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Tyrosine kinase inhibitor imatinib modulates the viability and apoptosis of castrate-resistant prostate cancer cells dependently on the glycolytic environment

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ABSTRACT

Aims: The tyrosine kinase inhibitor imatinib has been used in prostate cancer treatment with outcomes that did not follow the *in vitro* findings. The glycolytic environment has been shown to influence the efficacy of anti-cancer drugs. This study aimed to evaluate the effect of imatinib on cell viability, apoptosis, and metabolism in cell line models of castrate-resistant prostate cancer (CRPC) under hyperglycemic and hypoglycemic conditions.
Main methods: DU145 and PC3 CRPC cell lines were exposed to 20 μM imatinib under 5 mM (hypoglycemia) or 30 mM glucose (hyperglycemia) for 48–72 h. Cell viability was assessed by the MTS assay. The expression of apoptosis regulators and glycolytic metabolism-related proteins was analysed by Western blot, and the activity of caspase-3 and lactate dehydrogenase (LDH) was determined spectrophotometrically. Glucose consumption and lactate production were determined using biochemical assays.
Key findings: Imatinib decreased CRPC cells viability, whereas increasing apoptosis; effects only observed in hyperglycemic conditions. Glucose consumption and lactate production were significantly increased in imatinib-treated DU145 and PC3 cells, and independently of glucose availability. Accordingly, LDH expression and activity were significantly increased in response to imatinib.
Significance: Higher glucose availability improved the effectiveness of imatinib suppressing survival and growth of CRPC cells. It was also shown that imatinib treatment stimulated the glycolytic metabolism of CRPC cells. This study first demonstrated that a glucose-enriched environment intensifies the effect of imatinib, which stimulates the interest for testing this compound into the clinical setting, namely in hyperglycemia conditions (diabetic patients) or in co-administration with inhibitors of glycolytic metabolism.

1. Introduction

The advanced metastatic prostate cancer (PCa) is characterized by the loss of androgen responsiveness, reaching the stage of disease so-called castration-resistant prostate cancer (CRPC) [1]. Clinically this means the failure of classical androgen ablation therapies and corresponds to a usually lethal form of PCa [2,3].

Imatinib mesylate is a potent and selective inhibitor of receptors tyrosine kinase that has been used to treat gastrointestinal stromal tumors and leukemias [4,5]. Imatinib also has been tested in PCa treatment, but the results obtained were modest with this compound seeming to be ineffective controlling prostate-specific antigen levels and reducing tumor size [6,7]. Contrastingly, *in vitro* and animal experimentation studies showed that imatinib, alone or in combination with other drugs, have cytotoxic effects and sensitizes PCa cells to chemo- or radiotherapy [8–10]. Our research group investigated the cytotoxic effects of imatinib in cell line models of CRPC, DU145 and PC3 cells [11]. Imatinib was effective decreasing viability and increasing apoptosis of DU145 cells, whereas displaying opposite effects in PC3 cells [11]. These contradictory results were related to the distinct expression of c-KIT receptor isoforms [11], but also indicated that imatinib responses might depend on cell-specific or environmental conditions.

The glycolytic environment has been shown to influence the efficacy of various anti-cancer drugs in different types of cancer [12,13]. Although imatinib actions have been linked with alterations in glucose metabolism [14,15], the effect of this tyrosine kinase inhibitor dependently on the glycolytic environment has not been examined.

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On the other hand, the dependence on glycolysis is a recognized feature of cancer cells. This behavior, known as the Warburg's effect, is characterized by the use of glucose for lactate production instead of driven it to oxidative phosphorylation, what happens even in the presence of oxygen [16]. The lactate produced is exported into the extracellular space, acidifying the tumor microenvironment and promoting cancer cells growth and dissemination [17]. CRPC cells have been shown to display an increased glycolytic metabolism relatively to androgen-sensitive cells, with the glycolytic pattern being associated with progression and aggressiveness of disease [18–20].

Overall, the gathered information lead us to hypothesize that the imatinib effects controlling of PCa cells growth may depend on the glycolytic environment. The present study investigated the effect of imatinib in two cell line models of CRPC under conditions of different glucose availability (hyperglycemic *vs.* hypoglycemic). Alterations in cell viability, apoptosis and glycolytic metabolism were assessed. This included the expression analysis and activity measurement of several apoptosis regulators (Bcl-2, Bax, caspase-8, caspase-9 and caspase-3 proteins) and glycolytic metabolism-related proteins, namely glucose transporters (GLUTs), phosphofructokinase-1 (PFK1), lactate dehydrogenase (LDH) and monocarboxylate transporter 4 (MCT4).

2. Materials and methods

2.1. Cell lines and treatments

The human CRPC cell lines, DU145 and PC3, were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and maintained in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany) and penicillin (100 U /ml)/streptomycin (125 μg/ml)/amphotericin B (0,25 μg/ml) (SC-3690, Santa Cruz Biotechnology, Heidelberg, Germany), at 37 °C in an atmosphere equilibrated with 5% CO_2. Cells were maintained up to 60% confluence in all experiments and then the culture medium was replaced by glucose-free RPMI (R1383, Sigma-Aldrich) supplemented with 5 mM (hypoglycemia) or 30 mM of glucose (hyperglycemia) [21,22]. After additional 24 h, cells were exposed to a cytotoxic concentration (20 μM) of imatinib mesylate (CAS 220127–57-1, Santa Cruz Biotechnology) for 48 to 72 h, as recently described [10,11]. The experimental groups were as follow: i) 5 mM glucose without imatinib (imatinib (−) hypoglycemic group); ii) 5 mM glucose with imatinib (imatinib (+) hypoglycemic group); iii) 30 mM glucose without imatinib (imatinib (−) hyperglycemic group); and iv) 30 mM glucose with imatinib (imatinib (+) hyperglycemic group). For 72 h of treatment, control and treated-cells were harvested for protein extraction, and cell culture medium was collected for measurement of glucose and lactate concentration.

2.2. Cell viability assay

DU145 and PC3 cells were grown in 96-well plates, and cell viability was determined by the colorimetric CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI) at 48 and 72 h after treatment with imatinib in the presence of 5 mM or 30 mM glucose. The conversion of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2 M) tetrazolium compound to the colored formazan product was detected at 490 nm in the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA, USA). The relative number of viable cells in each experimental condition was calculated by normalizing the absorbance to that of the experimental group without imatinib (−) in hypoglycemic condition (5 mM glucose).

2.3. Quantification of glucose and lactate

The concentration of glucose and lactate in the cell culture medium was determined by spectrophotometric analysis using commercial kits (Spinreact, Girona, Spain) as described previously [18]. The glucose consumption and lactate production were calculated relatively to initial glucose and lactate concentrations at 0 h of treatment. All measurements were normalized to the total number of cells in each experimental condition.

2.4. Protein extraction

DU145 and PC3 cells were homogenized by pipetting in 60 μl of radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM EDTA) supplemented with 1% protease inhibitors cocktail (Sigma- Aldrich) and 10% PMSF (Sigma- Aldrich), kept on ice for 20 min with occasional mixing, and then centrifuged at 14,000 rpm for 20 min at 4 °C. Protein concentration was determined by the Bradford assay (Bio-Rad).

2.5. Western blot (WB)

Total proteins were resolved by SDS-PAGE on 12.5% gels and electrotransferred to a PVDF membrane (Bio-Rad). Membranes were incubated overnight at 4 °C with rabbit anti-Bcl-2 (1:1000, no. 2876; Cell Signaling Technology), rabbit anti-Bax (1:500, no. 2772, Cell Signaling Technology, Danvers, MA), rabbit anti-caspase-9 (1:500, H-170: sc-8355; Santa Cruz Biotechnology), mouse anti-caspase-8 (1:500, D-8: sc-5263; Santa Cruz Biotechnology), rabbit anti-GLUT1 (1:1000, CBL242, Millipore), rabbit anti-GLUT3 (1:2500, sc-30107, Santa Cruz Biotechnology), rabbit anti-PFK1 (1:1000, sc-67028, Santa Cruz Biotechnology), rabbit anti-LDH (1:10,000, Ab52488, Abcam, Cambridge, United Kingdom) or rabbit anti-MCT4 (1:1000, sc-50329, Santa Cruz Biotechnology) primary antibodies. Protein expression was normalized using a mouse anti-β-actin (1:1000, A5441, Sigma-Aldrich) antibody. Membranes were incubated with ECL substrate (Bio-Rad) for 5 min, and immunoreactive proteins were visualized with the ChemiDoc™ MP System (Bio-Rad). Band densities were obtained according to standard methods using the Image Lab software (Bio-Rad) and normalized by division with the respective β-actin band density.

2.6. Caspase-3 activity assay

The enzymatic activity of caspase-3 was determined spectrophotometrically at 405 nm by detecting the presence of the yellow product p-nitro-aniline (pNA), upon cleavage of caspase-3 substrate (Ac-DEVD-pNA). In brief, 50 μg of total protein extract was incubated overnight at 37 °C with reaction buffer (25 mM HEPES, 0.1% 3CHAPS, 10% sucrose, and 10 mM DTT, pH 7.5) and 200 μM of Ac-DEVD-pNA. The amount of generated pNA was calculated by extrapolation with a standard curve.

2.7. LDH activity

The enzymatic activity of LDH was determined using a commercial assay kit (Spinreact) following the manufacturers' instructions. LDH catalyses the reduction of pyruvate by NADH and the rate of decrease in concentration of NADH is proportional to the catalytic concentration of LDH present in the cells. The NADH concentration is measured spectrophotometrically at 340 nm using a xMarkTM Microplate Absorbance Spectrophotometer (Bio-Rad).

2.8. Statistical analysis

The statistical significance of differences between experimental groups was evaluated by one-way ANOVA followed by *Bonferroni post-hoc test*, using GraphPad Prism v6.00 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant. All experimental data are shown as mean ± standard error of the mean

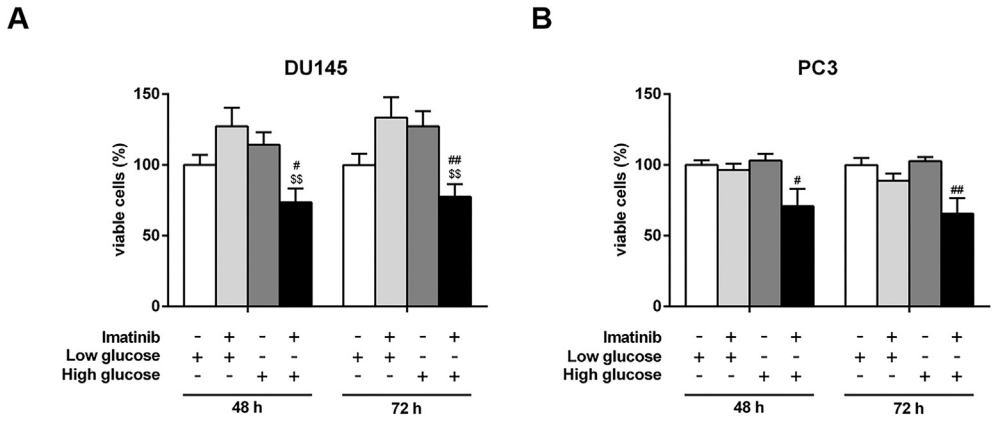

Fig. 1. Viability of DU145 (A) and PC3 (B) cells after treatment with imatinib (20 μM) at different glucose concentrations (5 and 30 mM) for 48 and 72 h determined by the MTS assay. Results are expressed as fold-variation relatively to imatinib (−) with low glucose (5 mM glucose). Error bars indicate mean ± S.E.M (n = 5). $^{\$}$ $P < 0.05$; $^{\$\$}$ $P < 0.01$; when compared with the imatinib (+) low glucose; $^{\#}$ $P < 0.05$; $^{\#\#}$ $P < 0.01$; when compared with the imatinib (−) high glucose.

(S.E.M).

3. Results

3.1. Imatinib decreased DU145 and PC3 cells viability under hyperglycemic conditions

The cytotoxic effects of imatinib in DU145 (Fig. 1A) and PC3 (Fig. 1B) cells under hypoglycemic (5 mM) and hyperglycemic (30 mM) conditions were evaluated by the MTS assay. Upon 48 h and 72 h of treatment with imatinib, the viability of DU145 cells was significantly decreased in the hyperglycemic group, 73,6 ± 9,8 and 77,4 ± 9,0 *vs.* 114,2 ± 8, 9 and 127,2 ± 10,7% in the imatinib (−) hyperglycemic condition, respectively (Fig. 1A). Moreover, DU145 viability in the imatinib (+) hyperglycemic group at 72 h was significantly decreased relatively to the imatinib (+) hypoglycemic group (~42% reduction). Also, the viability of PC3 cells was markedly reduced after 48 h and 72 h of treatment with imatinib in hyperglycemic conditions (73,9 ± 15,3 *vs.* 103,1 ± 4,7 ($P < 0,05$) and 65,6 ± 11,0 *vs.* 102,7 ± 2,9 ($P < 0,01$), respectively) (Fig. 1B). All the subsequent analysis on gene expression and cell metabolism were performed for 72 h of treatment.

3.2. Hyperglycemic conditions improved the pro-apoptotic effect of imatinib

In order to determine whether the diminished viability/proliferation of DU145 and PC3 cells in response to imatinib treatment is a consequence of augmented apoptosis the expression levels and activity of several apoptosis regulators were evaluated.

Bax and Bcl-2 proteins are, respectively, pro- and anti-apoptotic members of the Bcl-2 family of apoptosis regulators involved in the regulation of the intrinsic pathway of apoptosis [23]. Imatinib significantly decreased the expression of Bcl-2 in DU145 cells cultured in high glucose concentrations comparatively with the imatinib (−) 30 mM glucose (0,07 ± 0,02 *vs.* 0,17 ± 0,01, Fig. 2A) and imatinib (+) 5 mM glucose groups (0,07 ± 0,02 *vs.* 0,16 ± 0,02, Fig. 2A). In PC3 cells, Bcl-2 expression was also decreased in response to imatinib under hyperglycemic conditions (0,27 ± 0,04 *vs.* 0,45 ± 0,04 in the 30 mM glucose imatinib (−), $P < 0,05$, Fig. 2B). The Bax protein was only detected in PC3 cells and its expression was increased in hyperglycemic groups relatively to hypoglycemic conditions regardless of the presence of imatinib ($P < 0,05$, Fig. 2C).

Caspase-9 is the well-known activator of the intrinsic pathway of apoptosis, responsible for cleavage of the apoptosis effector caspase-3 [24]. However, caspase-9 expression remained unchanged in DU145-treated cells (Fig. 2D) whereas in PC3 it was augmented in the imatinib (−) group under hyperglycaemia relatively to the imatinib (−) in hypoglycaemia (0,60 ± 0,04 *vs.* 0,43 ± 0,04 $P < 0,05$ 1, Fig. 2E).

Caspase-3 can also be activated by caspase-8, the main mediator of the extrinsic pathway of apoptosis [25]. Hyperglycemia increased the expression of caspase-8 in DU145 cells ($P < 0,05$, Fig. 2F) independently of the presence of imatinib; caspase-8 expression was also augmented in PC3 cells under 30 mM glucose ($P < 0,05$, Fig. 2G).

Concerning the activation of caspase-3, at the convergence of intrinsic and extrinsic pathways and a remarkable end-point of apoptosis [26], it was observed that imatinib treatment significantly increased the activity of caspase-3 in both DU145 and PC3 cells. However, this effect only occurred in the hyperglycaemic conditions; 3396 ± 435,7 *vs.* 1824 ± 491,2 in imatinib (−) hyperglycaemic or 1274 ± 209,2 in imatinib (+) hypoglycaemic groups in DU145 cells (Fig. 2I); 6075 ± 990,2 *vs.* 2123 ± 311,7 in imatinib (−) hyperglycaemic or 2642 ± 321,3 in imatinib (+) hypoglycaemic groups in PC3 cells (Fig. 2J).

3.3. The glycolytic metabolism of DU145 and PC3 cells is altered by glycaemia conditions and imatinib

The glycolytic environment has been shown to modulate the response to therapy in different cancer cell types [12,13]. With this rational we investigated the glycolytic metabolism of DU145 and PC3 cells under treatment with imatinib in hyperglycemic and hypoglycemic conditions. Glucose consumption and lactate production were determined spectrophotometrically and WB analysis was performed to evaluate the protein levels of key transporters and enzymes involved in glucose metabolism.

Imatinib treatment increased glucose consumption in DU145 cells under hypoglycaemic conditions (57,5 ± 1,3 *vs.* 36,3 ± 0,4 in imatininb, (−) $P < 0,001$, Fig. 3A) whereas no differences were observed in hyperglycaemia. In PC3 cells glucose consumption was significantly augmented in response to imatinib both in hypoglycaemia and hyperglycaemia conditions (24,8 ± 0,5 *vs.* 15,5 ± 0,7 in imatinib (−) and 11,5 ± 0,4 *vs.* 5,6 ± 1,1 imatinib (−), respectively, Fig. 3B). On the other hand, it is note of worth that glucose consumption in DU145 and PC3 cells was significantly decreased in hyperglycemia conditions comparatively with the same experimental groups in hypoglycemia (Fig. 3A and B), with the exception of DU145 imatinib (−).

Regarding lactate production, it was increased in imatinib-treated cells in both glycemic conditions. Lactate production in DU145 cells in response to imatinib in hypoglycaemia varied from 24,4 ± 0,9 to 46,2 ± 2,4 ($P < 0,001$) whereas in hyperglycaemia conditions the variation was from 14,3 ± 0,9 to 22,6 ± 1,7 ($P < 0,05$) (Fig. 3C). In PC3 cells the imatinib treatment also increased the lactate production regardless of glycemic conditions (14,7 ± 0,7 in imatinib (+) *vs.* 9,7 ± 0,2 in imatininb (−) in 30 mM glucose and 14,4 ± 0,3 in imatinib (+) *vs.* 7,2 ± 0,5 in imatinib (−) in 5 mM glucose, $P < 0,001$ Fig. 3D). Moreover, the lactate production was decreased in hyperglycemic conditions relatively to hypoglycemia; 14,3 ± 0,9 *vs.* 24,4 ± 0,9 in imatinib (−) and 22,64 ± 1,68 *vs.* 46,17 ± 2,40 in

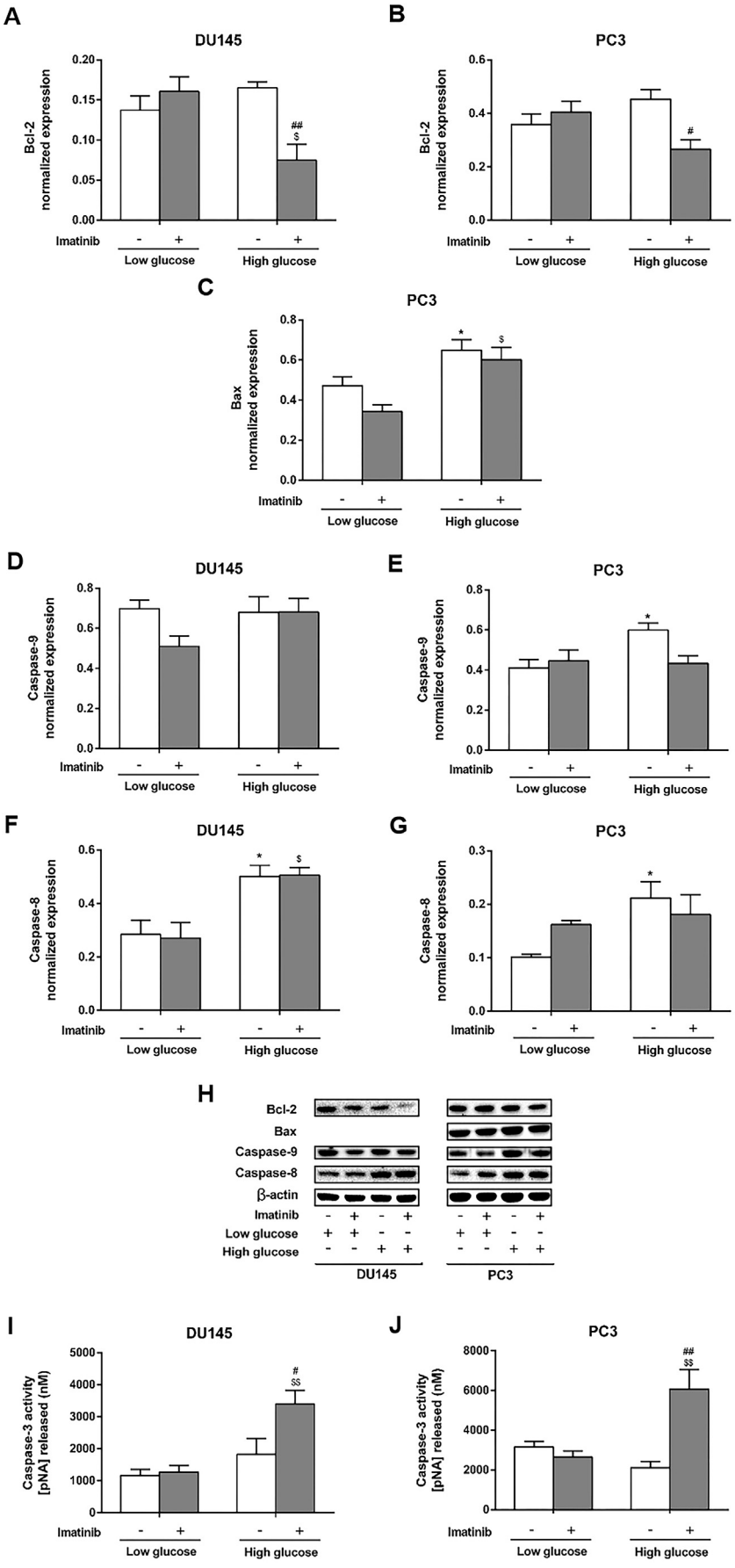

Fig. 2. Effect of imatinib (20 μM) on the expression of apoptosis regulators and caspase-3 activity in DU145 and PC3 cells under hypoglycemic (5 mM) and hyperglycemic conditions (30 mM) for 72 h of treatment. Protein levels of Bcl-2 (A, B), Bax (C), caspase-9 (D, E) and caspase-8 (F, G) were determined by WB analysis after normalization with β-actin. Representative immunoblots are shown in panel H. Caspase-3 activity (I, J) was measured spectrophotometrically by the release of the product pNA. Error bars indicate mean ± S.E.M (n = 5). * $P < 0.05$; when compared with the imatinib (−) low glucose; $^{\$}$ $P < 0.05$; $^{\$\$}$ $P < 0.01$; when compared with the imatinib (+) low glucose; $^{\#}$ $P < 0.05$; $^{\#\#}$ $P < 0.01$; when compared with the imatinib (−) high glucose.

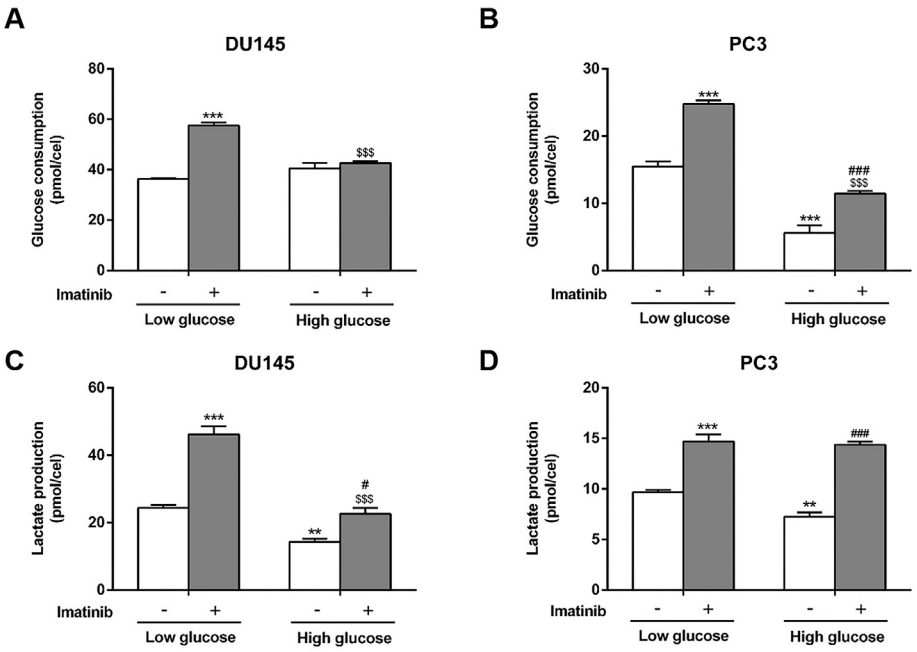

Fig. 3. Glucose consumption (A, B) and lactate production (C, D) in DU145 and PC3 cells treated with imatinib (20 μM) under hypoglycemic (5 mM) or hyperglycemic conditions (30 mM) for 72 h. Error bars indicate mean ± S.E.M (n = 5).** P < 0.01; *** P < 0.001 when compared with the imatinib (−) low glucose; $^{\$\$\$}$ P < 0.001 when compared with the imatinib (+) low glucose; $^{\#}$ P < 0.05; $^{\#\#\#}$ P < 0.001 when compared with the imatinib (−) high glucose.

imatinib (+) in DU145 cells (P < 0,01 and P < 0,001, respectively, Fig. 3C); 7,2 ± 0,5 *vs.* 9,7 ± 0,2 in imatinib (−) in PC3 cells (P < 0,01, Fig. 3D).

The transport of glucose across the plasma membrane, the first step of glycolytic process, is mediated by GLUTs, namely, the GLUT1 and GLUT3, which were previously identified in PCa cells [19,27]. The expression of GLUT1 was significantly decreased in hyperglycemic groups both in DU145 (0,09 ± 0,03 *vs.* 0,31 ± 0,05 in imatinib (−) and 0,08 ± 0,02 *vs.* 0,50 ± 0,09 in imatinib (+), Fig. 4A) and PC3 (0,05 ± 0,01 *vs.* 0,31 ± 0,01 in imatinib (−) and 0,06 ± 0,02 *vs.* 0,17 ± 0,04 in imatinib (+), Fig. 4B) cells. Also, a diminished expression of GLUT1 upon treatment with imatinib was observed in PC3 cells under hypoglycaemia (0,17 ± 0,04 *vs.* 0,31 ± 0,01, P < 0,01, Fig. 4B). Relatively to GLUT3 the results were the opposite, with the hyperglycemic conditions being associated to the increased expression of GLUT3. GLUT3 expression in imatinib-treated DU145 cells varied from 0,79 ± 0,10 in hypoglycaemia to 1,34 ± 0,03 in hyperglycaemia (P < 0,05, Fig. 4C). A similar pattern was observed in PC3 cells (Fig. 4D): imatinib (−), 0,54 ± 0,04 in hypoglycemia *vs.*0,82 ± 0,05 in hyperglycemia (P < 0,05); imatinib (+), 0,53 ± 0,06 in hypoglycemia *vs.* 0,86 ± 0,11 in hyperglycemia (P < 0,05).

An important step in the glycolytic flux is the conversion of fructose 6-phosphate into fructose 1,6-bisphosphate by PFK1, which is often considered a rate-limiting step in glycolysis [28]. However, no differences were observed in the expression of PFK1 among experimental groups in DU145 (Fig. 4E) and PC3 (Fig. 4F) cells. Other crucial enzyme in the glycolytic process is LDH, which mediates the production of lactate from pyruvate upon glucose metabolization [17]. LDH activity in DU145 cells, was increased in the imatinib (+) group in hyperglycaemia (49,1 ± 6,3 *vs.* 27,9 ± 1,6, P < 0,05, Fig. 5A). In the PC3 cell line, no effect of imatinib was observed but LDH activity diminished in the imatinib (−) group in hyperglycaemia (10,4 ± 0,8 *vs.* 15,3 ± 0,18, P < 0,01, Fig. 5B). We also identified an augmented expression of LDH in imatinib-treated DU145 and PC3 cells under hyperglycemia (respectively, 1,6 ± 0,1 *vs.* 1,2 ± 0,1, P < 0,05 (Fig. 5C) and 2,0 ± 0,3 *vs.* 1,4 ± 0,1, P < 0,05 (Fig. 5D)).

The lactate produced is exported to the extracellular space by a member of the MCTs family, the MCT4 that has been associated with PCa progression and poor prognosis [29,30]. In the DU145 cell line, MCT4 expression was significantly decreased in hyperglycemic groups (0,8 ± 0,1 *vs.* 1,7 ± 0,1 in imatinib (−) and 0,8 ± 0,1 *vs.* 1,3 ± 0,1 in imatinib (+), Fig. 5E). However, in imatinib-treated PC3 cells the expression of MCT4 was significantly increased in the hyperglycaemia group (0,9 ± 0,1 *vs.* 0,7 ± 0,1 in hypoglycaemia, Fig. 5F).

4. Discussion

The present study investigated the effect of imatinib on viability, apoptosis and glycolytic metabolism in two cell line models of CRPC under conditions of different glucose availability.

We observed that imatinib was effective in decreasing the viability of DU145 and PC3 cells, which was restricted to the 30 mM glucose groups (Fig. 1). The diminished viability of DU145 and PC3 cells in response to imatinib in hyperglycemic conditions was accompanied by the altered expression and activity of apoptosis regulators of both the intrinsic and extrinsic pathways (Fig. 2). This included the down-regulation of the anti-apoptotic Bcl-2 protein that prevents the release of cytochrome C into the cytosol and consequently inhibits apoptosis [31]; and the up-regulation of the pro-apoptotic protein Bax, at least in PC3 cells. Hyperglycemic conditions, independently of imatinib treatment, were also characterized by the increased expression of caspase-8 and caspase-9, the initiator caspases of the intrinsic and extrinsic pathways of apoptosis [32], respectively. However, glycaemia itself had no effect on DU145 or PC3 cells viability (Fig. 1).

Both caspase-8 and -9 can activate caspase-3, the known effector protein of the apoptotic process, which activity is considered a measure of the apoptosis rate [32,33]. The results obtained demonstrated that imatinib treatment, though not affecting caspase-8 and caspase-9 expression, strongly increased caspase-3 activity in DU145 and PC3 cells (Fig. 2I and J). Therefore, it is expected that caspase-8 or caspase-9 activity would be increased in response to imatinib despite no changes on protein expression were detected. Moreover, the effect of imatinib enhancing caspase-3 activity was only perceived in conditions if high-glucose availability, which is in accordance with the observed down-regulation of Bcl-2 and up-regulation of Bax. These findings showed that apoptosis is augmented in response to imatinib (high glucose), and are in line with the diminished cell viability observed in the imatinib (+) hyperglycemic groups (Fig. 1).

Altogether, our results revealed that imatinib is more effective inducing apoptosis and suppressing viability of CRPC lines in conditions

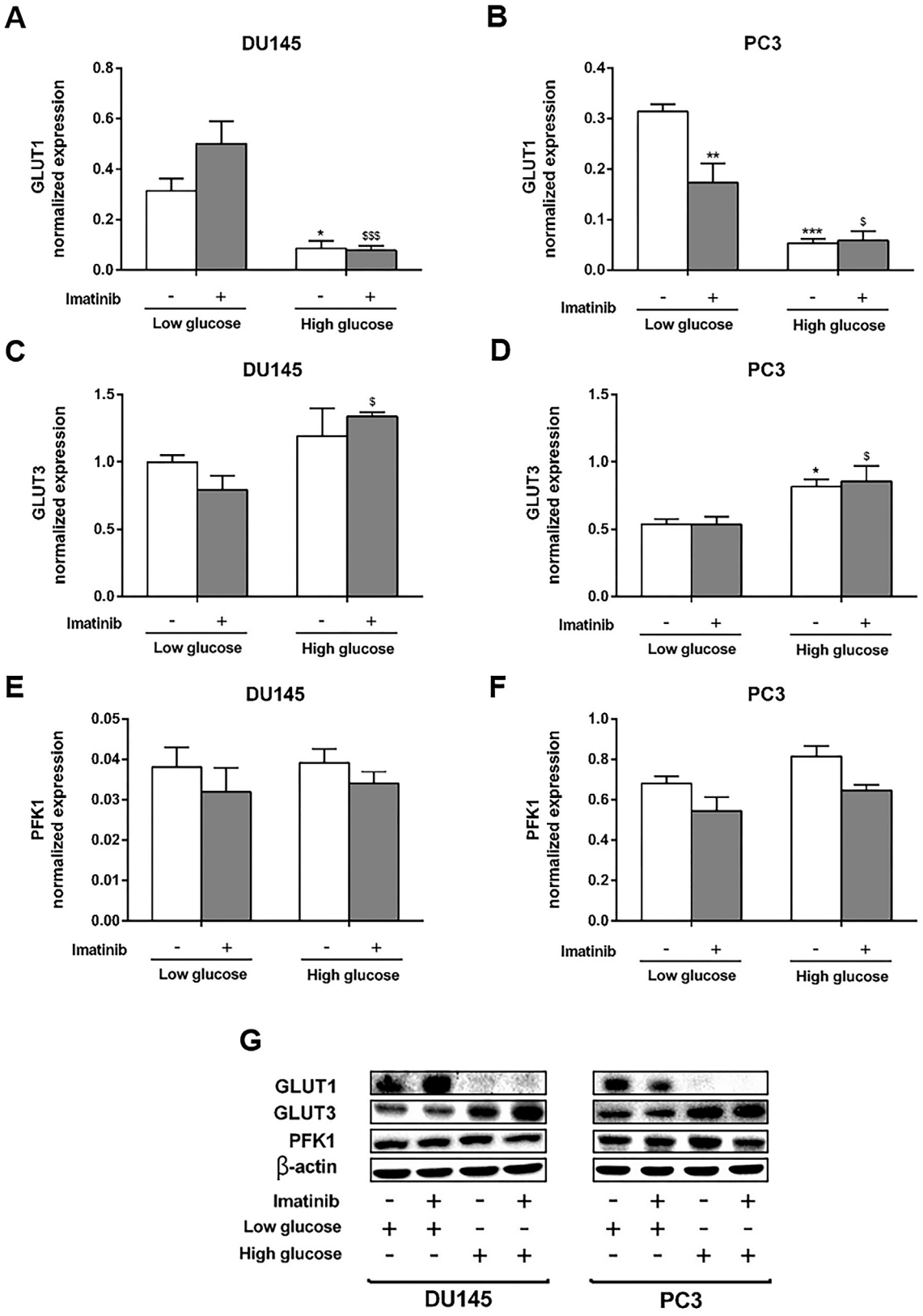

Fig. 4. Protein levels of glycolytic metabolism-associated proteins, GLUT1 (A, B), GLUT3 (C, D) and PFK1 (E, F) in DU145 and PC3 cells treated with imatinib (20 μM) under hypoglycemic (5 mM) or hyperglycemic conditions (30 mM) for 72 h. Expression was determined by WB analysis after normalization with β-actin. Representative immunoblots are shown in panel G. Error bars indicate mean ± S.E.M (n = 5). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared with the imatinib (−) low glucose; $^{\$}$ $P < 0.05$; $^{\$\$\$}$ $P < 0.001$ when compared with the imatinib (+) low glucose.

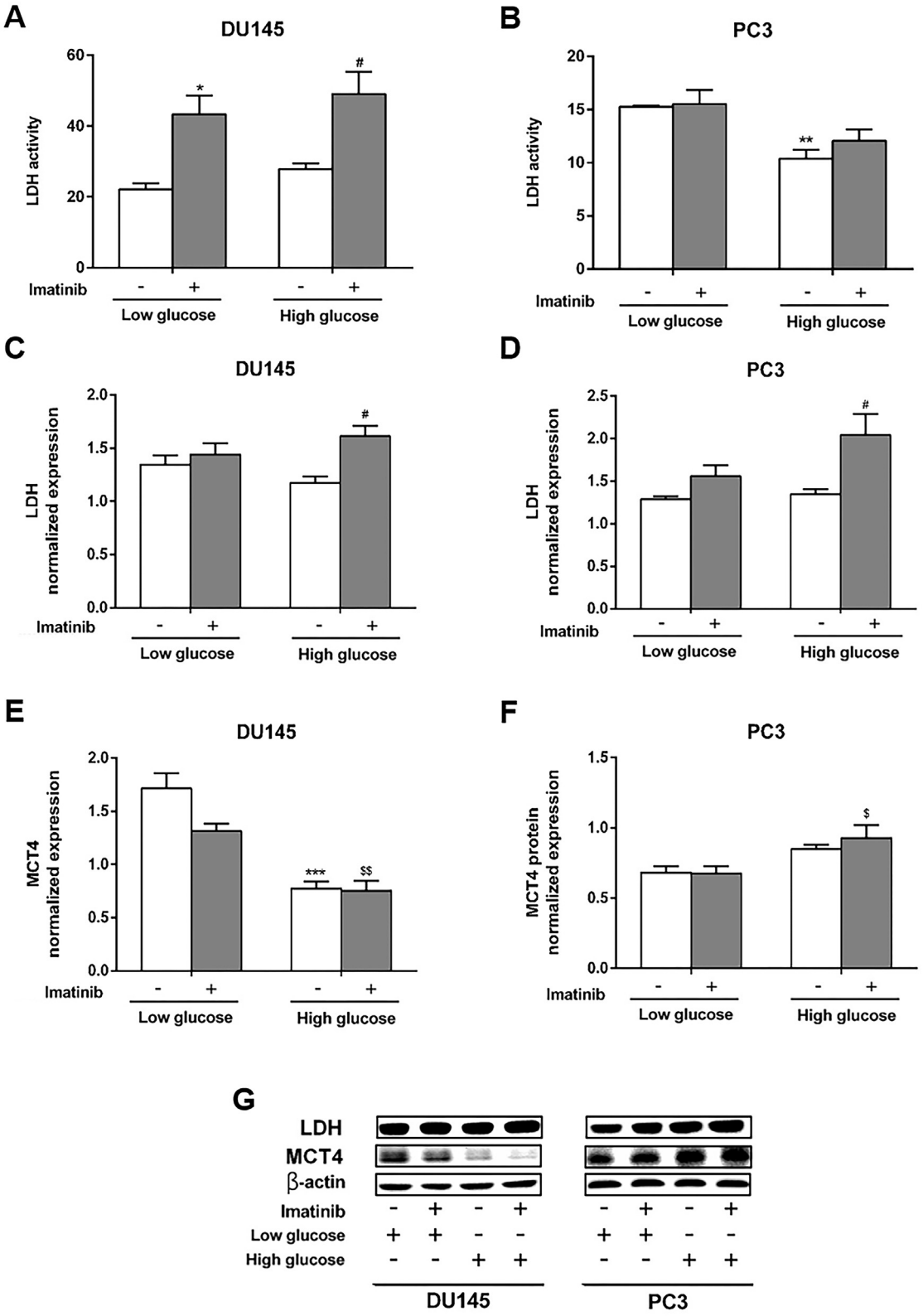

Fig. 5. LDH activity (A, B) and protein levels of LDH (C, D) and MCT4 (E, F) in DU145 and PC3 cells treated with imatinib (20 μM) under hypoglycemic (5 mM) or hyperglycemic conditions (30 mM) for 72 h. Representative immunoblots are shown in panel G. Error bars indicate mean ± S.E.M (n = 5).* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared with the imatinib (−) low glucose; $^{\$} P < 0.05$; $^{\$\$} P < 0.01$; when compared with the imatinib (+) low glucose; $^{\#} P < 0.05$ when compared with the imatinib (−) high glucose.

of hyperglycemia. The exact mechanisms by which hyperglycemia potentiates imatinib-induced apoptosis of PCa cells are still unknown, but in breast cancer MCF-7 cells it was shown that this glycemic condition increased the cytotoxicity of carboplatin and 5-fluorouracil by reducing the expression of P-glycoprotein and increasing oxidative stress levels [13].

Glycaemia and the glycolytic status of the tumor microenvironment have been shown to influence the response of PCa cells to other therapeutic targets. For example, hyperglycaemia was shown to induce chemoresistance of PCa cells to the taxane docetaxel [21]. Nevertheless, at least for our knowledge, this is the first study addressing the effect of the tyrosine kinase inhibitor imatinib under conditions of different glucose availability. The present findings highlight for the complex functional basis of imatinib therapy in PCa, which seems to depend on the environmental conditions and cell-specificities. Previous studies also have reported the distinct effects of imatinib controlling proliferation and apoptosis of DU145 and PC3 cells [11].

Despite not always in agreement with the *in vitro* findings, several epidemiological association studies have been indicating diabetes as a protective or a good prognostic factor in PCa [34–40]. Our results showed that higher levels of glucose, the main serum alteration in diabetic patients, potentiated the effects of imatinib decreasing PCa cell viability and survival, which raises the curiosity about the efficacy of imatinib for treatment of castration-resistant diabetic patients.

Recent evidence has also highlighted for the fact that imatinib treatment provokes alterations in blood glucose levels of cancer patients with/without diabetes [41,42]. Imatinib, as well as Dasatinib, another tyrosine kinase inhibitor widely used for treatment of leukaemia, were related with scenarios of hypoglycaemia [14,15,41]. Moreover, modifications of fasting glucose in chronic myeloid leukaemia patients have been considered as the first sign of resistance to imatinib [43]. It was also shown that chronic myeloid leukaemia cells resistant to imatinib have increased glycolytic activity and lactate production relatively to sensitive-imatinib cells [44], which is typical of cancer cells with more aggressive phenotypes [19]. Taking into account the influence of imatinib over glucose handling, we evaluated its effect modulating the glycolytic metabolism of CRPC cells in conditions of hypo- and hyperglycemia.

Glucose consumption was augmented in DU145 and PC3 cells treated with imatinib (Fig. 3A and B), with results more pronounced in low glucose groups. This trend was somehow expected since high glucose availability has been shown to diminish glucose consumption in different cell types [45–47].

The uptake of glucose from the extracellular medium is carried out by members of GLUTs family, which in PCa cells has been indicated to be a task of GLUT1 and GLUT3 [19,27]. GLUT1 expression was diminished in DU145 cells in hyperglycemic conditions (Fig. 4A) comparatively with the 5 mM glucose imatinib-treated groups, which is in accordance with the reduced consumption of glucose observed in these conditions. However, despite the increased glucose consumption observed in imatinb (+) groups, no significant alterations were found on GLUT1 or GLUT3 expression (Fig. 4), which may suggest that other GLUTs can be involved. A likely candidate would be the GLUT12, as this transporter was recently identified as an androgen target gene and the GLUT responsible for the androgenic control of glucose uptake and PCa cell growth [48].

Also, it is curious the observed shift on GLUT1 and GLUT3 expression in response to glucose availability; GLUT1 expression was decreased in high glucose conditions whereas GLUT3 levels were increased. These results are supported by other studies showing the differential expression of GLUTs dependently on glucose concentrations [49,50]. At the moment, it is not clear how imatinib facilitates entry of glucose into cancer cells, and how imatinib treatment contributes to decreasing blood glucose levels in cancer patients [14,15,41]. However, in light of the results obtained herein in PCa cells is liable to suggest that the increased glycolytic activity driven by imatinib may sustain the diminution of blood glucose concentrations.

Another crucial step in the glycolytic process is the irreversible conversion of fructose 6-phosphate to fructose 1,6-bisphosphate, a reaction catalysed by PFK1, but no differences could be observed on PFK1 expression levels, related with imatinib treatment or glycaemic conditions.

The glycolytic flux ends with the conversion of pyruvate to lactate through the activity of LDH, and the export of lactate to the extracellular space. Following the effects observed on glucose consumption, imatinib treatment increased lactate production in DU145 and PC3 cells (Fig. 3) under different glycemic conditions. Moreover, the augmented lactate production was accompanied by the enhanced expression of LDH in both cell lines (hyperglycemia, Fig. 5C and D), and by the increased enzyme activity in DU145 (Fig. 5A).

The MCTs are the molecular partners involved in the export of lactate to the extracellular space, which in the case of PCa cells has suggested to be a role of the MCT4. MCT4 has been shown to be expressed in DU145 and PC3 cells and in tumor samples of CRPC patients [19,30,51]. Despite the alterations in lactate production, imatinib treatment did not show to alter MCT4 expression levels (Fig. 5E and F). Nevertheless, hyperglycemia conditions induced a down-regulation of MCT4 expression in DU145 cells, which was consistent with the lower production of lactate when cultured in 30 mM glucose comparatively with the culture medium containing 5 mM glucose.

Overall, imatinib stimulated glucose consumption and lactate production in CRPC, independently of glucose availability, due to the increased expression and activity of LDH. These findings are particularly relevant considering the previous studies showing that the increased expression of LDH and export of lactate are associated with the resistance of cancer cells to treatment [52]. Indeed, increased levels of lactate in the tumor microenvironment have been shown to favor tumor growth, invasion, and aggressiveness, as well as, suppressing the immune system [52–54]. Co-treatment of CRPC cells with imatinib and an LDH inhibitor might be the next step to investigate the effectiveness of this tyrosine kinase inhibitor for treatment of PCa in hyperglycemia conditions.

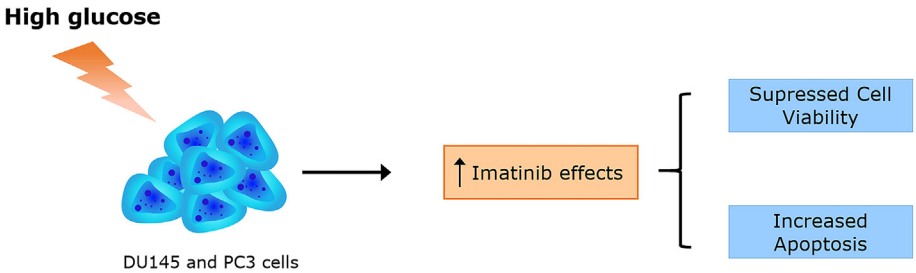

Fig. 6. The glycolytic environment modulated the effect of the tyrosine kinase inhibitor imatinib in CRPC cells. High glucose availability potentiated imatinib actions suppressing viability and enhancing apoptosis of DU145 and PC3 cells.

5. Conclusion

It was demonstrated that imatinib suppressed the viability of DU145 and PC3 cells whereas inducing apoptosis, which was restricted to a glucose enriched environment (Fig. 6). This was the first study addressing the effect of this compound under conditions of different glucose availability and allowed to conclude that hyperglycaemia increases the effectiveness of imatinib suppressing growth and survival of CRPC cells. The obtained results also highlighted for the complexity of the functional basis underlying the response to imatinib therapy. In addition, imatinib treatment stimulated glucose consumption and lactate production, which can be linked with cancer cells aggressiveness and mechanisms of resistance. Altogether, the present work provided a set of pre-clinical findings that raise the curiosity about the use of imatinib for treatment of CRPC diabetic patients, as well as of its administration simultaneously with inhibitors of glycolytic metabolism.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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