. All tests were repeated three times. The most cytotoxic compound was chosen for further evaluation using the IC50 value. The time-dependent effects on cancer cell proliferation were measured as previously described.[11,12] Briefly, PC-3 (1×10^5) or LNCaP cells (2.4×10^5) were seeded in 6-well plates in RPMI containing 10% FBS and the corresponding compound at its IC50 value. Trypsinized cells were collected from compound- or vehicle-treated wells and the number of viable cells was counted with a haemocytometer at 24-h intervals for a period of 96 h. All trials were carried out in triplicate.

Cell adhesion, migration and invasion assays

The xCELLigence Real-Time Cell Analyzer (Applied Science Roche, Mannheim, Germany) was used to measure cell adhesion and followed a procedure previously presented.[11] Briefly, cell attachment and spreading of cultured cells was automatically analysed by registering the coefficient via microelectrodes placed on the bottom of a special fibronectin-coated 96-well plate as electrical impedance in the cell index.[13] The effect of BZT on the cell migration was determined by the wound-scrape repair assay in confluent cells.[14] For testing the effect on cell invasion, we followed the Boyden chamber technique as previously described using LNCaP cells (1×10^5 cells/ml).[11]

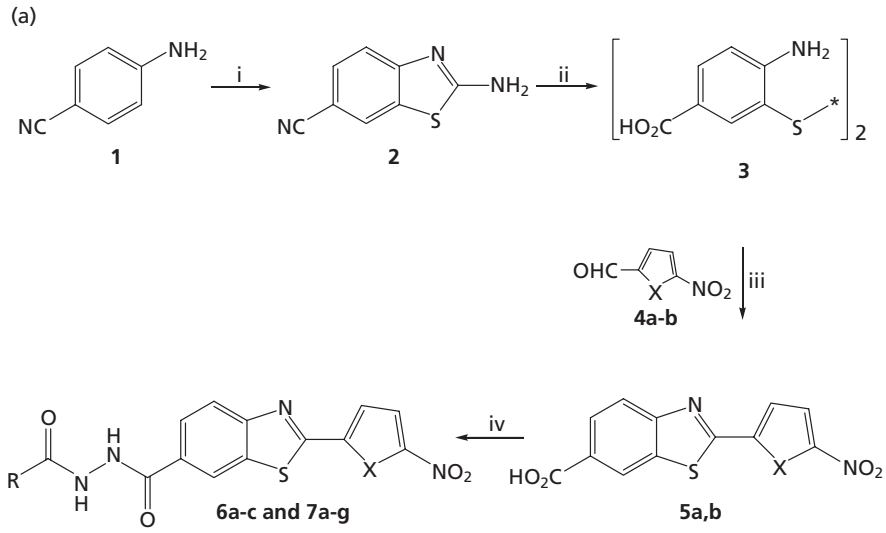
Matrix metalloproteinase zymography

Cultured human PCa cells (PC-3 and LNCaP cells, 80% confluence in 6-well plates) were incubated with the BZT compound at its respective IC50 value in serum-free medium (24 h at 37°C). This protocol was specified in previous reports for measuring the gelatinolytic activity of MMP-2 and MMP-9 in conditioned media using ImageJ Software for Windows.[11] As molecular weight markers we used pure human MMP-9 and MMP-2 proteins.

Chicken chorioallantoic membrane angiogenesis assay

The chicken chorioallantoic membrane (CAM) assay was used to demonstrate the anti-angiogenic effect of the BZT

(a)



(b)

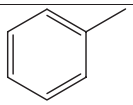
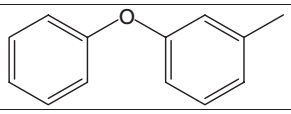
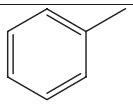
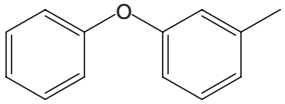
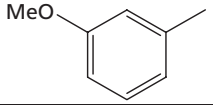
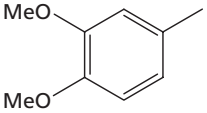
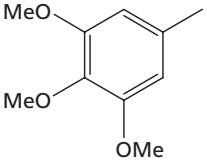
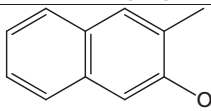
Compound	R	X
6a	H	S
6b		S
6c		S
7a	H	O
7b		O
7c		O
7d		O
7e		O
7f		O
7g		O

Figure 1 Scheme of synthesis of benzothiazole derivatives (**6a–c**, **7a-g**). i, NH_4SCN, Br_2, rt; ii, 30% KOH/MeOH, Δ, HCl; iii, nitrobenzene, Δ; iv, $SOCl_2$, benzene, DMF, Δ, aryl acid hydrazides CH_2Cl_2, DMAP, 0°C → rt, X, O, S, R = H, phenyl, naphthyl.

derivative.[15] For this purpose, fertilized chicken eggs (Lohmann Tierzucht, Cuxhaven, Germany) were incubated at 37°C in constant humidity for 10 days. The CAMs were revealed by a rectangle in the eggshell and small silicone rings containing vehicle control or the BZT compound (at IC50 20 μg/ml) were placed onto the CAM. The two groups were compared and the number of blood vessels after 72 h of treatment was recorded by a Kappa digital camera system.

Measurement of clonogenic activity

We evaluated the anchorage-independent growth of cells grown in agar.[11,16] This included incubation of the PC-3 and LNCaP cells (1×10^5) with the vehicle or the BZT compound at its IC50 concentration on a semi-solid agar layer (0.6%). After 14 days, clones were stained with crystal violet and visualized by light microscopy to determine the total number of colonies and the relative colony sizes.

Assay of *in vitro* and *in vivo* toxicity

To evaluate the *in vitro* toxicological effect of the most active BZT derivative, **6a**, we used a model based on the lysis of red blood cells (RBCs), measuring the haemoglobin released in the supernatant fraction.[17] RBCs in 50% Alsever's solution were centrifuged at 800*g* for 10 min and then washed with saline solution. The BZT compound (5–5000 μg/ml) was incubated with a 2% final suspension of RBCs at 37°C for 45 min. The release of haemoglobin by an equal number of RBCs by hypotonic lysis in 0.05 volumes of water was used as a 100% positive control, while RBCs treated with saline solution served as negative controls. Results were expressed as the concentration at which half of the RBCs lysed (LyticC50).

To investigate the *in vivo* acute toxicological effect of the BZT, a brine shrimp (*Artemia salina*) bioassay was used.[18] In brief, artificial seawater was prepared by dissolving sea salt in distilled water (38 g/l). The seawater and a teaspoon of brine shrimp eggs were added to a small tank, one half of which was covered and the other half remained uncovered to allow the penetration of light that attracts hatched shrimps. The tank containing the brine shrimp eggs was left at room temperature for 24 h to allow the eggs to hatch. The BZT compound was dissolved in DMSO and diluted with artificial seawater so that the final concentration of DMSO did not exceed 0.05%. Different concentrations of the compound were prepared, in triplicate 96-well plates (50 μl, 5–5000 μg/ml). Brine shrimp larvae (nauplii, 10–20, 150 μl) were added to each well and then covered with parafilm at room temperature for 24 h.[19] After this period, the number of dead and surviving brine shrimps was recorded using light microscopy. Each experiment was replicated three times and the average values were determined. The toxicity was determined from the 50% lethal concentration values (LC50) as determined by probit analysis.[18]

PC-3 xenografts

All animal protocols were in accordance with the German animal protection laws and guidelines for the use of living animals for scientific purposes and were approved by the Landesamt für Gesundheit und Soziales in Berlin. Male athymic nude BALB/c (nu/nu) mice (5–7 weeks old; Taconic Europe, Ejby, Denmark) were housed under microbiological monitored conditions according to the recommendations of the Federation of European Laboratory Animal Science Associations Working group.[20] We injected a suspension of 2×10^6 PC-3 cells in BD Matrigel Matrix High Concentration (Becton Dickinson Biosciences, Heidelberg, Germany) (0.1 ml, 1:1 ratio) subcutaneously into the left flank of each mouse. The tumour growth was measured each day using digital calipers and the tumour volumes were calculated using the formula: $V = \pi/6 \times 1.69 (\text{length} \times \text{width})^{3/2}$.[21] Ten randomized mice per group were treated at the moment when tumours were palpable (size approx. 250 mm^3). We treated the mice by intraperitoneal injection, either with a suspension of the DMSO-dissolved BZT (saline solution–tween 20, 2%) to a final dose of 5 mg/kg or with vehicle every 24 h for 21 uninterrupted days.[22] This dose was chosen according to our previous studies with other organic compounds that showed comparative IC50 values for PC-3 cells. At the end of the study, Mice were killed and tumours were removed and measured.

Statistical analysis

The SPSS Statistics 19 (IBM, Chicago, IL, USA) and GraphPad Prism 5.04 (GraphPad Software, San Diego, CA, USA) software packages were used for statistical analysis. Where applicable, the distribution of data was tested using the Sharp–Wilk normality test (GraphPad $software). Data were analysed using the Student's *t*-test and analysis of variance approaches (one-way, two-way and repeated measures). Bonferroni correction or the Games–Howell test for multiple comparisons were applied in case of homogeneity or inhomogeneity of variances (Levene test; sphericity estimate epsilon). $P < 0.05$ (two-tailed) was accepted as indicating statistical significance. All data are given as arithmetic means ± standard error of the mean (SEM). The equation $Y = Bottom + (Top - Bottom)/(1 + 10^{(logIC50-X)*Hillslope})$ (GraphPad Software) was used to calculate IC50 values by nonlinear regression of experimental

data, where X was the logarithm of concentration and Y was the response.

Results

Effect of benzothiazole derivatives on cell viability and proliferation of PCa cells

The newly synthesized BZT derivatives shown in Figure 1 demonstrated inhibition of the viability of human PCa cell lines PC-3 and LNCaP as well as the non-tumour cells ARPE-19 and HK-2 (Table 1). These effects were compared with those of the reference anti-tumour agent, dequalinium (DQ), as previously described by others.[22–24] Compounds **6a**, **7a**, **7b**, **7d** and **7e** showed cytotoxic characteristics in a dose-dependent manner, inhibiting PC-3 cell viability at IC50 concentrations of <50 μg/ml. Furthermore, compounds **6a** and **7a** were cytotoxic to LNCaP cells at IC50 concentrations of <30 μg/ml. Special attention was paid to compound **6a**, *N*′-formyl-2-(5-nitrothiophen-2-yl)benzothiazole-6-carbohydrazide, because this compound demonstrated the strongest inhibitory effect, comparable with that of the anti-tumour agent DQ. This BZT analogue, hereafter referred to as BZT **6a**, was also more toxic to tumour cells (PC-3 and LNCaP) than non-tumour cell lines (ARPE-19, HK-2) (Table 1). Although in most cases the DQ control agent was also more toxic to tumour cells than normal cell lines, this compound was more toxic in PC-3 than ARPE-19 cells. These results show that BZT **6a** showed toxic effects to tumour cell lines with lower toxicity to normal cells and in general, the specificity was higher than the control, DQ. Therefore, the following studies were performed with compound **6a**. Figure 2a,b provides a detailed overview of the dose- and time-dependent effects of this BZT derivative on the PC-3 and LNCaP cells.

Effect of benzothiazole derivative 6a on the adhesion, spreading, migration and invasion of PCa cells

Compound **6a** inhibited the attachment and spreading of PC-3 cells at its IC50 for up to 3 h after incubation (Figure 2c). As shown by the wound-healing assay, cell migration was also decreased in PC-3 cell cultures treated with the BZT derivative **6a** in comparison with vehicle-treated cultures (Figure 2d). In addition, the BZT derivative significantly reduced the invasion of LNCaP cells in the Boyden chamber assay after 18 h of incubation to 80.2 ± 1.7% compared with **6a**-free controls (100.4 ± 2.9%).

Effect of benzothiazole derivative 6a on neoangiogenesis and MMP activity

The CAM assay was used to evaluate the effect of the BZT derivative **6a**. A complex network of small and tiny blood vessels fills the entire chorioallantoic membrane of chicken embryos. This usually homogeneously distributed microvasculature was seen in the vehicle-treated CAMs (Figure 3a). In contrast, we observed a degenerated microvasculature in the **6a**-treated CAMs. These CAMs showed avascular fields with degenerated microvessels (Figure 3b). In addition, the number of blood vessels was significantly reduced (Figure 3c), suggesting the potential anti-angiogenic properties of this compound.

MMP-9 was strongly expressed in PC-3 cells while the expression of this enzyme was clearly lower in LNCaP cells (data not shown), as previously reported. Compound **6a** was able to reduce the MMP-9 activity in the conditioned medium of the PC-3 cultures to 14.2 ± 0.8% (Figure 4) and also reduced MMP-9 activity in LNCaP cells. We did not

Table 1 Cytotoxic effect of benzothiazole derivatives on prostate tumour and non-tumour cells indicated as half-inhibitory concentration IC50

	IC50 (μg/ml)[b]			
Derivative[a]	PC-3	LNCaP	ARPE-19	HK-2
6a	19.9 ± 1.17†,**	11.2 ± 0.79***	119.4 ± 4.33	36.4 ± 1.53
6b	>50	>50	n.d.	n.d.
6c	>50	>50	n.d.	n.d.
7a	32.8 ± 2.08	29.9 ± 1.38	n.d.	n.d.
7b	36.8 ± 4.34	40.3 ± 1.17	n.d.	n.d.
7c	>50	>50	n.d.	n.d.
7d	29.1 ± 3.20	16.8 ± 2.48	n.d.	n.d.
7e	42.6 ± 5.04	>50	n.d.	n.d.
7f	>50	>50	n.d.	n.d.
7g	>50	>50	n.d.	n.d.
Dequalinium	24.6 ± 2.22	4.94 ± 0.66	18.1 ± 1.26	42.4 ± 1.52

n.d. = not determined. [a]Structures of the numbered derivatives are given in Figure 1. [b]Data represent means ± SEM of three different experiments. †$P < 0.05$ compared with HK2; **$P < 0.01$ and ***$P < 0.001$ compared with the control substance dequalinium and ARPE-19 cells.

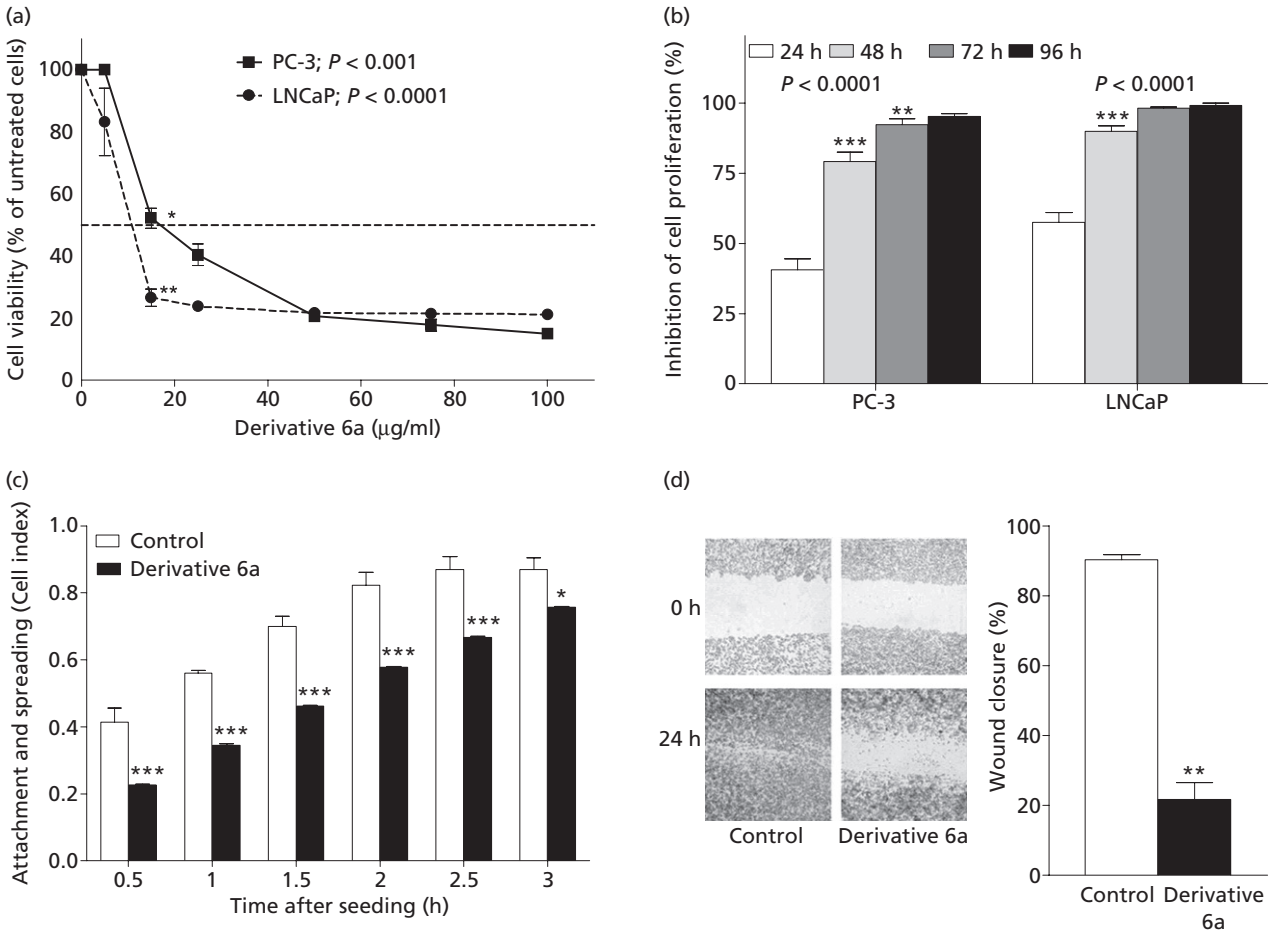


Figure 2 Effect of the benzothiazole derivative **6a** on cellular characteristics of PC-3 and LNCaP cells. (a) Dose–response curves. (b) Time–response effects at IC50. (c) Effect on adhesion and spreading of PC-3 cells. PC-3 cells were incubated with the compound at its IC50 concentration of 20 μg/ml or control vehicle (medium–dimethyl sulfoxide, 0.2%) and applied in triplicate onto the fibronectin–coated sensors of the xCELLigence analyzer. Their attachment and spreading were quantified by real-time cell electronic sensing as cell index after treatment. (d) Effect in a single-scrape wound model. The pictures are representative images of PC-3 cells captured at the time of wounding and after 24 h. The percentage of wound area closed at 24 h post treatment (IC50 concentration of 20 μg/ml or control vehicle) was calculated. All results are means ± SEM of three independent experiments. The SEM values were partly very small so that the error bars do not partly exceed the size of the points that indicate the used benzothiazole concentration in the figure. Significances, calculated in (a) by one-way analysis of variance and the post-hoc test according to Games–Howell (with equal variances not assumed), in (b) by one-way analysis of variance, Bonferroni corrected testing (with homogeneity of variances according to the Levene test), in (c) as two-factor study with repeated measures of one factor (with homogeneity of variances according to the Levene test) and in (d) as Student's *t*-test: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with the previous concentrations and time in (a) and (b), respectively, and to control vehicle in (c) and (d).

detect any MMP-2 activity in either of the cell lines tested, as was also previously reported.[11,25]

Effect of benzothiazole derivative 6a on the clonogenicity of PCa cells

Anchorage-independent growth of cell lines is strongly associated with transformation and is highly correlated with *in vivo* tumorigenicity, invasiveness and tumour growth in mice.[26] After 2 weeks of incubation, significant growth-forming colonies in soft agar were observed in vehicle-treated tumour cells, as has been previously reported.[11,22] Colonies were either reduced in size and number or in some cases completely absent in tumour cells treated with compound **6a** (Table 2; Appendix S1, Figure SA1), demonstrating the anti-cancer action of this BZT derivative.

In vitro and *in vivo* toxicological effects of benzothiazole derivative 6a

To determine the toxicity of compound **6a**, two models were used: the haemolysis effect on RBCs and the lethality

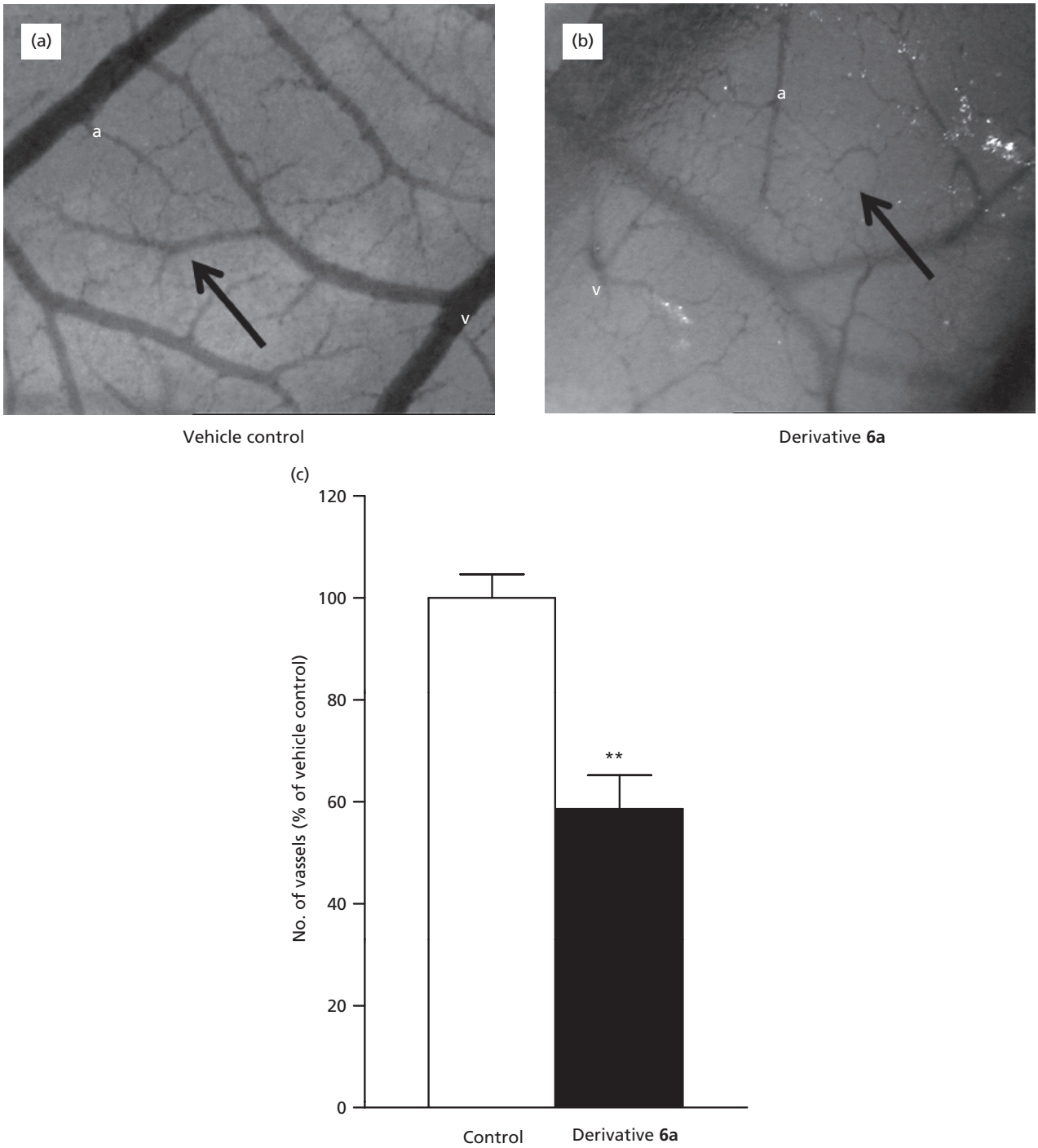


Figure 3 Anti-angiogenic effect of benzothiazole derivative **6a**. Vehicle control or compound **6a** (20 μg/ml) were inoculated onto the chicken chorioallantoic membrane (CAM) on day 10 of chick embryo development and the effect was evaluated 72 h later. Representative microphotographs (×25) are shown on day 13. (a) Control CAM. Blood vessels are arranged symmetrically and have characteristic branches (arrow). (b) Compound **6a**-treated CAM. Compound **6a** leads to the degeneration of the vascular network shown as vessel-free areas and vessel branching (arrow). Also the number of blood vessels is significantly reduced in the compound-**6a** treated CAMs (c). Blood vessels were counted in four sections of each CAM and changes are presented as percentage means ± SEM in relation to control vehicle in four independent experiments. Significances, calculated by Student's *t*-test: $^{**}P < 0.01$ compared with control. Arteries and veins are shown by 'a' and 'v', respectively.

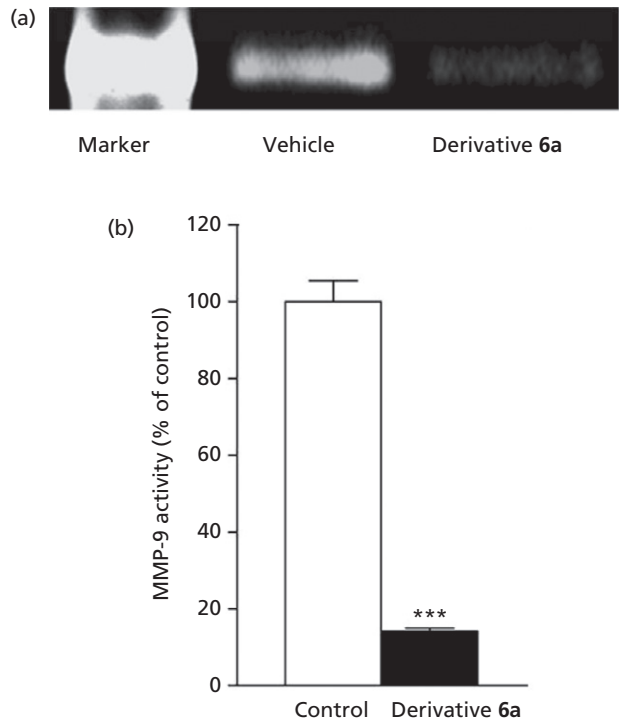


Figure 4 Activity of MMP-9 metalloproteinase by gelatin zymography in PC-3 cell after exposure to benzothiazole derivative **6a** at IC50 for 24 h. Conditioned medium prepared from subconfluent cultures were collected, resolved in non-reducing gels containing gelatin (1 mg/ml) and processed for zones of gel degradation activity. (a) A representative experiment is shown. (b) The results were quantified in relation to control vehicle and are presented as means ± SEM of percentage of activity of three different experiments. Significance, calculated by the Student's *t*-test: ***$P < 0.001$ compared with vehicle control.

Table 2 Inhibition of clonogenic potential by benzothiazole derivative **6a**

Measure[a]	Controls	Derivative **6a**
Colony formation (%)		
PC-3 cells	100 ± 17.6	0.51 ± 0.25**
LNCaP cells	100 ± 18.3	0
Relative colony size		
PC-3 cells	1 ± 0.30	0.02 ± 0.01**
LNCaP cells	1 ± 0.05	0

[a]Colony formation and size relative to non-treated PC-3 and LNCaP quantified by light microscopy are given as means ± SEM of three independent experiments. **$P < 0.01$ compared with control (vehicle).

assay on brine shrimp (Table 3). The results show lower overall toxicity of BZT **6a** compared with the control agent DQ, as the concentration required to achieve appreciable toxic response on erythrocytes was higher for BZT **6a** than for DQ. This effect was also evident when BZT **6a** was tested in the brine shrimp assay. Thus, both toxicological evaluations showed that BZT **6a** was less toxic than control DQ. In addition, it is important to note the higher concentrations needed to induce toxic effects according to these assays compared with the cytotoxic IC50 in tumour cells (Table 1).

Table 3 *In vitro* and *in vivo* toxicological profile of benzothiazole derivative **6a**

Compound	LyticC50 (μg/ml)	LethalC50 (μg/ml)
Derivative **6a**	>500	101.48 ± 3.60***
Dequalinium	>200	37.26 ± 2.59

Results are expressed as means ± SEM of three different experiments. LyticC50, half-lytic concentration on red blood cells; lethalC50, half-lethal concentration on *Artemia salina* (brine shrimps). ***$P < 0.001$ compared with dequalinium. Each experiment was performed five different times.

Effect of benzothiazole derivative 6a on tumour growth of PC-3 xenografts

The *in vivo* effect of compound **6a** (5 mg/kg, i.p.) was examined by the evaluation of the tumour development in PC-3 xenografts. The treatment was started when tumours were palpable, corresponding to a size of approximately 250 mm^3. Significantly reduced tumour growth from day 11 until the end of the treatment (Figure 5) was observed for mice treated with compound **6a** compared with vehicle-treated mice. All mice were observed and weighed daily and this data showed that the compound dosage was well tolerated in all mice, as supported by the *in vitro* toxicological experiments. Volumes of the harvested tumours at the end of the study showed significant differences between the treated and control mice (450 mm^3 ± 53 vs 718 mm^3 ± 82; $P = 0.018$) and confirmed the *in vitro* inhibitory effect of this compound on PCa cells.

Discussion

Over the past ten years BZT-related structures have been shown to be potential new therapies with different effects against infections, osteoarthritis and cancer, involving different mechanisms of action.[27–29] As possible chemotherapeutics, BZT analogues, such as tetrahydrobenzothiazoles, have shown anti-tumour properties through inhibition of p53 transcriptional activity and induction of mitochondrial-mediated apoptosis.[30] BZT-related compounds have shown pro-apoptotic effects on tumour cells by inducing the expression of different proteins, including the extrinsic apoptosis-initiating receptor TNFRSF6.[31] BZT compounds have shown *in vitro* cytotoxicity on human cancer cell lines through caspase-dependent and caspase-independent pathways inducing apoptosis.[32] Several

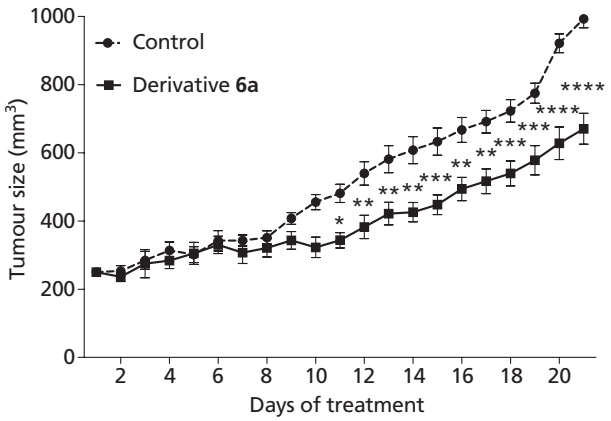


Figure 5 *In vivo* anti-tumour activity of benzothiazole derivative **6a** in PC-3 xenografts. One week following tumour cell injection, when the tumours were palpable (around 250 mm^3), mice were treated every day (5 mg/kg, i.p.). The tumour volume was determined every day using a digital caliper. Values are given as means ± SEM, $n = 10$ for each group. Significances, calculated as analysis of variance-based two-factor study with repeated measures of one factor with Bonferroni correction for post-hoc tests (according to the sphericity criterion epsilon with indicating equality of variances of the differences between measurements): $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ compared with control vehicle.

analogues of these structures, such as 2-(4-aminophenyl) benzothiazoles (CJM-126), represent highly selective classes of anti-tumour agents that elicit potent growth inhibition in several human-derived cancer cell lines.[33,34] Also, BZT-related compounds have shown mutagenic and genotoxic effects on tumour cells by inducing the expressions of different proteins, including the DNA-damage response genes CDKN1A (p21/Cip1) and DNA binding protein 2.[35]

BZT compounds are also aryl hydrocarbon receptor agonists, resulting in the induction of CYP1A1 and causing the generation of electrophilic reactive species which form DNA adducts, producing cell death by activation of the intrinsic and extrinsic apoptotic machinery.[7] There is a constitutive expression of CYP1A (CYP1A, CYP1A1 and CYP1A2) in PCa cancer cell lines PC-3 and LNCaP, whereas normal prostate cells have no detectable CYP1A1. Thus, hormonal and cancer-specific factors affect the expression and induction of the phase I metabolic enzymes in prostate cells.[36] Indeed, in tumour cells CYP1A1 induces the metabolism and biotransformation of BZT structures to their reactive toxic species, which are responsible for DNA adduct formation and cell death, in addition to their metabolism to inactive metabolites.[7] Thus, selective over-expression of CYP1A1 in cancer cells is advantageous for tumour-specific BZT drug design for anti-cancer therapy.[37]

The BZT compound Phortress (NSC 710305) is a novel, potent and selective experimental anti-tumour agent that is synthesized as a prodrug and has already been introduced in clinical trials.[7] Its mechanism of action involves the biotransformation of 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole to generate electrophilic reactive species, which covalently bind to DNA, causing lethal damage to sensitive tumour cells.[38,39] Indeed, dose- and time-dependent DNA damage *in vitro* and *in vivo* was also described,[39] while the growth of breast (MCF-7) and ovarian (IGROV-1) xenograft tumours was significantly retarded by this compound.[38]

Our results confirm the biological activity of the BZT structure. We have shown the potential anti-tumour effect of a new series of these analogues in human PCa PC-3 and LNCaP cells (Table 1). The most effective compound tested *in vitro* was BZT **6a**, whose effects were dose- and time-dependent (Figure 2a,b). The time-dependency became apparent between 24–96 h following drug exposure, inhibiting almost 100% of the cell growth.

The absence of an aromatic substitution in the carbohydrazide group could be the reason for the structure–activity relationship of BZT compounds. In this context, compounds **6a** and **7a**, which lack aromatic ring substitution and contain only an *N*′-formyl group, were the most active structures against the tumour cell lines tested. However, the substitution of a nitrothiophene instead of a nitrofuran in position 2 of the BZT nucleus seems to favour the cytotoxic activity, since compound **6a** was more active than **7a** (Table 1). We also noted that as the group substitutions increased in volume, the *in vitro* anti-tumour effect decreased, since **6a** > **6b**, **6c** and **7a** > **7b** > **7d** > **7**, **7f**, **7g**, **7c**, suggesting a possible size-dependent activity of these molecules.

The BZT compound **6a** also exhibited stronger inhibitory effects against tumour than against non-tumour cells, showing higher cytotoxicity to the human PCa cell lines compared with non-tumour ARPE-19 and HK-2 cells. Indeed, the selectivity of this structure was also superior compared with the effects observed for DQ (Table 1). It is also important to note the possible presence of different metabolites of BZT compound **6a**. Since we found interesting results in human prostate tumour cells, the evaluation of BZT **6a** in metabolically competent cells, ideally in liver cells and primary hepatocyte cultures rich in drug-metabolizing and biotransformation enzymes, will be necessary in further studies.[40]

Tumour progression is a multi-step process in which adhesion plays a pivotal role in the development of recurrent, invasive and distant metastasis. In this context, tumour cells must enter into the blood or lymphatic circulation; this involves the loss of intercellular adhesion and renders adhesion molecules likely participants in the development of metastatic disease. Evidence to date suggests that adhesion is associated with invasion and metastasis in

a variety of human malignancies.[41] Our results show that PC-3 cell adherence to the specific adhesive substrate fibronectin was inhibited by compound **6a**. Due to the fact that adhesion is related to many extracellular matrix (ECM) components (e.g. fibronectin, collagens, laminin and vitronectin with integrins),[42] there are many possibilities for an interaction between ECM components and the BZT derivative **6a**.

New tissue formation, invasion, angiogenesis and cell motility and migration also represent attractive targets for the development of new anti-tumour compounds.[43] Our results show that the BZT derivative **6a** inhibited cell migration and invasion as well as neoangiogenesis. Neoangiogenesis is an essential part of tumour progression. Tumours induce blood vessel growth by secreting different factors, such as vascular endothelial growth factor, leading to capillary growth and allowing growth and metastasis of tumours by supplying the required nutrients. Compounds that target the tumour vasculature by inhibition of angiogenesis could lead to potential therapies against cancer progression.[44] Our results also show that compound **6a** causes a degeneration in the vascular complex by preventing the formation of new blood vessels, which was clearly evident after 72 h of compound incubation, demonstrating a strong inhibitory effect on neoangiogenesis, as has already been shown by our group for other potential antineoplastic agents.[11,22]

Most of the cells in multicellular organisms are surrounded by a complex mixture of a three-dimensional network assembled from multiple components, including collagens, non-collagenous glycoproteins, elastin, proteoglycans and matricellular proteins in an organ-specific manner, which makes up the ECM. The anchorage between cells and the ECM represents an essential process for cell survival. MMPs are involved in the breakdown of ECM in normal physiological processes, such as embryonic development, reproduction and tissue repair, as well as in pathologies such as arthritis and cancer.[45] In arthritis, these proteases represent an interesting target against inflammation and BZT derivatives have been proposed as potential compounds for the allosteric inhibition of MMP-13, which is involved in articular cartilage turnover and cartilage pathophysiology associated with osteoarthritis.[29] On the other hand, tumour cells have the ability to invade other tissues and to spread to different organs and the degradation of the ECM by MMPs, such as MMP-9, represents a key step for accomplishing these events and even defines the degree of tumour malignancy.[46] MMP-9 is highly expressed in cancer cells and is involved in the release of growth factors that promote tumour development by increasing migration, invasion and angiogenesis, leading to the malignant behaviour of carcinomas.[47] Inhibitors of this enzyme could represent possible drugs against tumour development. Our results showed for the first time that a BZT derivative decreased the activity of MMP-9 in human PCa PC-3 cells, suggesting that this enzyme could be a molecular target of compound **6a** and its probable molecular mechanisms of action. Thus, the effects of this derivative on angiogenesis could also be due to the inhibition of MMP-9, in addition to the other multiple steps of tumour development, such as proliferation, adhesion, migration and invasion as shown in this study.

In vitro anchorage-independent growth of cells is one of the most specific criteria for assessing the cell's potential for expression of a transformed phenotype *in vivo*. This property is highly correlated with the ability of the cells to form tumours in athymic nude mice.[48] Compound **6a** strongly inhibited anchorage-independent growth in human tumour cell lines, supporting our hypothesis that this BZT could decrease the development of a tumour-like phenotype *in vivo*.

For detailed evaluations of new structures with potential biological properties, it is necessary to understand their toxicological properties. BZT **6a** showed low toxicity *in vitro* with no significant RBC lytic effects reported throughout the different concentrations of compound tested; low *in vivo* toxicity was shown due to the elevated concentration necessary to induce brine shrimp death (Table 3). In addition, BZT **6a** was less toxic than the control anti-tumour reagent, DQ, against the non-tumour cells ARPE-19 and HK-2 (Table 1).

Due to our promising results *in vitro* and the previously mentioned low toxic effects we decided to evaluate the effects of compound **6a** in PCa xenografts. We found that tumour growth and volume of the explanted tumours after termination of the experiments were significantly decreased in mice treated with compound **6a** as compared with vehicle control, and that this effect was sustained over the time. The daily dose of 5 mg/kg was well tolerated by the mice and no side effects were observed. Probably, the anti-tumour effect could be enhanced by increasing the dose, as has been shown in studies with other BZT derivatives with doses up to 20 mg/kg.[39] According to our experience with other organic compounds and comparative IC50 values, a dose up to 5 mg/kg should be tested.[22] However, the rigorous interpretation of the animal protection law by the local authority did not permit a dose–effect trial for this first preclinical study.

Conclusions

The results of this work demonstrated the anti-tumour activity of new BZT derivatives, particularly the compound **6a** (*N*′-formyl-2-(5-nitrothiophen-2-yl)benzothiazole-6-carbohydrazide), which was highly efficient *in vitro* and *in vivo*. The inhibitory effect of this compound on multiple

essential cell biological functions characteristic of PCa cells and its low toxic effects make this derivative attractive as a potential multi-target drug for PCa. We conclude that this promising compound warrants further detailed analyses in PCa research to elucidate the molecular mechanism of action in more detail.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. N′-formyl-2-(5-nitrothiophen-2-yl)benzothiazole-6-carbohydrazide as potential antitumor agent for prostate cancer in experimental studies.