Metformin enhances the antiproliferative and apoptotic effect of bicalutamide in prostate cancer

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BACKGROUND: Prostate cancer incidence and mortality vary dramatically by geographical location. Both are higher in developed countries. Some attribute this to westernized lifestyles of high-energy diets and limited physical activity with consequent obesity. Obesity and obesity-related diseases like diabetes cause hyperinsulinaemia, which upregulates pro-survival cell signalling. Previous work revealed diet-induced hyperinsulinaemia enhances prostate cancer xenograft growth *in vivo*. Metformin, an antidiabetic medication, reduces hyperinsulinaemia and also exhibits antineoplastic properties. Herein, we assess the potential additive benefit of combining bicalutamide antiandrogen therapy with metformin, *in vitro* and *in vivo*.
METHODS: Using clonogenic assays, we assessed the effect of bicalutamide and/or metformin on clonogenicity in prostate cancer cell lines. Western blot and cell cycle analyses were used to elucidate mechanisms of interaction between the drugs in androgen receptor (AR)-positive (LNCaP) and AR-negative (PC3) cell lines. The combination treatment regimen was assessed *in vivo* using an LNCaP murine xenograft model.
RESULTS: Micromolar bicalutamide or millimolar metformin caused a significant dose-dependent reduction in clonogenicity ($P<0.001$). Combination treatment further significantly reduced clonogenicity ($P<0.005$) with greater effects in AR-positive cells. Western blot and cell cycle analyses suggested differing mechanisms of interaction in AR-positive and -negative cell lines. Following combination treatment, LNCaP cells exhibited an altered cell proliferation (decreased phospho mammalian target of rapamycin expression) and perturbed cell cycle kinetics (G1/S cell cycle arrest). PC3 cells showed evidence of enhanced apoptosis (increased Bcl-2-associated X protein and decreased total caspase 3 expression). Markedly diminished tumour growth occurred following combination treatment *in vivo* ($P<0.001$).
CONCLUSION: Combining bicalutamide and metformin significantly reduces prostate cancer cell growth further than either monotherapy. In AR-positive cells, this effect appeared to be mediated by reducing proliferation rates, whereas in AR-negative cells the combination treatment appeared to promote apoptosis. This combination drug regimen may improve prostate-cancer-specific survival by the direct antineoplastic properties outlined.

Keywords: prostate neoplasm; metformin; bicalutamide; diabetes mellitus; obesity; drug therapy (combination)

INTRODUCTION

The incidence of prostate cancer has risen dramatically over the past three decades, with over 215 000 new diagnoses and 32 000 deaths now occurring per annum in the United States of America.[1] Simultaneously, the incidence of obesity has risen dramatically, particularly in so called developed countries.[2] It is estimated that by 2020, 40% of the American population will be classified as obese (body mass index $>30\,kg\,m^{-2}$). A strong correlation has been described between obesity and colorectal and endometrial cancer.[3] However, evidence of a direct link between prostate cancer diagnosis and obesity is less conclusive.[4] This may be explained, in part, by the differential effect of obesity on the development of high- and low-grade prostatic tumours. Several authors report obesity to be a risk factor for high-grade disease, whereas excess weight appears protective against low-grade tumours.[5–8] Given that obese individuals are known to exhibit hyperinsulinaemia, one plausible mechanism by which obesity may influence the development and/or progression of prostate cancer relates to the proproliferative effects of insulin and the related insulin-like growth factors (IGF). Pre-clinical studies from our own laboratory showed mice bearing prostate cancer tumour xenografts fed on a high-carbohydrate high-fat, designed to induce hyperinsulinaemia, exhibited a significantly greater tumour growth than mice fed on an isocaloric low-carbohydrate high-fat diet.[9]

A rapid escalation in both obesity and diabetes diagnoses has occurred over the last 30 years.[10,11] The incidence of non-insulin-dependent diabetes has particularly increased.[12] Metformin, an oral biguanide, has been used for several decades to treat non-insulin-dependent diabetes. Although classically described as an insulin-sensitizing agent, there is no clear consensus regarding its modes of action. In the liver, metformin reduces serum glucose levels by inhibiting glycogenolysis and gluconeogenesis.[13,14] Its activity in peripheral tissues is less clearly defined. Inhibition of hepatic glycogenolysis and gluconeogenesis is mediated by the activation of adenosine monophosphate kinase (AMPK). AMPK is a

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highly conserved protein kinase, which functions to maintain both whole-body and cellular energy homeostasis.[15] The net effect of metformin use is to lower serum glucose levels and consequently inhibit the development of chronic hyperinsulinaemia.

As well as exhibiting antidiabetic activity, metformin has recently been shown to exert antineoplastic effects in a number of tumour systems, including the prostate, ovarian, breast, colorectal and endometrial carcinoma models.[15–22] Potential mechanisms of action for these antineoplastic effects include direct antiproliferative activity mediated by metformin-induced activation of AMPK, with a subsequent downstream inhibition of the mammalian target of rapamycin (mTOR)/S6 kinase pathway, or direct inhibition of insulin/IGF-mediated cellular proliferation.

Given the accumulating data regarding the antineoplastic capacity of metformin in pre-clinical prostate cancer models, we set out to assess the use of metformin as an adjunct to bicalutamide, an established hormonal therapy used in the treatment of prostate cancer. Bicalutamide is a non-steroidal antiandrogen, which functions by blocking the androgen receptor (AR). It exerts its effect predominantly through induction of a G1/S phase arrest of the cell cycle.[23,24] We hypothesized that combining metformin with bicalutamide may have an additive antiproliferative effect by each drug's differing mechanisms of action. Thus, we sought to determine whether combining metformin and bicalutamide would inhibit prostate cancer cell growth using *in vitro* and *in vivo* prostate cancer models. We also proposed to assess whether any potential beneficial effect was AR-dependent by utilizing prostate cancer cell lines of differing AR expression.

MATERIALS AND METHODS

Cell lines

Three human prostate cancer cell lines (DU145, LNCaP and PC3) were obtained from the American Type Culture Collection (Rockville, MA, USA). Human prostate cancer cell line, PC3AR2, was a generous gift from Dr Ted Brown, Mount Sinai Hospital, Toronto, ON, Canada. PC3AR2 cells are PC3 cells transfected with full-length functional AR.[25] Thus, LNCaP and PC3AR2 cells express functional AR, whereas PC3 and DU145 cells do not. Cells were cultured at 37 °C in a 5% CO_2 incubator in the following media: LNCaP cells, RPMI 1640 medium (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (Sigma, St Louis, MO, USA), 0.3 mg ml^{-1} L-glutamine and 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Invitrogen); PC3 and DU145 cells, Dulbecco's minimal essential medium/F12 (Invitrogen) with 10% fetal bovine serum supplemented with 0.3 mg ml^{-1} L-glutamine and 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin; PC3AR2 cells, RPMI 1640 medium supplemented with 5% fetal bovine serum, 0.3 mg ml^{-1} L-glutamine, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, Fungizone (250 μg ml^{-1} amphotericin B and 250 μg ml^{-1} deoxycholate, Invitrogen) and 100 μg ml^{-1} hygromycin B (Invitrogen).

Chemicals

Metformin (Sigma, Oakville, Ontario, Canada) was supplied by Dr Michael Pollak, McGill University, Montreal, QC, Canada. A working concentration (1 M) was formulated by dissolving the drug in appropriate cell culture medium. Bicalutamide was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. A working solution (10 mM) was created by dissolving the compound in dimethyl sulfoxide (DMSO, Sigma). All other chemicals were obtained from Sigma, USA, unless otherwise specified.

Clonogenic assays

Confluent cells were trypsinized and seeded at densities ranging from 400 to 4000 cells per 10 cm petridish, depending on the cell line. Cells were allowed to adhere for 24 h before application of treatment with either metformin alone (0.01–10 mM), bicalutamide alone (0.01–10 μM) or a combination of the two drugs. In the combination treatment regimen, the dose of bicalutamide was fixed (at the minimum dose detected to inhibit colony formation when used as monotherapy) and the dose of metformin varied (0.01–10 mM). Treatments were applied daily for 5 days without media change. Colonies developed over 9–14 days, and were stained with crystal violet and manually counted. Each assay was internally controlled using untreated cells (cell media or 0.01% DMSO carrier). Relative plating efficiencies were expressed as percentages relative to the plating efficiency of untreated cells. All experiments were performed in triplicate. In view of the light sensitivity of metformin assays were performed in a darkened environment.

Flow cytometry

Cell cycle profiles were determined in LNCaP and PC3 cells by flow cytometry. Cells were labelled with anti-bromodeoxyuridine, fluorescein isothiocyanate and propidium iodide to determine the distribution of cells in differing phases of the cell cycle. Asynchronously growing cells (5×10^5 cells per plate) were plated in 10 cm petridishes and treated for 48 h with metformin (0.1 mM) and/or bicalutamide (0.1 μM for LNCaP and 10 μM for PC3 cells). Control plates were treated with vehicle alone (cell culture media or 0.01% DMSO). Cells were pulse-labelled with bromodeoxyuridine for 2 h before harvesting. As a negative control, a no-bromodeoxyuridine control was included. Cells were trypsinized, fixed in ice-cold 70% ethanol and stored at −20 °C until further analysis. Cells were subsequently washed in buffer [phosphate-buffered saline (Invitrogen) and 0.5% Tween-20] and treated with 2 N HCl for 20 min to expose labelled DNA. Cells were incubated for 1 h on ice with anti-bromodeoxyuridine-conjugated fluorescein isothiocyanate (DAKO, Mississauga, Ontario, Canada). Cells were washed, centrifuged and resuspended in 10 μg ml^{-1} propidium iodide, and allowed to incubate for 30 min on ice. Samples were filtered through a nylon mesh and cell cycle analysis performed on the FACSCalibur flow cytometer using the Cell Quest Pro software package (Becton Dickinson, Franklin Lakes, NJ, USA). Experiments were performed in duplicate.

Western blot analyses

LNCaP or PC3 prostate cancer cells were exposed to metformin (0.1 mM) and/or bicalutamide (0.1 μM for LNCaP and 10 μM for PC3 cells) for 48 h before cell lysis [NP-40 lysis buffer with inhibitors (leupeptin/pepstatin, aprotinin and phenylmethanesulfonylflouride), SDS, deoxychalate and EDTA]. Protein levels were quantified using the Bradford protein assay (Biorad, Hercules, CA, USA) before loading into gradient SDS gels for electrophoresis. Following overnight transfer, membranes were probed to assess: (i) receptor expression levels (AR), (ii) pathways downstream of AR and AMPK (PSA, AMPK, phospho-AMPK (threonine 172), mTOR, phospho-mTOR) and (iii) markers of apoptosis (Bcl-2-associated X protein, B-cell lymphoma extra large and caspase-3). AR and PSA antibodies were purchased from Santa Cruz, USA and the remaining antibodies were purchased from Cell Signaling, Danvers, MA, USA. Image quantification software (ImageJ, US National Institute of Health, Bethesda, MA, USA) was used to semiquantitatively determine protein expression levels, relative to β-actin.[26] All western blot experiments were performed at least in duplicate.

Effect of metformin and/or bicalutamide on prostate cancer cells *in vivo*

Ethical approval for the *in vivo* component of this work was obtained from the University of Toronto Animal Research Ethics Board. All work was conducted in accordance with the established guidelines and protocols approved by the Canadian Council on Animal Care (CACC). Six-week-old male nu/nu athymic nude mice were purchased from Harlan Laboratories, Mississauga, Ontario, Canada. Following a week of acclimatization, 8×10^5 LNCaP cells resuspended in 100 μl matrigel solution (BD Biosciences, Franklin Lakes, NJ, USA) were inoculated subcutaneously, unilaterally, into the flank of each mouse, under inhalational (isofluorane) general anaesthesia. Mice were subsequently commenced on a high-carbohydrate, high-fat (HC-HF) diet, previously shown to induce hyperinsulinaemia and

promote tumour growth (Purina test diet 5382, St Louis, MO, USA).[9] Tumour volume was determined using the formula: $\pi/6 \times (\text{tumour width})^2 \times \text{tumour length}$. When tumours achieved an average volume of 100 mm^3 (day 11), mice were randomized into four treatment groups; control ($n = 16$, injected with vehicle only), metformin alone ($n = 8$), bicalutamide alone ($n = 9$) or combined metformin and bicalutamide ($n = 8$). Metformin was dissolved in cell culture medium and was administered at a dose of 50 mg kg^{-1}. Bicalutamide was dissolved in DMSO and was also administered at a dose of 100 μg kg^{-1}. Both drugs were administered on days 12, 14, 16, 19, 21 and 23 by intraperitoneal injection, with the appropriate diluent made up to a total volume of 200 μl. Despite a prior bicalutamide dose finding study in CD1 mice, 8/9 mice receiving bicalutamide as monotherapy and 3/8 mice receiving bicalutamide in combination with metformin experienced profound toxicity resulting in death. The only difference to the prior dosing experiments was the use of larger volumes of DMSO utilized to create a consistent 200-μl injection volume. The surviving mice that had not received treatment with metformin on day 1 were redistributed to create a second cohort of mice for the bicalutamide group, leaving six mice in the control group, eight mice in the metformin-only group, six mice in the bicalutamide-alone group and five mice in the combination treatment group. Subsequent injections of the same dose of bicalutamide in a lower volume of DMSO did not cause toxicity. Mice were weighed three times per week with simultaneous tumour measurement. Mice with tumours exceeding 17 mm diameter were culled in accordance with CACC guidelines. Serum samples were obtained by saphenous vein puncture during (i) acclimatization, (ii) before active treatment and (iii) during active treatment. At termination of the experiment (day 54), serum samples were collected by direct cardiac puncture. The serum samples were analysed using commercially obtained assay kits to determine IGF1 (Active Mouse/Rat IGF ELISA kit, Diagnostic Systems Laboratories, Webster, TX, USA) and PSA (Quantikine Human Kallikrein 3/PSA immunoassay kit, R & D Systems, Minneapolis, MN, USA) levels. Metformin levels were also assayed during the active treatment phase of the study, using high-performance liquid chromatography.

Statistics

Analysis of between-group variations for all *in vitro* experiments were assessed using Student's *t* testing. Analyses of between-group variations at specific time points in the *in vivo* experiments was performed using Student's *t* testing. Repeated-measures analysis of variance was used to assess for differences between whole treatment groups over time. Statistical analyses were performed using SAS software, version 8 (SAS Institute, Cary, NC, USA).

RESULTS

Combining metformin with bicalutamide significantly reduces colony formation rates in prostate cancer cell lines, compared with either monotherapy alone

Clonogenic assays were used to assess the effect of metformin and/or bicalutamide on clonogenicity in prostate cancer cell lines (Figure 1). Exposure to millimolar doses of metformin caused a significant dose-dependent reduction in colony formation in all four cell lines ($P < 0.05$). Exposure to bicalutamide caused a significant reduction in colony formation rates in AR-positive cell lines only ($P < 0.00001$). In LNCaP cells, combining varying millimolar doses of metformin and 0.1 μM bicalutamide resulted in a further significant reduction in the colony formation, compared with treatment with either monotherapy ($P < 0.003$). In PC3AR2 cells, higher doses of bicalutamide (1 μM) were required to achieve a further significant reduction in the colony formation in comparison with monotherapy ($P < 0.004$). In PC3 cells further significant reduction in colony formation with combination treatment was only achieved with exposure to high doses of bicalutamide, 10 μM ($P < 0.02$). DU145 cells exhibited no further reduction in colony formation rates following combination treatment.

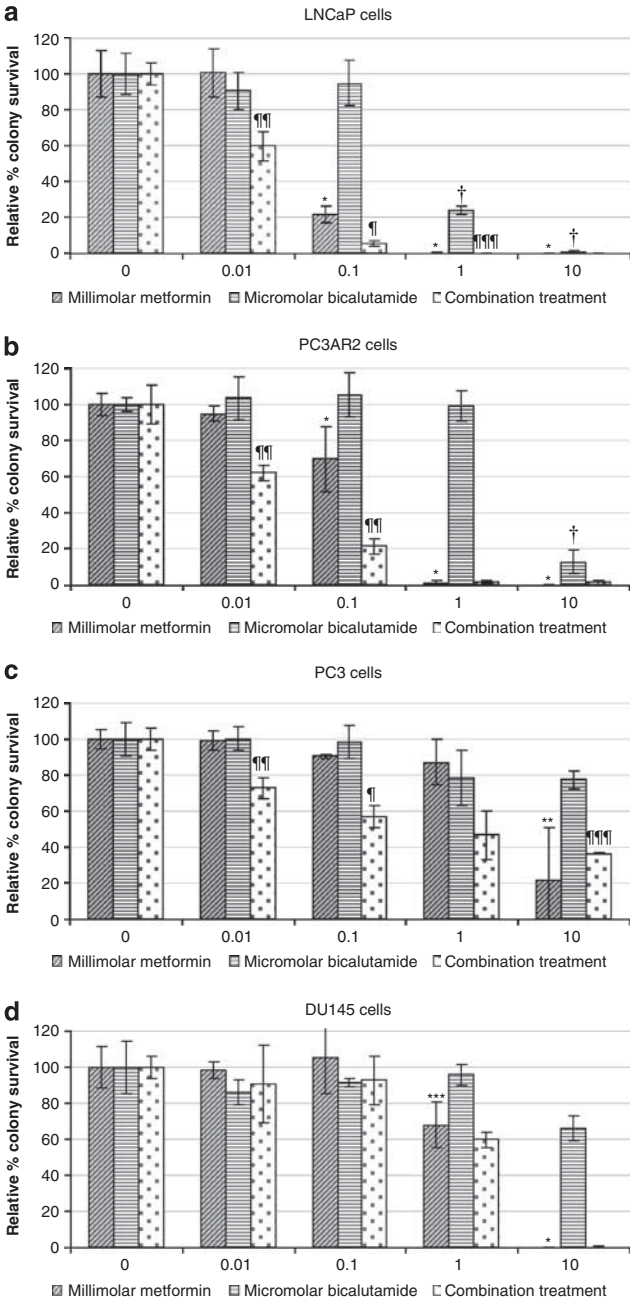

Figure 1. Clonogenic assays showing the effect of daily treatment for 5 days with either varying doses of mM metformin (diagonal striped bars), varying doses of μM bicalutamide (horizontal striped bars) or combination treatment with varying doses of mM metformin and, 0.1 μM bicalutamide in LNCaP cells (**a**), 1 μM bicalutamide in PC3AR2 cells (**b**), 10 μM bicalutamide in PC3 cells (**c**) and DU145 cells (**d**). Significant reduction in colony formation rate seen compared with untreated cells $^{*}P < 0.0005$; $^{**}P < 0.005$; $^{***}P < 0.05$ for metformin and $^{\dagger}P < 0.0005$ for bicalutamide, or compared with either monotherapy for combination treatment $^{\P}P < 0.0005$; $^{\P\P}P < 0.005$ and $^{\P\P\P}P < 0.05$.

Metformin induces a G1/S phase arrest of the cell cycle

The effect of metformin and/or bicalutamide on cell cycle distribution was assessed using flow cytometry. Following 48 h of treatment with 0.1 mM metformin either as monotherapy or in combination with 0.1 μM bicalutamide, LNCaP cells showed a significant decrease in the proportion of cells in the S phase of the

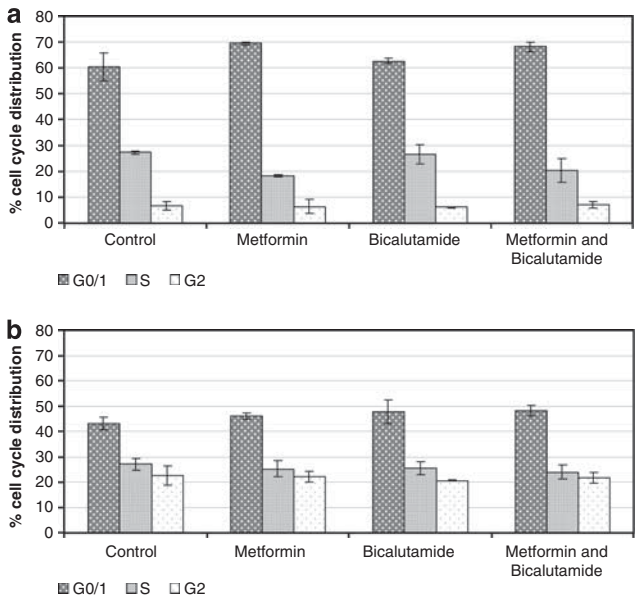

Figure 2. (**a**) Alteration of proportion of cells in differing phases of the cell cycle in LNCaP cells treated with 0.01 mM metformin, 0.1 μM bicalutamide or a combination of 0.01 mM metformin and 0.1 μM bicalutamide. A significant decrease of cells in the S phase, with a concomitant increase of cells in G0/1, occurred following treatment with metformin, $P<0.001$ or combination treatment, $P=0.03$, consistent with a G1/S cell cycle arrest. Treatment with bicalutamide did not exert any significant effect on the proportion of cells in S phase of the cell cycle. (**b**) No alteration of proportion of cells in differing phases of the cell cycle was observed in PC3 cells treated with 0.1 mM metformin, 10 μM bicalutamide or a combination of 0.1 mM metformin and 10 μM bicalutamide.

cell cycle, consistent with a G1/S cell cycle arrest, Figure 2a ($P<0.03$). Bicalutamide alone had no effect on cell cycle distribution and the combination regimen did not further decrease the proportion of cells in S phase, relative to treatment with metformin alone. No change in cell cycle distribution was observed following the treatment of PC3 cells with either monotherapy or the combination treatment regimen (Figure 2b).

Western blotting was used to assess the potential mechanisms of interaction between metformin and bicalutamide. Thus, a variety of proliferative and apoptotic signalling proteins were assessed, following treatment with metformin and/or bicalutamide.

Treatment with metformin and/or bicalutamide has minimal or no effect on AR expression, whereas exposure to metformin reduces PSA expression in LNCaP cells

Exposure of LNCaP cells to 0.1 μM bicalutamide caused a minimal reduction in AR expression (Figure 3a). Exposure of LNCaP cells to 0.1 mM metformin, either as monotherapy or in combination with 0.1 μM bicalutamide did not alter AR expression. Conversely, exposure of LNCaP cells to 0.1 mM metformin (either as monotherapy or in combination with 0.1 μM bicalutamide) resulted in decreased PSA expression. Exposure of LNCaP cells to 0.1 μM bicalutamide monotherapy led to a minimal reduction in PSA expression. As expected, PC3 cells expressed neither AR nor PSA.

Combining metformin and bicalutamide downregulates proliferative signalling proteins in LNCaP cells and promotes apoptosis in PC3 cells

Exposure of LNCaP cells to either 0.1 mM metformin or 0.1 μM bicalutamide resulted in two- and threefold increase in phosphorylation of AMPK, respectively (depicted in Figure 3b as the ratio of phospho-AMPK:total AMPK). Combination treatment with 0.1 mM metformin and 0.1 μM bicalutamide resulted in enhanced AMPK phosphorylation (4.5-fold relative to untreated cells). As activation of AMPK inhibits the proproliferative function of mTOR, we next assessed the level of phosphorylated mTOR. Exposure of LNCaP cells to 0.1 mM metformin, 0.1 μM bicalutamide or a combination of the two resulted in decreased phosphorylated mTOR levels, indicating that the direct antiproliferative effects of these treatments are mediated via AMPK/mTOR signalling.

In PC3 cells 1 mM metformin, either as monotherapy or in combination with 10 μM bicalutamide resulted in activation (phosphorylation) of AMPK (Figure 3b). Conversely, 10 μM bicalutamide monotherapy had no effect on AMPK phosphorylation. In contrast to LNCaP cells, the combination treatment regime did not result in further activation of AMPK. Bicalutamide, either as monotherapy (10 μM) or in combination with 1 mM metformin, activated mTOR, as evidenced by an increase ratio of phospho-mTOR to mTOR, indicating that the reduced colony formation rates seen using clonogenic assay are unlikely mediated via mTOR-mediated antiproliferative signalling.

LNCaP cells treated with a combination of 0.1 mM metformin and 0.1 μM bicalutamide showed a minimal increase in the level of total caspase 3 (Figure 3c). Total caspase 3 levels were unaffected by treatment with either 0.1 mM metformin or 0.1 μM bicalutamide monotherapy. Neither Bcl-2-associated X protein nor B-cell lymphoma extra large levels were altered by treatment with either monotherapy or the combination treatment regimen. In summary, no evidence of apoptosis was observed in LNCaP cells treated with 0.1 mM metformin and/or 0.1 μM bicalutamide.

Conversely, PC3 cells showed evidence of apoptosis, following treatment with either monotherapy or the combination treatment regimen (Figure 3c). Metformin (1 mM) or bicalutamide (10 μM) resulted in a twofold reduction in total caspase 3 levels. Combination treatment with 1 mM metformin and 10 μM bicalutamide substantially reduced total caspase 3 expression (ninefold reduction relative to untreated cells). B-cell lymphoma extra large levels were unaffected by treatment with either monotherapy or the combination treatment regimen; however, Bcl-2-associated X protein levels were increased following exposure to either monotherapy or the combination treatment regimen. The ratio of the proapoptotic protein, Bcl-2-associated X protein, to the antiapoptotic protein, B-cell lymphoma extra large, revealed a 1.5-fold increased expression in cells treated with 1 mM metformin and/or 10 μM bicalutamide relative to untreated cells, consistent with apoptosis.

Treatment of LNCaP prostate cancer xenografts with metformin and bicalutamide significantly reduces tumour volumes

A prostate cancer xenograft model was used to assess the potential growth-inhibitory effect of metformin and/or bicalutamide *in vivo*. Treatment with metformin and/or bicalutamide was well tolerated. All mice consistently maintained their body weight in response to feeding with the HC-HF diet, irrespective of allocated treatment group (data not shown). No significant difference in mean body weights existed between treatment groups at termination of the experiment ($P=0.12$). Initial analyses using Student's *t* testing revealed a significantly reduced tumour growth in mice treated with the combination regimen relative to all other treatment groups ($P<0.05$) in the time period immediately following active treatment (day 25 and day 27), Figure 4. To avoid the use of multiple Student's *t* testing, repeated-measures analysis of variance was utilized to assess for significant differences in tumour volumes between the groups over the whole duration of the study (days 0–54). This analysis revealed highly significant differences to exist between the four treatment groups ($P<0.00001$). Further pairwise testing indicated no significant difference in tumour volume in mice treated with

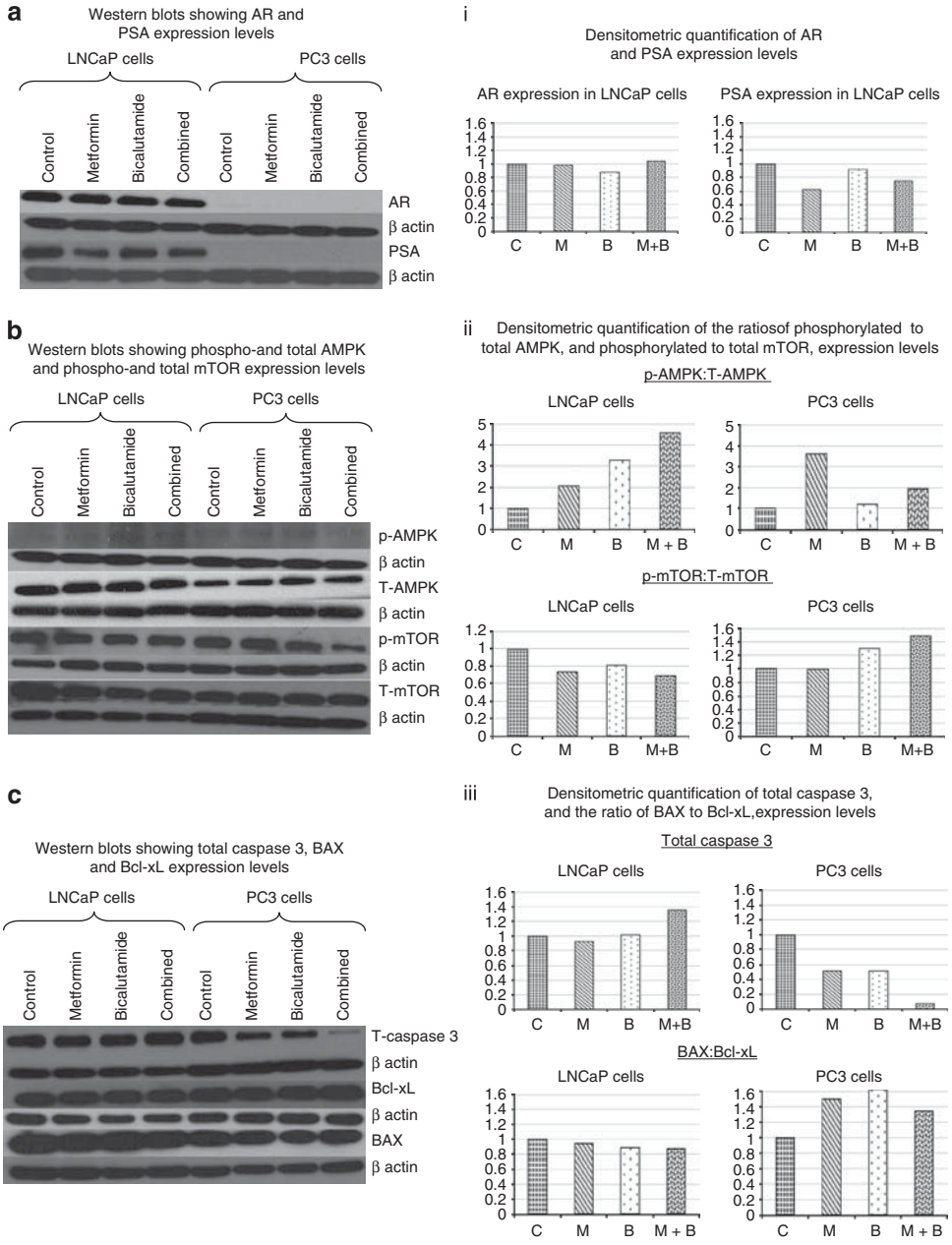

Figure 3. Western blots showing levels of expression of (**a**) androgen receptor (AR) and PSA, (**b**) phospho- and total adenosine monophosphate kinase (AMPK), phospho- and total mammalian target of rapamycin (mTOR) and (**c**) total caspase 3, Bcl-2-associated X protein (BAX) and B-cell lymphoma extra large (Bcl-xL), following 24 h exposure of (i) LNCaP cells to either no treatment, 0.1 mM metformin (M), 0.1 μM bicalutamide (B) or combined 0.1 mM metformin and 0.1 μM bicalutamide (M + B) and (ii) PC3 cells to either no treatment, 1 mM metformin (M), 10 μM bicalutamide (B) or combined 1 mM metformin and 10 μM bicalutamide (M + B). Associated bar histograms (i–iii) depict densitometric quantification of levels of isolated protein expression or the ratio of expression of associated proteins for the individual blots shown, relative to β actin expression.

metformin when compared with untreated control mice ($P = 0.15$). Conversely, mice treated with bicalutamide had significantly smaller tumours than untreated control mice ($P = 0.02$), and mice treated with a combination of metformin and bicalutamide had highly significantly smaller tumours than untreated mice, see Figure 4 ($P < 0.0004$).

An HC-HF diet significantly increases serum IGF1 levels and treatment with metformin and/or bicalutamide significantly reduce serum IGF1 levels

To assess the effect of the HC-HF diet on IGF signalling, along with the subsequent effect of our differing treatment regimens we performed serum analyses of IGF1 levels before commencement of the diet, before commencement of treatment and during the active treatment phase of the study, using pooled serum samples from the groups. Serum IGF1 levels rose significantly following commencement of the HC-HF diet (554 ng ml^{-1} pre-diet versus 609 ng ml^{-1} pretreatment versus 643 ng ml^{-1} in control mice during active treatment phase, $P < 0.01$). Treatment with metformin, bicalutamide or a combination of the two significantly reduced IGF1 levels in comparison with untreated control mice (566 ng ml^{-1} versus 514 ng ml^{-1} versus 418 ng ml^{-1}, respectively, $P < 0.001$), Figure 5a. The reduction in IGF1 levels consequent upon combination treatment was also significant when compared

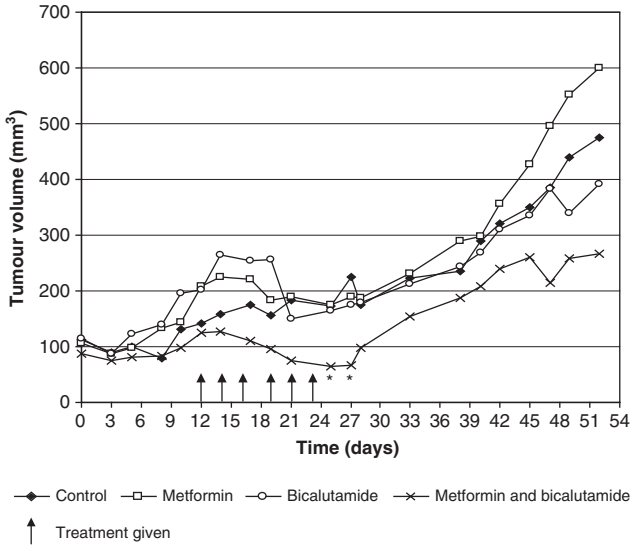

Figure 4. Variation in tumour volume over time for four differing treatment groups, either control mice or those treated with metformin alone (50 mg kg^{-1}), bicalutamide alone (100 μg kg^{-1}) or combined metformin and bicalutamide. Using Student's *t* testing at individual time points revealed significantly reduced tumour growth in the combination group compared with all other groups at days 25 and 27 (*$P<0.05$). Using repeated-measures analysis of variance to assess variation in tumour volume across the whole time period of the *in vivo* study, mice treated with the combination regimen exhibited a significantly reduced tumour growth ($P<0.00001$).

with treatment with either metformin or bicalutamide alone ($P<0.0001$).

Combination treatment with metformin and bicalutamide significantly reduces serum PSA levels

PSA levels were ascertained using serum obtained at experiment termination, day 54 (Figure 5b). Serum PSA was significantly elevated in all treatment groups at the experiment termination, compared with levels obtained either before commencement of the HC-HF diet or pretreatment ($P<0.001$). Treatment with metformin did not reduce terminal serum PSA levels significantly ($P=0.97$), whereas treatment with bicalutamide or the combination of metformin and bicalutamide significantly reduced terminal serum PSA levels (34.0 ng ml^{-1} and 19.0 ng ml^{-1}, respectively, $P<0.002$). The serum PSA level for the combination treatment regimen was significantly lower than that for either monotherapy ($P<0.002$).

High-performance liquid chromatography was used to assess serum metformin levels during the active treatment phase of the experiment. Mice treated with metformin (either as a monotherapy or in combination with bicalutamide) exhibited mean serum metformin levels ranging from 24.1 to 131.6 μM, depending on treatment groups assessed. These readings are of the same order of magnitude as serum levels achieved with therapeutic metformin doses utilized in humans (10–20 μM).[27]

DISCUSSION

There is mounting evidence for the detrimental effect of diabetes- or obesity-induced hyperinsulinaemia on a variety of malignancies.[3] Metformin, a treatment that lowers blood glucose levels, prevents chronic hyperinsulinaemia. In this study, we assessed the role of metformin as an adjunct to bicalutamide, a known prostate cancer therapy, using *in vitro* and *in vivo* models of prostate cancer.

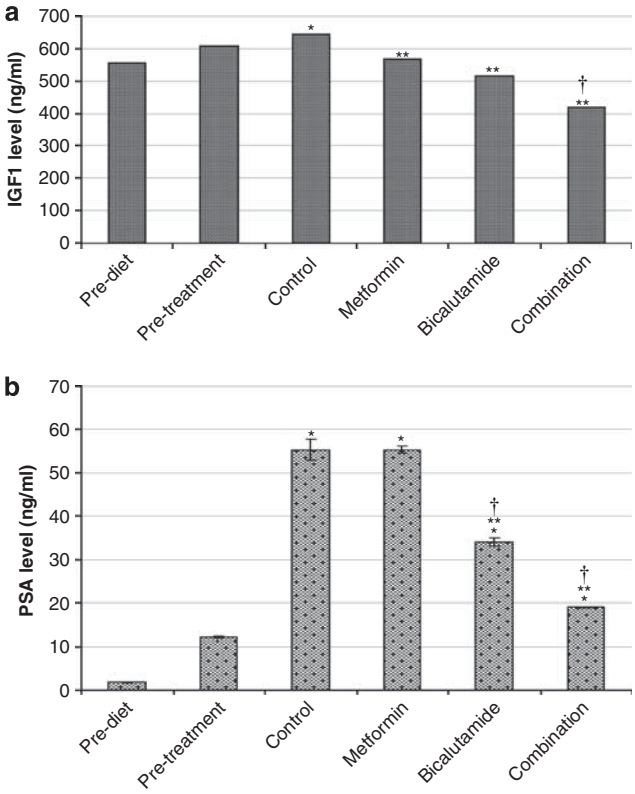

Figure 5. (**a**) Variation in serum insulin-like growth factors (IGF)1 levels in mice, before commencement of high-carbohydrate high-fat diet, before commencement of treatment and during treatment with either metformin, bicalutamide or a combination of metformin and bicalutamide. * denotes a significant increase in IGF1 level in comparison with either pretreatment levels and ** denotes a significant decrease in IGF1 levels in comparison with control mice, $P<0.001$ and † denotes a further significant decrease in the IGF1 level compared with treatment with either monotherapy. (**b**) Variation in serum PSA levels in mice either before commencement of high-carbohydrate high-fat diet, before commencement of treatment and at study termination, following treatment with either metformin, bicalutamide or a combination of metformin and bicalutamide. * denotes significant increase in terminal serum PSA level in comparison with either pre-diet and pretreatment levels, ** denotes significant decrease in terminal serum PSA levels in comparison with control mice PSA levels and † denotes a further significant decrease in terminal serum PSA levels compared with other monotherapy serum PSA levels, $P<0.001$.

Our *in vitro* results showed that combining metformin with bicalutamide significantly reduces prostate cancer cell survival, particularly in cells expressing a functional AR. Mechanistic studies using flow cytometry and western blotting suggested that this effect was mediated by a direct antiproliferative effect in AR-positive cells, as evidenced by activation of phospho-AMPK with subsequent inhibition of downstream mTOR signalling and altered cycle distribution with a G1/S cell cycle arrest. Similar to our findings in AR-positive cells, several investigators have shown that metformin functions by activating AMPK with subsequent downstream inhibition of proproliferative mTOR signalling.[17,18,20] In AR-negative cells, we also found metformin capable of activating AMPK; however, this did not translate into reduced mTOR signalling. Bicalutamide had no effect on AMPK phosphorylation, suggesting that a functional AR is required for bicalutamide-mediated AMPK phosphorylation. Thus, using clonogenic assays in PC3 cells, we found no evidence that the combination regimen modulated markers of proliferation in the same way as seen in the AR-positive LNCaP prostate cancer cell line. Further evaluation

using alternative proliferation assays would be beneficial in consolidating this finding.

In AR-negative cells, diminished prostate cancer cell survival appeared to be mediated through enhanced levels of apoptosis. Evidence for a proapoptotic effect of metformin is mixed. Cantrell *et al.*[22,28] showed that metformin promoted apoptosis in endometrial carcinoma cells whereas others have been unable to recapitulate this finding.[16] Bicalutamide is widely accepted as a proapoptotic agent, although the mechanism by which it induces apoptosis remains unclear and likely differs depending on androgen sensitivity.[29] We found that in AR-negative cells, both metformin and bicalutamide reduced levels of total caspase 3 and also increased expression of the proapoptotic factor B-cell lymphoma extra large. No evidence of an apoptotic effect was found in cells expressing a functioning AR. It is however unclear whether the lack of AR alone is responsible for the switch to apoptosis in AR-negative cell lines, or whether the 10–100-fold higher doses of metformin and bicalutamide utilized in AR-negative cells altered the mechanism of action of the evaluated compounds. Other studies have shown that the proapoptotic effect of metformin is only seen when high doses of the drug are utilized.[22]

The *in vitro* model used in these studies did not assess the potential indirect effects of metformin that is, those mediated by inhibition of hyperinsulinaemia. This was more easily assessed using our *in vivo* xenograft model. We, along with others, have shown that feeding mice on a HC-HF diet causes hyperinsulinaemia and this promotes growth of prostate cancer xenografts.[9,19] Herein, we again confirmed that serum IGF1 levels were significantly increased by commencement of the HC-HF diet. Treatment with either metformin or bicalutamide significantly reduced serum IGF1 levels with the combination regimen, causing a further significant reduction in the level of IGF1. Interestingly, monotherapy with metformin did not reduce tumour volume (a finding that we have previously reported for the 50 mg kg^{-1} dose of metformin used in this study[30]) whereas monotherapy with bicalutamide significantly reduced tumour volume ($P = 0.02$) and combination therapy reduced tumour volume further ($P < 0.001$). Serum PSA levels obtained at the termination of the experiment mirrored the tumour volume results, further corroborating our findings. Results from our *in vivo* study suggest that the additive antiproliferative effects of combining metformin and bicalutamide are dependent on modulation of the functioning AR. Further evaluation of the effects of the combination regimen using an AR-negative xenograft model are planned to further elucidate the mechanism of interaction between these two agents.

CONCLUSION

We have demonstrated an additive effect of metformin on the action of bicalutamide in prostate cancer *in vivo* and *in vitro*. This effect is mediated by several pathways. In AR-positive cells, a direct antiproliferative effect occurs characterized by the activation of phospho-AMPK with subsequent inhibition of downstream mTOR signalling and altered cycle distribution with a G1/S cell cycle arrest. In AR-negative cells, apoptosis was induced, albeit only at higher concentrations of both drugs. These studies support the potential role of metformin as an adjunct to androgen deprivation therapy, as well as a potential prostate cancer prevention agent.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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