Polyamines and prostatic cancer

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Abstract

The importance of polyamines in prostatic growth and differentiation has prompted studies to evaluate the clinical relevance of the ornithine decarboxylase/polyamine system in prostatic cancer. These studies show that differences in biological behaviour of prostatic (cancer) cells are associated with changes in polyamine levels and/or the activity of their metabolic enzymes. Faulty antizyme regulation of polyamine homoeostasis may play an important role in the growth and progression of prostatic carcinoma. Treatment of human prostate carcinoma cells with inhibitors of polyamine metabolic enzymes or polyamine analogues induces cell growth arrest or (apoptotic) cell death. Our recent *in vitro* studies using conformationally restricted polyamine analogues show that these compounds inhibit cell growth, probably by inducing antizyme-mediated degradation of ornithine decarboxylase. Sensitivity of human prostate cancer cells for these compounds was increased in the absence of androgens. These results suggest that these analogues might have chemotherapeutic potential in case prostatic cancer has become androgen-independent. Pilot data in an *in vivo* model show that these analogues have effects on tumour cell proliferation, vascularity, blood perfusion and tissue hypoxia. Overall, these studies show that polyamines may serve as important biomarkers of prostatic malignancy and provide a promising target for chemotherapy of prostatic cancer.

Introduction

The prostate and prostatic secretions have played an important role in our current understanding of polyamines. The prostate has one of the highest polyamine concentrations of any tissue. The initial identification of one of the polyamines, i.e. spermine, was reported in human semen as early as the 17th century by Antoni Van Leeuwenhoek. In rat prostate, spermidine is the predominant polyamine, whereas in the human prostate spermine is present in high amounts. Studies on castration-induced regression and testosterone-stimulated regrowth of the rat prostate showed that prostatic polyamines are under the control of androgens [1–3]. The activities of the polyamine biosynthetic enzymes ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase and spermidine synthase are induced by androgens in a co-ordinated way and expression of these enzymes is primarily localized to the glandular epithelial cells of the prostate [3–5]. Studies on the murine ODC gene revealed that the ODC promoter contains an androgen-responsive element-like sequence that can bind to the androgen receptor *in vitro* [6].

It is apparent that functions of ODC and polyamines in the prostate are related to cellular proliferation and secretory activities. The functional significance of seminal polyamines is still unclear. The highly cationic polyamines would encounter difficulties in penetrating into the spermatozoa before being utilized. Proton magnetic resonance spectroscopy (MRS) studies of human prostatic fluids indicate that spermine is linked to citrate, also present in remarkably high concentrations in the prostate [7]. Complex formation with the negatively charged acid citrate would enable polyamines to penetrate in spermatozoa. Seminal spermine possibly modulates sperm fertilization competence and the acrosome reaction [8] or may have other functions in the regulation of seminal clotting or prevention of bacterial growth in the urinary tract [9].

Since polyamines are involved in prostatic growth and differentiation, monitoring of polyamines and their metabolic enzymes in prostatic tissue may be useful in the diagnosis and prognosis of prostate cancer. Furthermore, interference with polyamine homoeostasis may be a modality for chemotherapy in prostate cancer.

Polyamines as biomarkers for malignant behaviour in prostate cancer

Recently it was shown that overexpression of ODC can cause cellular transformation, suggesting that the enzyme can be considered as a proto-oncogene product [10–12]. Studies on the rat and human prostate-derived tumours showed that ODC activity was substantially higher in the more malignant sublines [13,14]. Immunoblot analysis of tissue specimens of patients with prostate cancer showed a significantly elevated protein expression of ODC in the cancerous tissues as compared with the benign tissues [15]. Moreover, studies on the expression levels of the ODC gene

Key words: antizyme, polyamine, polyamine analogue, prostatic cancer.

Abbreviations used: ODC, ornithine decarboxylase; DFMO, difluoromethylornithine; SSAT, spermidine/spermine-N^1-acetyltransferase; MRS, magnetic resonance spectroscopy; BE-3-3-3, N^1,N^{11}-diethylnorspermine; BE-4-4-4-4, 1,19-di-(ethyl-amino)-5,10,15-triazononadecane; BE-3-7-3, bis(ethylamino)-4,12-diazapentadecane; CPE-3-3-3, N^1-ethyl-N^{11}-[(cyclopropyl)methyl]-4,8-diazaundecane; CHE-3-3-3, N^1-ethyl-N^{11}-[(cycloheptyl)methyl]-4,8-diazaundecane; BIS, 1,12-diaziridinyl-4,9-diazadodecane.

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in a series of 23 human prostate cancers dissected from radical prostatectomy specimens revealed significantly higher ODC mRNA levels in tumours compared with the benign tissue [16].

Polyamine measurements in prostatic cancer cell lines with different degrees of differentiation showed that less-differentiated cell lines contained lower spermine concentrations. In patients, we found a similar correlation between spermine levels and the degree of differentiation in biopsies from prostates [17]. Comparison of polyamine levels in normal, benign and malignant tissues of human prostate indicated that in normal and benign hyperplastic prostatic tissues a high content of spermine occurs whereas in tumour tissue, especially in prostatic carcinoma with metastases, spermine levels are reduced. Hence, a dramatic decrease of the prostatic spermine content could indicate a conversion of prostatic tissue from a benign to a malignant phenotype. Smith et al. [18] reported that spermine inhibited the growth of prostatic carcinoma *in vitro* as well as *in vivo*. Hence, the comparatively slow growth of prostatic carcinoma at its primary site, the prostatic gland, may be attributed to the high spermine levels (10–12 mM) present within the prostatic microenvironment. Escape from this regulation may emerge from altered inducibility of enzymes regulating polyamine biosynthesis in tumour cells and/or an acquired insensitivity towards spermine. Recent evidence for this supposition has been reported in a study by Koike et al. [19], showing a differential sensitivity of rat prostatic cancer cell lines towards spermine. Exposure to spermine induced cell cycle arrest and apoptosis in the poorly metastatic AT2.1 cell line but not in the highly metastatic AT3.1 cell line. A possible explanation for the resistance to spermine may be found in the different induction of antizyme in spermine-sensitive and spermine-insensitive cells. In the spermine-insensitive cells, ODC antizyme levels were not up-regulated, thereby failing to inhibit and degrade ODC. Antizyme levels are also reduced in other types of carcinoma, indicating that antizyme possibly acts as a tumour-suppressing factor [20].

Polyamine homoeostasis as target for chemotherapy of prostate cancer

Since the ODC/polyamine system is important for prostatic cell growth and function, interference with ODC activity could provide a promising target for chemotherapy of prostatic cancer. Difluoromethylornithine (DFMO), the most widely studied inhibitor of ODC, has striking inhibitory effects on the growth of cultured prostatic cancer cells [13,21]. DFMO-inhibited growth of PC-3, PC-82 and androgen-stimulated LNCaP cells could be reversed by exogenously added polyamines or their acetylated derivatives [21]. Further experiments showed that the intracellular event leading to this polyamine-mediated restoration of growth was ultimately the recompensation of intracellular spermidine levels. These results suggest that spermidine may be the key polyamine in the regulation of cell growth of prostatic (cancer) cells. DFMO was highly tolerable in animals and humans [22,23] but failed to induce a dramatic effect on the growth of transplantable rat and human prostatic tumours probably due to compensatory uptake of polyamines from extracellular sources. In spite of the limited success so far, future studies on DFMO combined with other forms of therapy might still point to the effectiveness of DFMO as a therapeutic agent.

The growing awareness that ODC plays an important role in carcinogenesis has increased the interest in the development of DFMO as a possible chemoprevention agent [23,24]. DFMO treatment inhibited carcinogen-induced cancer development in a number of rodent models including the transgenic adenocarcinoma mouse prostate ('TRAMP') model [23,25]. Furthermore, ODC activity is inhibited by several compounds that possess chemopreventive properties, e.g. non-steroidal inflammatory drugs [23], green tea polyphenols [26], seed extracts from *Asteracantha longifolia* [27] and sandalwood oil [28].

A promising alternative to the use of inhibitors of polyamine biosynthetic enzymes is the application of synthetic polyamine analogues. Symmetrically substituted bis(ethyl) analogues of spermine, i.e. N^1,N^{11}-diethylnorspermine (BE-3-3-3; also known as BENSpm or DENSpm), 1,19-di-(ethyl-amino)-5,10,15-triazononadecane (BE-4-4-4-4) and bis(ethylamino)-4,12-diazapentadecane (BE-3-7-3), non-symmetrically substituted alkylated analogues, i.e. N^1-ethyl-N^{11}-[(cyclopropyl)methyl]-4,8-diazaundecane (CPE-3-3-3) and N^1-ethyl-N^{11}-[(cycloheptyl)methyl]-4,8-diazaundecane (CHE-3-3-3) and the spermine analogue 1,12-diaziridinyl-4,9-diazadodecane (BIS), have been tested in various human prostatic carcinoma cell lines [14,29–32] (Table 1).

From these studies it can be concluded that these analogues have differential effects on cell growth and polyamine homoeostasis in different prostatic carcinoma cells, i.e. the androgen-independent DU-145 cells were the most sensitive, whereas the well-differentiated androgen-dependent LNCaP cells were relatively insensitive. Treatment with these analogues evoked intracellular accumulation of the analogue and various regulatory responses, e.g. down-regulation of polyamine biosynthesis, the induction of the catabolic enzyme spermidine/spermine-N^1-acetyltransferase (SSAT), and the depletion or decrease of the natural polyamines.

Recently, Marton and co-workers developed new compounds that are based on BE-4-4-4-4 but are restricted in their chain flexibility [33,34]. We studied the activity of some of these compounds in our panel of prostatic cancer cell lines *in vitro* (DU-145, PC-3, LNCaP and PC-346C; J.C. Romijn, R.G. Schipper, V.M.J.I. Cuijpers, L.J. Marton, B. Frydman, A. Valasinas, V.K. Reddy and A.A.J. Verhofstad, unpublished work). All compounds had considerable cytotoxicity against all cell lines tested. The hormone-unresponsive DU-145 was the most sensitive cell line, whereas the hormone-responsive cell lines LNCaP and PC-346C were less sensitive. The sensitivity of PC-346C cells for the polyamine analogues

Table 1 | Effects of polyamine analogues on human prostatic cancer cells

AdoMetDC, S-adenosylmethionine decarboxylase; SSAT, spermidine/spermine-N1-acetyltransferase; ID50, drug dose which inhibited growth to 50% of control; –, data not available; na, no apoptosis; a, apoptosis; nsa, not significantly affected; ↑, increased, ↓, decreased. Abbreviations for the analogues are given in the text.

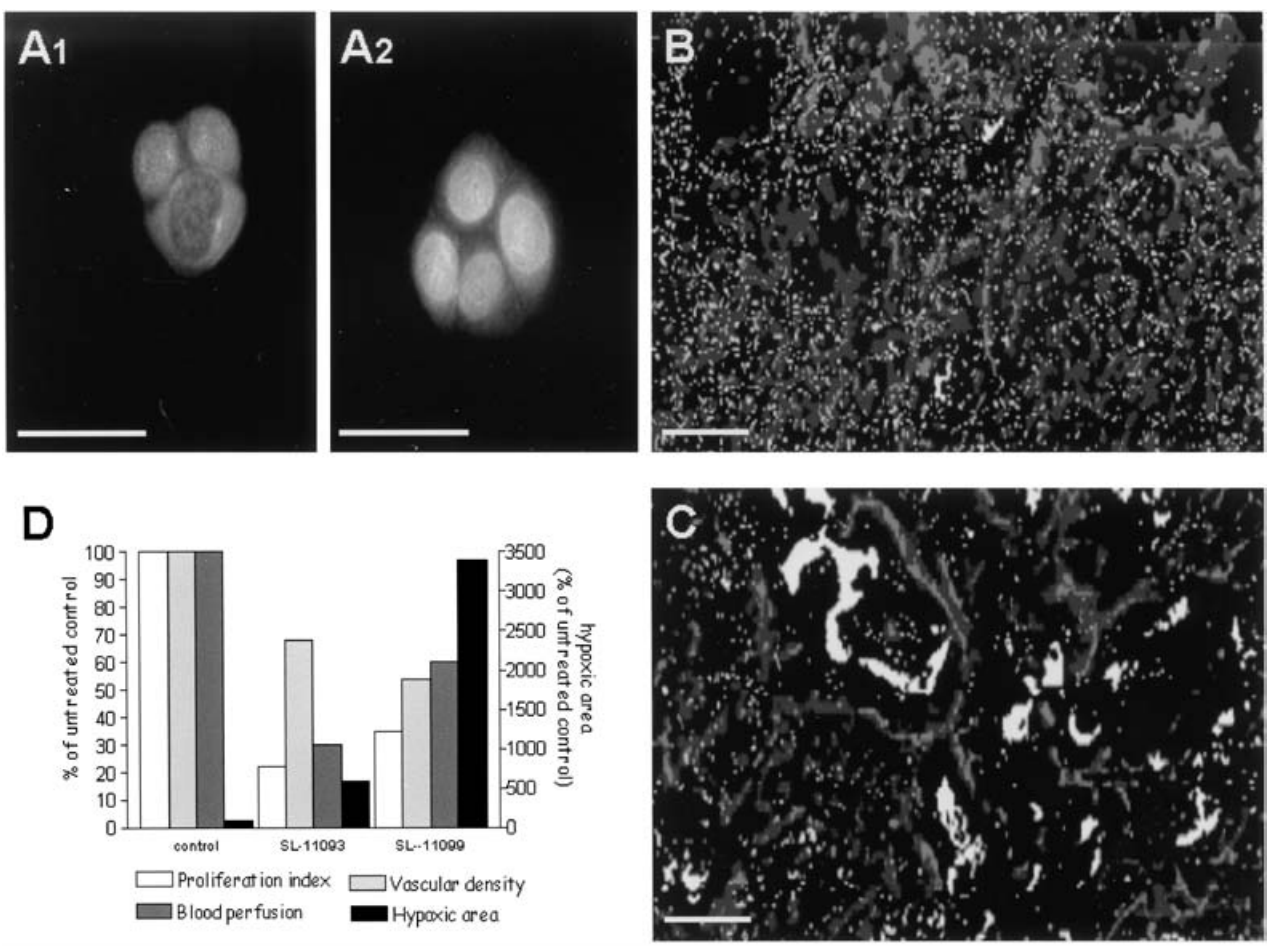
Analogue	Cell line . . .	DU-145	PC-3	LNCaP	TSU-PR1	DuPro-1	Reference
ID50 value (μM)							
BE-3-3-3		0.01–0.05	0.5–10	0.1	–	–	[29]
		3.2 na	–	–	1.6 na	6 na	[30]
		1 na	28 na	>1000 na	–	–	[31]
		0.3–1 na	1–3 na	1–3 a	–	–	[37]
		3 na	65 na	–	62 na	–	[14]
BE-4-4-4-4		0.01–0.05 a	0.5–10	0.1	–	–	[29]
		0.7 na	–	–	0.4 na	0.07 na	[30]
cis-unsaturated		0.9–0.5	0.07–0.5	0.2 to >31		0.2 to >31	[33]
conformationally restricted		0.1 to >31	0.1–2.8	0.6 to >31		0.7 to >31	[34]
BE-3-7-3		0.01–0.05	0.5–10	0.1	–	–	[29]
BIS		1.2 a	0.7 a	–	–	–	[40]
CPE-3-3-3		0.3–1 a	1–3 a	1–3 a	–	–	[37]
CHE-3-3-3		0.3–1 a	1–3 a	3 a	–	–	[37]
Polyamine pools							
BE-3-3-3		↓	↓	↓	–	–	[31]
		↑	↓	↓	–	–	[37]
		↓	↓	–	↓	–	[14]
BE-4-4-4-4		↓	↓	↓	–	–	[29]
cis-unsaturated		↓	↓	↓		↓	[33]
conformationally restricted		↓	↓	↓		↓	[34]
BE-3-7-3		↓	↓	↓	–	–	[29]
CPE-3-3-3		↓	↓	↓	–	–	[37]
CHE-3-3-3		nsa	↓	nsa	–	–	[37]
ODC/AdoMetDC activity							
BE-3-3-3		↓	↓	↓	–	–	[31]
		nsa	nsa	nsa	–	–	[37]
		↓	↓	–	↓	–	[14]
CPE-3-3-3		nsa	nsa	nsa	–	–	[37]
CHE-3-3-3		nsa	nsa	nsa	–	–	[37]
SSAT activity							
BE-3-3-3		↑	↑	↑	–	–	[31]
		↑	↑	↑	–	–	[37]
		↑	↑	–	↑	–	[14]
BE-4-4-4-4		nsa	–	–	nsa	nsa	[30]
CPE-3-3-3		↑	↑	↑	–	–	[37]
CHE-3-3-3		nsa	nsa	nsa	–	–	[37]

increased in the absence of androgens in the culture medium. These preliminary results suggest that these analogues might have chemotherapeutic potential in prostate cancer in cases where hormone therapy has failed. These analogues have been shown to be effective inducers of antizyme [35], which targets ODC to the nucleus for degradation [36]. In PC-346C cells, antizyme immunoreactivity was increased in the nucleus after treatment with the analogues (Figure $1A_2$). These data indicate that, at least partly, polyamine analogues may act on cell growth by inducing antizyme-mediated degradation of ODC.

BE-3-3-3, BE-4-4-4-4 and BIS have also been tested *in vivo*, using nude mice bearing xenograft tumours derived from DU-145, PC-3 or DuPro-1 cells [14,29,30]. In all

Figure 1 | Effect of polyamine analogues on antizyme induction and markers of tumour progression

(**A**) Immunostaining of antizyme in PC-346C cells treated with (A2) or without conformationally restricted (CR) polyamine analogue (A1). Antizyme staining is increased in the nucleus after CR-polyamine analogue treatment. Bar = 5 μm. (**B**–**D**) Immunocytochemical detection and quantitative analysis of markers of proliferation (incorporation of BrdU), vascular density (9F1), blood perfusion (Hoechst 333) and tissue hypoxia (pimonidazole). Quantitative analysis (**D**) of xenografts of PC-346 treated with (**C**) or without (**B**) CR-polyamine analogues shows that the analogues decrease the number of proliferating cells (light-grey dots), vascular density (light-grey areas) and blood perfusion (dark-grey areas), whereas tissue hypoxia increases (white areas). Bar = 500 μM.



tumours treated the analogue was taken up and inhibited tumour growth. Levels of spermidine and spermine were found to decrease whereas levels of putrescine were not affected. A possible explanation for the lack of polyamine depletion *in vivo* is the uptake of polyamines from extracellular sources, present in an *in vivo* system, in particular via polyamine uptake from the gastrointestinal tract. The results in nude mice bearing xenograft tumours support the idea that polyamine analogues act on cell growth not merely by depleting the natural polyamines but by exerting their own dysfunctional effects on cell growth as well.

Currently, we are performing pilot *in vivo* studies with conformationally restricted analogues on PC-346 xenografts in athymic mice. Preliminary studies on the short-term effects (after 1 week of treatment with analogue) show that these analogues have inhibiting effects on proliferation, vascular density and blood perfusion but have a stimulating effect on tissue hypoxia (Figures 1B–1D). These initial data suggest that polyamine analogues not only inhibit growth but also progression (e.g. angiogenesis) of prostate tumours.

Until now, the exact mode of action of polyamine analogues remains unclear, but inhibition of cell respiration and interaction with chromatin, probably resulting in dysfunctional changes, have been suggested as possible mechanisms. The huge induction of SSAT, which is directly or indirectly related to the sensitivity of the cells for the drugs, can have potential chemotherapeutic benefits. Several working mechanisms by which SSAT induction inhibits cell growth have been suggested, e.g. increased excretion and/or catabolism could rapidly deplete the intracellular polyamine

pools, and acetylation of the polyamines could reduce their positive charges and therefore reduce their ability to interact with negatively charged macromolecules, such as DNA. However, the relationship between induction of SSAT and inhibition of cell growth is not yet fully understood since BE-4-4-4-4 and CHE-3-3-3, in contrast to BE-3-3-3, exert their growth inhibitory effects without apparent induction of SSAT activity [37].

Some research groups have investigated the mechanism of cell death induced by polyamine analogues and found that the cytotoxicity of some of these analogues is actually based on their ability to induce apoptotic cell death. The observed apoptosis-inducing effects of some polyamine analogues are in agreement with the merging evidence that polyamines are actively involved in apoptotic cell death [38]. Changes in polyamine homoeostasis have been reported during cell death of nerve cells, in programmed cell death of embryonic cells and in various *in vitro* models of apoptosis. Furthermore, interference with polyamine homoeostasis modulates processes of cell death in a cell-type specific way. Much ambiguity exists over the mechanisms by which polyamines mediate apoptosis since they have been shown to act as promoting, modulating or even protective agents in apoptosis.

Concluding remarks

The studies reviewed here clearly show that polyamines are of crucial importance for the regulation of cell proliferation and differentiation of prostatic glandular epithelial cells. Our preliminary studies as well as data from others show that alterations in cell kinetic behaviour of prostatic (cancer) cells are accompanied with changes of polyamine levels and/or their metabolic enzymes. Prostatic cancer cell proliferation requires an increased rate of polyamine biosynthesis, in particular increased levels of the polyamines putrescine and spermidine. On the contrary, spermine seems to influence the functional, secretory state of the prostatic epithelium. This is in accordance with previous suggestions that a decrease in spermidine level and/or an increase in spermine usually indicate a shift of cells from a proliferation state into a state with a higher degree of differentiation. The prostate has a large glandular epithelial cell volume with large lumina where secretory products, like spermine and citrate, are concentrated. Consequently, changes in the cell organization resulting in decreased luminal volumes reduce the amount of secreted compounds. In line with this, normal and benign hyperplastic prostatic tissues are characterized by high levels of spermine while in human prostatic carcinoma spermine levels are low. Hence, a strong decrease in the concentration of spermine in the prostate could indicate a conversion of prostatic cells from a benign into a malignant phenotype. Based on these considerations, it seems appropriate to further explore the potential of polyamines as biomarkers for malignant behaviour in prostate cancer. Preliminary data show that MRS provides a powerful, non-invasive and spatially resolved method for the *in vivo* detection of polyamines and other metabolites in prostatic tissue [17,39]. We anticipate that MRS techniques will mature into valuable diagnostic tools in prostatic cancer and that the high-resolution magic-angle spinning methodology will be useful to monitor polyamines in *ex vivo* tissue specimens.

Interference with polyamine homoeostasis as a tool to inhibit tumour growth has been studied with various polyamine biosynthesis inhibitors and polyamine analogues. Inhibition of polyamine biosynthesis proved to be mostly cytostatic, since growth inhibition could be reversed by inhibitor withdrawal or polyamine repletion. These reversible cytostatic effects explain the limited success of these compounds in tumour growth inhibition *in vivo* attributed to the presence of exogenous polyamines, allowing tumour cells to proliferate at a normal rate even if endogenous *de novo* production of polyamines is blocked. Most likely is that specific inhibitors that block the entry of exogenous polyamines into cells may provide better possibilities for therapeutic intervention in the polyamine metabolism. Manipulation of polyamine body pools, for instance by dietary means, may have additional value in the chemotherapy and chemoprevention of prostate cancer.

Compared with polyamine biosynthesis inhibitors, polyamine analogues may have even more clinical value, since these compounds can be cytotoxic. As shown by our group and others it is evident that polyamine analogues are effective growth inhibitors of different phenotypes of human prostatic carcinoma cells and, furthermore, are able to inhibit the *in vivo* growth of small as well as more developed prostatic tumours. Currently, some of the analogues are under phase I or II clinical investigation with promising results.

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