



Article

ROS-Fueled Allies: STAT3, PKM2, and HIF-1 α Influencing Energy Metabolism in Hormone-Independent Cancers

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Abstract

Hormone-independent breast and prostate cancers represent highly aggressive malignancies characterized by profound metabolic reprogramming, elevated oxidative stress, and loss of sensitivity to endocrine therapies. Increasing evidence indicates that tumor progression and metabolic plasticity are sustained by interconnected signaling networks linking transcriptional regulation to energy metabolism. Among these, the STAT3–PKM2–HIF-1 α signaling axis, functionally reinforced by reactive oxygen species (ROS), has been proposed as a central regulator of the Warburg phenotype and cellular adaptation to adverse microenvironmental conditions. Using androgen-independent prostate cancer (DU145) and triple-negative breast cancer (KPL-4) cell lines, we demonstrated constitutive activation and reciprocal regulation of STAT3, PKM2, and HIF-1 α . Pharmacological inhibition of STAT3, stabilization of tetrameric PKM2 by L-serine, and ROS scavenging with N-acetylcysteine significantly reduced STAT3 phosphorylation, PKM2 nuclear translocation, and HIF-1 α stabilization. These molecular effects were accompanied by decreased intracellular ROS levels, reduced lactate production, increased pyruvate levels, and a metabolic shift toward oxidative phosphorylation. Functionally, treated cells exhibited reduced Ki-67 expression and impaired clonogenic capacity. Our results identify the STAT3–PKM2–HIF-1 α /ROS axis as a key determinant of metabolic and phenotypic plasticity in hormone-independent breast and prostate cancers, highlighting its potential as a molecular target for therapeutic modulation of cancer-associated metabolic phenotypes.

Keywords: STAT3; PKM2; HIF-1 α ; oxidative stress; Warburg effect; hormone-resistant cancer; energy metabolism



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1. Introduction

Cancer represents a major global health challenge. Estimates from the World Health Organization, World Bank, Directory of Radiotherapy Centers, UNDP, and IARC indicate that, by 2040, there will be approximately 29.9 million new cancer cases and 15.3 million cancer-related deaths worldwide, underscoring the significant impact of this disease [1].

Among the most common cancers are those with hormone-dependent carcinogenesis, particularly breast and prostate cancers, which account for a large proportion of incidence in the United States and are leading causes of mortality [2,3]. Endocrine therapy is the primary treatment for both malignancies, prolonging survival and improving quality of life [4].

A major challenge in oncological research is that breast and prostate cancers frequently progress to a hormone-resistant state, leading to more aggressive diseases. Multiple molecular mechanisms drive this resistance [5], mostly involving alterations in hormone-specific pathways—estrogen receptor (ER) and progesterone receptor (PR) axis in breast cancer and the androgen receptor (AR) axis in prostate and breast cancer [6,7], and the activation of bypass pathways that allow tumor growth independent of estrogen or androgen [8–11]. Breast cancer may also lose ER expression, rendering ER-targeted therapies ineffective [12], whereas prostate cancer often shows AR amplification, making cells hypersensitive to low androgen levels and able to proliferate even when androgen levels are substantially suppressed by standard hormonal therapies [13,14].

To elucidate the mechanisms underlying the development of endocrine resistance, research has focused on crosstalk between hormone receptors and other signaling cascades [15,16]. However, the tumor microenvironment, which is composed of hormones, growth factors, cytokines, ROS, and a variety of metabolites, can also influence tumor development and size [17,18]. Hormones, growth factors, and cytokines mediate signaling crosstalk with ER–AR pathways [7]. ROS, indicators of oxidative stress, contribute to all stages of tumorigenesis by promoting DNA damage, suppressing immunity, stimulating angiogenesis, and mimicking hypoxic conditions that favor drug resistance [19,20]. Metabolites in the tumor microenvironment reflect the relative activity of metabolic pathways that cancer cells reprogram according to tissue context and disease stage [21].

This metabolic flexibility enables adaptation to therapy-induced environmental changes, promoting drug resistance and reduced treatment efficacy [22,23].

Lactate is a key metabolite that accumulates in neoplastic tissues at concentrations exceeding 40 mM, well above physiological levels [24]. This reflects the Warburg effect, driven by oncogenes such as c-Myc and STAT3 that upregulate glycolytic enzymes including HK2 and PKM2 [25]. In hormone-independent tumors, this process likely involves the STAT3–PKM2–HIF-1 α protein circuit known as “Warburg’s vicious circle” [26]. STAT3, PKM2, and HIF-1 α play interconnected roles in carcinogenesis. STAT3 is constitutively active in most cancers and drives tumor development through canonical and non-canonical pathways [27]. PKM2 is frequently overexpressed in tumors and promotes proliferation and metastasis through its functional switch between a dimeric (nuclear) form, which promotes tumor growth, and a tetrameric (cytosolic) form, which sustains glycolysis under hypoxic or hyperproliferative conditions [28]. HIF-1 α , stabilized under hypoxia, regulates genes involved in glycolysis, survival, angiogenesis, and therapy resistance, thereby supporting tumor progression [29,30].

This vicious circle can be initiated by negative allosteric regulation of PKM2, which promotes its transition from the cytosolic tetramer to the nuclear dimeric form. In the nucleus, PKM2 acts as a protein kinase and phosphorylates STAT3 at Tyr705, activating its canonical pathway [31,32]. STAT3 then upregulates HIF-1 α expression, which in turn, induces PKM2 transcription, thereby sustaining a self-amplifying loop [26]. This circuit is closely interconnected with ROS, which modulate the activity of all three proteins and may function as integral components of the loop [33–35]. Given the central role of metabolic reprogramming in maintaining malignant phenotypes, targeting this axis through selective metabolites, specific inhibitors, or modulation of ROS may represent a promising therapeutic strategy in hormone-independent tumors. Such interventions could

partially restore physiological metabolic programs and potentially re-sensitize tumors to conventional therapies [36].

In this study, the androgen-independent prostate cancer cell line DU145 (AR[−]) and the triple-negative breast cancer cell line KPL-4 (ER[−]/PR[−]/HER2[−]) were used. Both cell lines are highly aggressive, lacking hormonal receptors, and are unresponsive to hormone-based therapies and exhibit a strong dependence on Warburg-type metabolism [37–40]. High Ki-67 expression further supports their proliferative phenotype [41]. The STAT3–PKM2–HIF-1 α loop was investigated by assessing protein activation through specific phosphorylation events and subcellular localization. STAT3 activation was evaluated by phosphorylation at Y705, indicative of canonical signaling and nuclear localization, and phosphorylation at S727, associated with non-canonical, oxidative stress-induced mitochondrial signaling, which contributes to metabolic regulation linked to the Warburg phenotype [42–44]. Nuclear localization of PKM2 and HIF-1 α was also analyzed, as both proteins exert transcriptional functions within the loop, with PKM2 additionally acting as a nuclear kinase for STAT3 [45–47]. Based on existing evidence, we hypothesize that this functional circuit is active in DU145 and KPL-4 cells and that oxidative stress contributes to its maintenance.

2. Results

2.1. Serine, N-acetylcysteine, and S3I-201 as Modulators of the Protein Loop

Tumor cells, in response to their intrinsic proliferative demands and/or to adapt to adverse microenvironmental conditions, exhibit a remarkable capacity for metabolic remodeling, culminating in the reconfiguration of their bioenergetic profile. Specifically, they can induce a transition from oxidative phosphorylation (OxPhos) to aerobic glycolysis, a phenomenon known as the Warburg effect [48].

This high metabolic plasticity suggests that tumor growth and progression could potentially be slowed or inhibited by promoting a metabolic switch toward the reactivation of oxidative metabolism. Such a reversal can be achieved either through the direct targeting of key enzymes in major metabolic pathways or by modulating the signaling pathways that regulate metabolic programs, with the ultimate goal of restoring physiologically directed metabolic fluxes [49,50].

To this end, considering the cellular functions of the three proteins constituting the loop in tandem with reactive oxygen species (ROS), this metabolic transition could be managed by modulating the components of the loop and the ROS.

Our strategy to inhibit or negatively modulate the loop activation focused on the respective roles of the three protein components and ROS in its activation. We primarily targeted STAT3, the core or “start” of the loop, PKM2, which acts as both a kinase and a transcription factor, and ROS, which activate all three proteins. HIF-1 α was not considered a primary inhibition target but rather a marker of the hypoxia-like state and the outcome of loop activation, as its production and stabilization depend on the presence of ROS and the activation of STAT3 and PKM2 [51].

Based on these premises, our experiments utilized compounds with inhibitory activity toward STAT3 and ROS, and a positive allosteric modulator for PKM2.

L-serine, acting as a positive allosteric modulator of PKM2, stabilizes the tetrameric form of the enzyme, which is highly catalytically efficient and more closely associated with glycolytic flux directed toward oxidative metabolism [52]. This strategy reflects the enzyme’s physiological regulation. As a non-essential amino acid, serine provides one-carbon units necessary for the synthesis of nucleotides, glutathione, tetrahydrofolate, cysteine, glycine, phospholipids, and porphyrins [53], and its metabolic pathways are frequently upregulated in highly aggressive tumors, such as hormone-resistant prostate and breast

cancers [54,55]. Additionally, serine acts as a metabolic hub linking glycolysis, the Krebs cycle, OxPhos, and biosynthetic pathways by modulating PKM2. Serine stabilizes the PKM2 tetramer (the cytosolic isoform with higher catalytic efficiency in converting Phosphoenolpyruvate (PEP) to pyruvate), thereby promoting efficient oxidative glycolysis [56]. In the absence of serine, the less efficient dimeric form of PKM2 predominates, which is associated with the Warburg phenotype, and following nuclear translocation, can promote the transcription of HIF-1, as well as the phosphorylation of STAT3 [57]. Serine administration is, therefore, intended to reduce loop activity and promote the reactivation of oxidative metabolism.

N-acetylcysteine (NAC), an antioxidant molecule derived from cysteine and a precursor of glutathione [58], was employed to reduce ROS levels, which are major mediators of oxidative stress. ROS accumulation promotes a pseudo-hypoxic state, stabilizing HIF-1, by inhibiting prolyl hydroxylases responsible for its degradation [59]. Under these conditions, HIF-1 functions as a transcription factor, inducing the expression of glycolytic genes such as PKM2, LDH, and VEGF [60]. ROS also activate the non-canonical STAT3 pathway via phosphorylation at S727, promoting mitochondrial localization and contributing to the Warburg phenotype [61]. In the case of PKM2, ROS facilitate the stabilization of its nuclear dimeric isoform [62], which cooperates with STAT3 to enhance HIF-1 expression [26]. Restoring physiological ROS levels through NAC, therefore, promotes metabolic loop inactivation and oxidative metabolism reactivation, potentially reducing tumor aggressiveness and increasing therapeutic sensitivity.

In parallel with microenvironmental modulation, given the critical role of STAT3 as a central node and trigger of the loop, the STAT3 inhibitor S3I-201 was employed to directly block its phosphorylation, dimerization, and nuclear translocation. This compound, primarily known for inhibiting phosphorylation at Y705, also affects S727 and is widely used as a standard in STAT3 research [63]. Its inclusion in the experimental design serves a dual purpose: to provide a reference for inhibition and to confirm the central role of STAT3 as the core of the loop, integrating multiple oncogenic signaling pathways (cytokines, EGF, and hormones).

Finally, HIF-1 was considered a marker of the pseudo-hypoxic state and a functional output of loop activation, as its stabilization depends on ROS levels and the activity of STAT3 and PKM2.

Based on these premises, cells from the two lines were treated with NAC at a concentration of 1 mM, serine at 1 mM, and S3I-201 at 100 μ M [64–66]. Concentrations were selected based on the literature data, while the 48 h treatment duration was experimentally determined to be optimal for subsequent analyses.

2.2. Immunofluorescence and Western Blotting Analysis for the Assessment of Loop Modulation

DU-145 and KPL-4 cell lines were separately treated with serine, NAC, and S3I-201 and compared with untreated control samples. Following treatment, the activation status and cellular localization of the analyzed proteins were assessed by immunofluorescence and western blotting on both total and nuclear protein fractions.

Immunofluorescence and western blotting analyses, shown in Figures 1–4, yielded consistent results for all proteins examined. Figure 1 shows immunofluorescence (Figure 1A–C) and western blotting (Figure 1B,D) analyses of STAT3 phosphorylated at tyrosine 705, a marker of canonical pathway activation. In both cell lines, STAT3 phosphorylation and its nuclear localization were reduced following treatment, with a more pronounced effect observed upon NAC and S3I-201 exposure. Figure 2 reports immunofluorescence (Figure 2A–C) and western blotting (Figure 2B,D) results for STAT3 phosphorylated at serine 727, indicative of non-canonical pathway activation. Accordingly, phosphorylation at

serine 727 and mitochondrial localization decreased in response to the treatments. Figure 3 presents immunofluorescence (Figure 3A–C) and western blotting (Figure 3B–D) data for PKM2, demonstrating that the treatments reduced the nuclear translocation of the protein. Figure 4 shows immunofluorescence (Figure 4A–C) and western blotting (Figure 4B–D) analyses related to HIF-1 α , indicating that the treatments restored the physiological degradation of HIF-1 α .

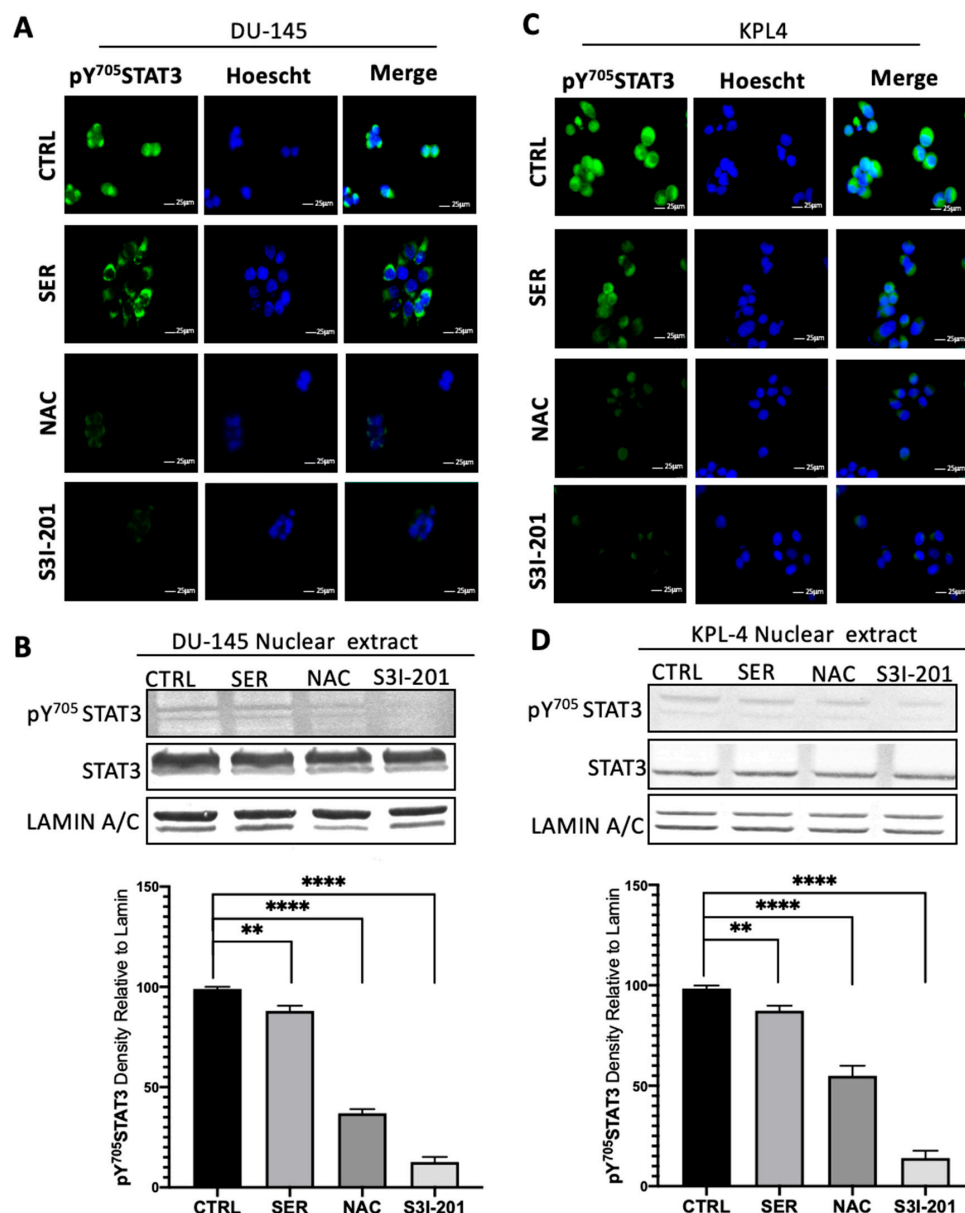


Figure 1. Cellular distribution of pY705STAT3 analyzed by immunofluorescence in DU-145 (A) and KPL-4 (C) cells treated for 48 h with 1 mM of serine, 1 mM of N-acetylcysteine (NAC), and 100 μ M of S3I-20 for 24 h. Images clearly show a reduction in STAT3 nuclear localization in cells treated with serine (SER), NAC, and S3I-201 with respect to control (CTRL). Immunoblot on DU-145 (B) and on KPL-4 (D) nuclear extract and the relative densitometries evidenced a decrease in band intensity of pY705STAT3 upon treatment with serine, NAC, and S3I-201. Phosphorylation levels were referred to the amount of total STAT3 present in each sample and were compared to the control. Experiments were repeated three times with similar results, and obtained values are reported as mean and standard deviation. Statistical analysis was performed with GraphPad Prisma software version 8.2.1 using ANOVA followed by Tukey's post hoc test. Statistically significant differences (** $p < 0.01$; **** $p < 0.0001$) are marked with asterisks and are referred to the control.

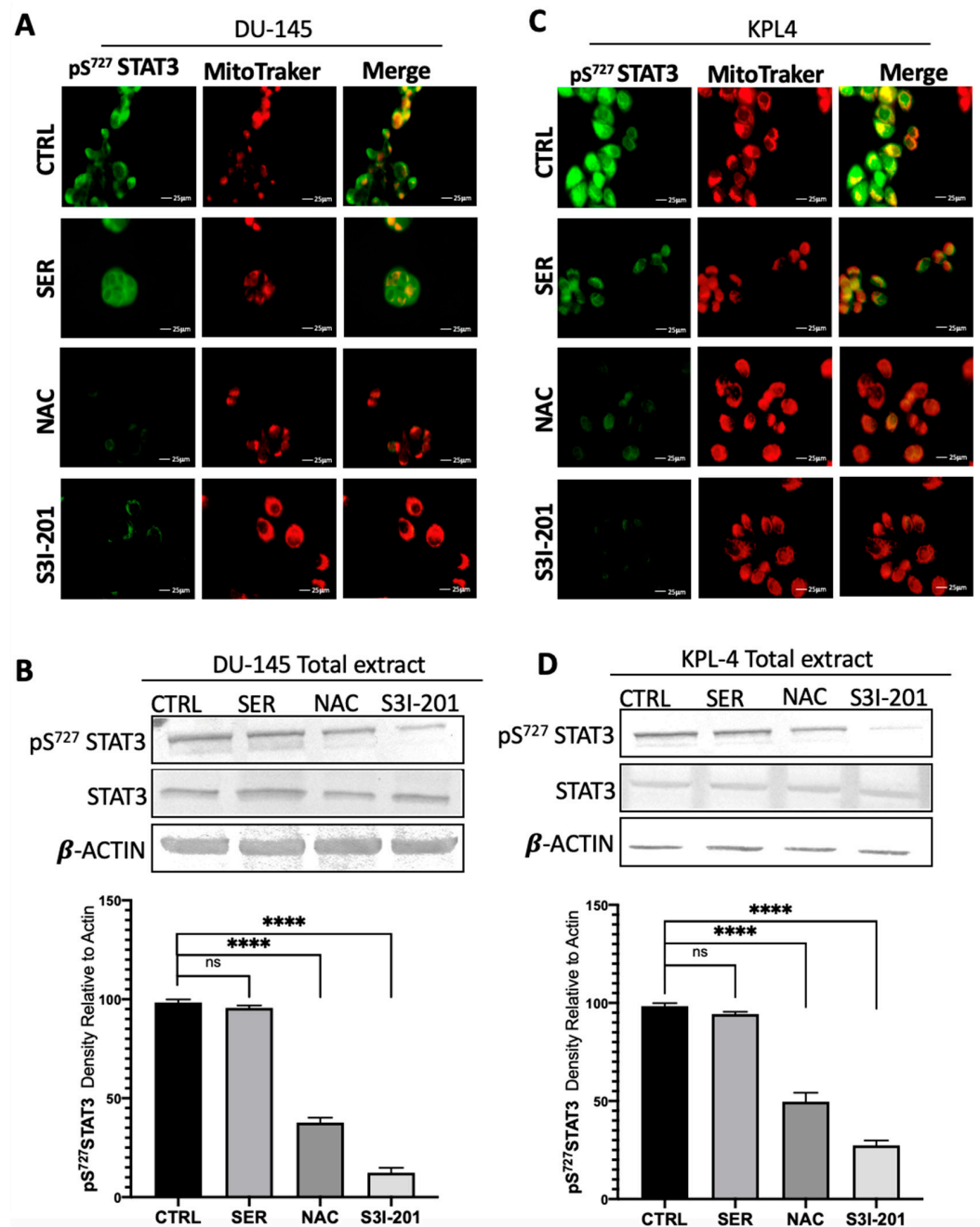


Figure 2. Cellular distribution of pS^{727/75}STAT3 analyzed by immunofluorescence in DU-145 (A) and KPL-4 (C) cells treated for 48 h with 1 mM of serine (SER), 1 mM of N-acetylcysteine (NAC), and 100 μM of S3I-20 for 24 h. Images clearly show a reduction in STAT3 mitochondrial localization in cells treated with NAC and S3I-201 with respect to control (CTRL). Immunoblot on DU-145 (B) and on KPL-4 (D) total extract and the relative densitometries evidenced a decrease in band intensity of pS^{727/75}STAT3 upon treatment with serine, NAC, and S3I-201. Phosphorylation levels were referred to the amount of total STAT3 present in each sample and were compared to control. Experiments were repeated three times with similar results, and obtained values are reported as mean and standard deviation. Statistical analysis was performed with GraphPad Prisma software version 8.2.1 using ANOVA followed by Tukey's post hoc test. Statistically significant differences (ns not statistically significant, **** $p < 0.0001$) are marked with asterisks and are referred to the control.

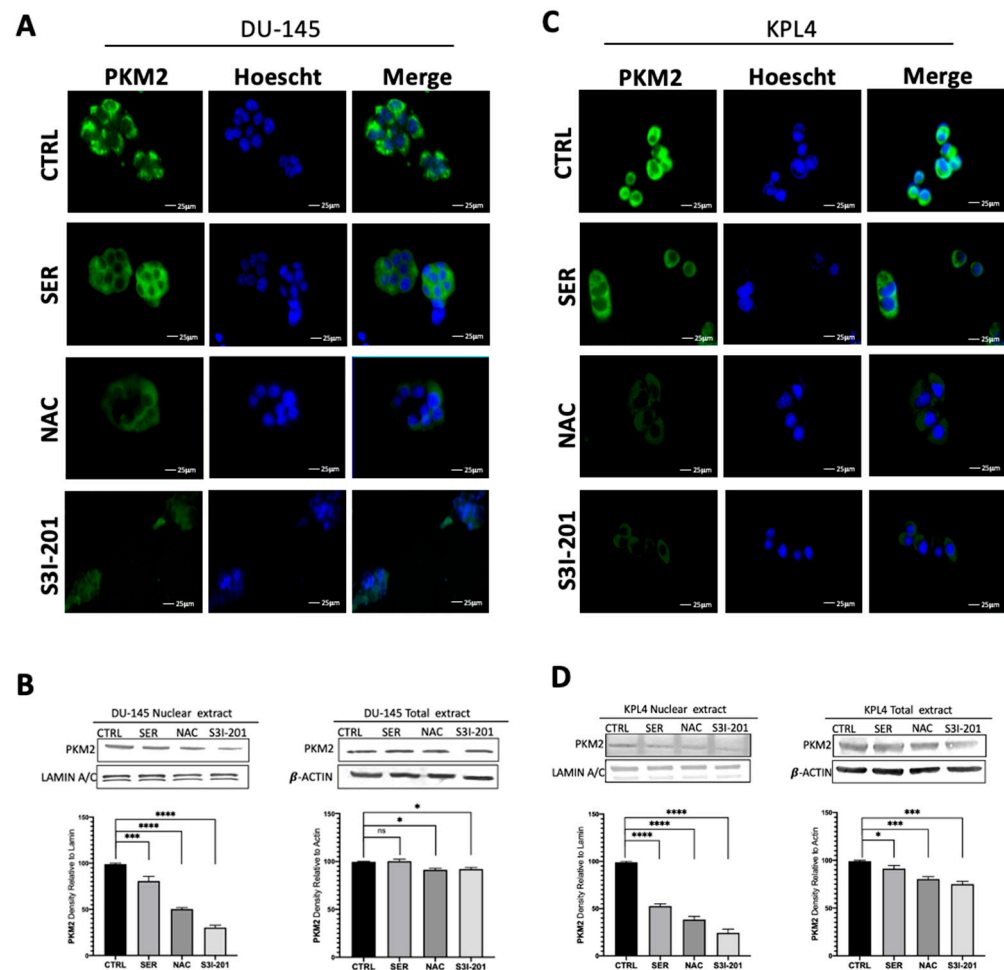


Figure 3. Cellular distribution of PKM2 analyzed by immunofluorescence in DU-145 (A) and KPL-4 (C) cells treated for 48 h with 1 mM of serine (SER), 1 mM of N-acetylcysteine (NAC), and 100 μ M of S3I-20 for 24 h. Images clearly show a reduction in PKM2 nuclear localization in cells treated with NAC and S3I-201 with respect to control (CTRL). Immunoblot on DU-145 (B) and KPL-4 (D) of nuclear and total extract and the relative densitometries evidenced a decrease in nuclear localization of PKM2 upon treatments. Protein expression levels were referred to β -actin for total extract and to Lamin A/C for nuclear extract and were compared to control. Experiments were repeated three times with similar results, and obtained values are reported as mean and standard deviation. Statistical analysis was performed with GraphPad Prism software version 8.2.1 using ANOVA followed by Tukey's post hoc test. Statistically significant differences (ns not statistically significant, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$) are marked with asterisks and are referred to the control.

2.3. Effect of STAT3 Inhibition on the Loop

In samples treated with the STAT3 inhibitor, STAT3 was effectively inhibited, as indicated by the absence of phosphorylation on both Y705 and S727 residues. Consistently, STAT3 levels were markedly reduced and almost undetectable in both the nuclear and mitochondrial fractions. Regarding PKM2, the results were consistent with STAT3 inhibition, confirming its central role in the loop. A reduction in total PKM2 expression levels was observed, with a more pronounced decrease in the nuclear fraction. This finding supports the hypothesis that inhibition of STAT3, acting as a transcription factor for HIF-1 α , which in turn, regulates PKM2 expression, leads to the downregulation of PKM2. Moreover, since S3I-201 also inhibits the mitochondrial activity of STAT3, thereby reducing its contribution to the Warburg effect, it can be expected that a cellular condition favors the cytosolic tetrameric isoform of PKM2 rather than the nuclear dimeric form. Finally, HIF-1 α expres-

sion was also markedly reduced. As the HIF-1 α gene is directly regulated by the canonical STAT3 pathway, and its half-life depends on oxygen levels and ROS concentration, both linked to S727 phosphorylation and STAT3 mitochondrial activity, the inhibition of both phosphorylation sites by S3I-201 results in a significant decrease in HIF-1 α levels, both in total protein content and within the nuclear compartment [67,68].

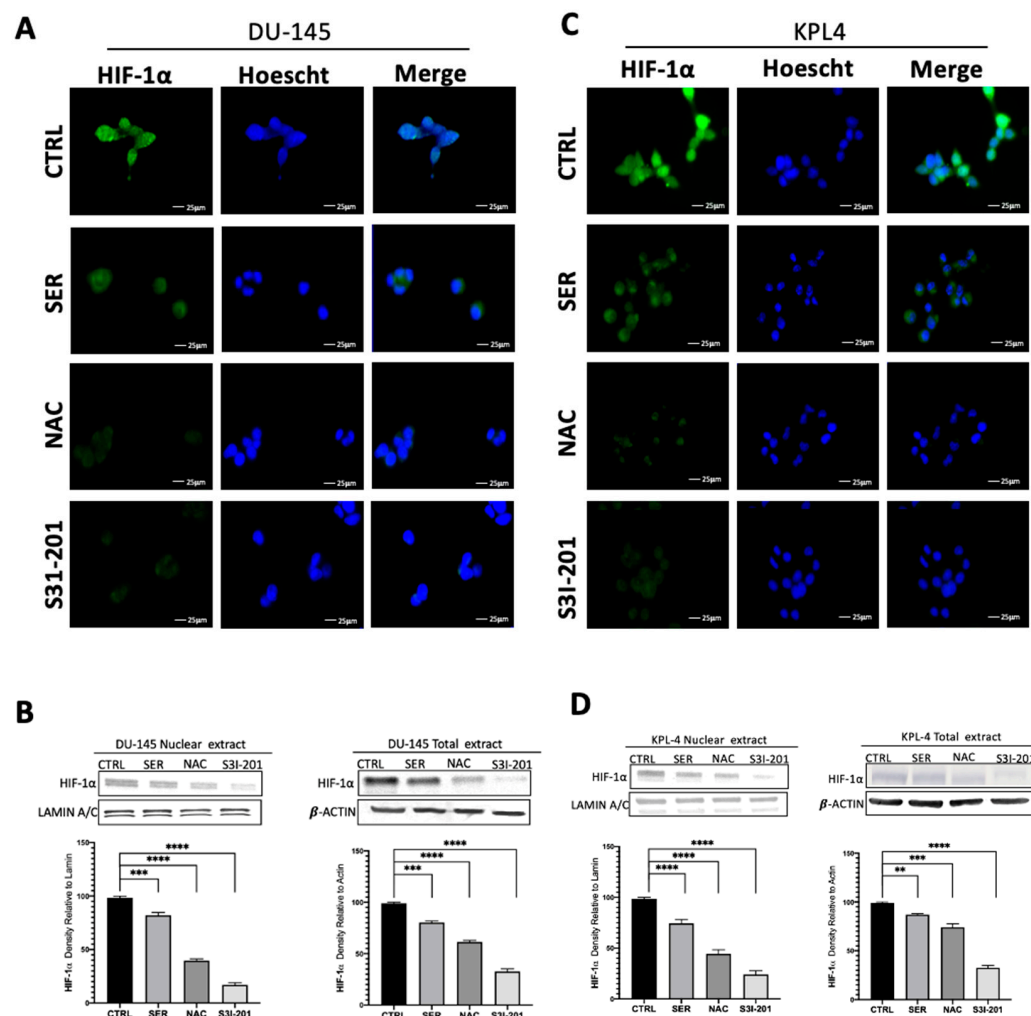


Figure 4. Cellular distribution of HIF-1 α analyzed by immunofluorescence in DU-145 (A) and KPL-4 (C) cells treated for 48 h with 1 mM of serine (SER), 1 mM of N-acetylcysteine (NAC), and 100 μ M of S3I-20 for 24 h. Images clearly show a reduction in HIF-1 α in cells treated with NAC and S3I-201 with respect to control (CTRL). Immunoblot on DU-145 (B) and KPL-4 (D) of nuclear and total extract and the relative densitometries evidenced a decrease in HIF-1 α expression upon treatments. Protein expression levels were referred to β -actin for total extract and to Lamin A/C for nuclear extract and were compared to control. Experiments were repeated three times with similar results, and obtained values are reported as mean and standard deviation. Statistical analysis was performed with GraphPad Prism software version 8.2.1 using ANOVA followed by Tukey's post hoc test. Statistically significant differences (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) are marked with asterisks and are referred to the control.

2.4. Effect of Serine on the Loop

Serine, acting as a positive allosteric regulator of PKM2, stabilizes the cytosolic tetrameric form of the enzyme, thereby preventing its nuclear translocation, where PKM2 functions as a kinase for STAT3, phosphorylating it on Y705, and as a transcriptional co-factor for HIF-1 α . Consequently, treatment with serine was expected to result in reduced phosphorylation of STAT3 at Y705 and its nuclear translocation. Furthermore, since the

tetrameric PKM2 isoform promotes the reactivation of oxidative metabolism, a concomitant decrease in STAT3 phosphorylation at S727 was also anticipated. Both cell lines treated with serine exhibited a marked reduction in STAT3 phosphorylation at both residues (Y705 and S727), accompanied by lower STAT3 expression levels in nuclear and mitochondrial fractions. PKM2, as hypothesized, did not translocate to the nucleus and was clearly detectable in the total protein fraction, whereas its levels were significantly reduced in the nuclear extracts. HIF-1 α expression was also reduced in serine-treated samples compared to controls, both in total and nuclear protein fractions, consistent with the role of PKM2 as one of its transcriptional regulators. The inhibitory effect of serine on HIF-1 α expression was less pronounced than that observed following STAT3 inhibition, likely because STAT3 itself contributes to HIF-1 α expression and stability through multiple mechanisms, resulting in a more significant inhibitory effect. Indeed, HIF-1 α expression is directly regulated by the canonical STAT3 pathway, and its half-life is influenced by oxygen levels and the presence of reactive species. This regulation is associated with STAT3 phosphorylation at S727 and its mitochondrial activity. Treatment with S3I-201 inhibits both phosphorylations, leading to a pronounced decrease in HIF-1 α levels in both total and nuclear protein fractions [69].

2.5. Effect of NAC on the Loop

ROS, markers of oxidative stress, were considered elements acting in tandem with the analyzed loop, since, as previously described, they represent hallmarks of more aggressive tumor phenotypes or advanced stages of carcinogenesis. Moreover, ROS contribute to the activation of all three proteins within the loop, thereby functioning as its “fuel.” Consequently, treatment with the antioxidant NAC was employed to reduce intracellular ROS levels, restoring a more balanced redox state. The results obtained demonstrate that, in both cell lines, STAT3 exhibited a reduction in phosphorylation at Y705 and S727 compared to the control, and decreased localization within both the nuclear and mitochondrial compartments. PKM2 was predominantly detected in the total protein fraction, with minimal nuclear presence. A similar trend was observed for HIF-1 α , though with a more pronounced reduction, consistent with its transcriptional regulation by STAT3 and PKM2, and its stabilization by ROS. Thus, the depletion of ROS not only limits the activation of STAT3 and PKM2 but also promotes HIF-1 α degradation, collectively resulting in a marked decrease in its overall expression.

2.6. Detection ROS

ROS were defined as the driving force of the signaling loop; therefore, it was of particular interest to determine whether the treatments selected to inhibit the loop also affected ROS production. Both cell lines were subjected to the same treatments, and after 48 h, intracellular ROS levels were revealed using the CellROX™ Green Reagent Kit (Thermo Fisher Scientific, Rodano, Italy) (Figure 5A,B). The most pronounced reduction in fluorescence was observed following NAC treatment, as expected. However, decreased fluorescence was also evident in cells treated with serine or S3I-201. This finding is particularly relevant, as it highlights the interplay between oxidative stress and the STAT3/PKM2/HIF-1 α loop. Indeed, inhibiting ROS exerts a clear inhibitory effect on the loop, while conversely, inhibition of loop components results in reduced ROS production. These observations support the existence of a positive cooperative relationship between oxidative stress and loop activation, which may underlie the maintenance of a fermentative metabolic phenotype and cellular conditions characteristic of the later stages of carcinogenesis, namely, tumor promotion, progression, metastasis, and chemoresistance.

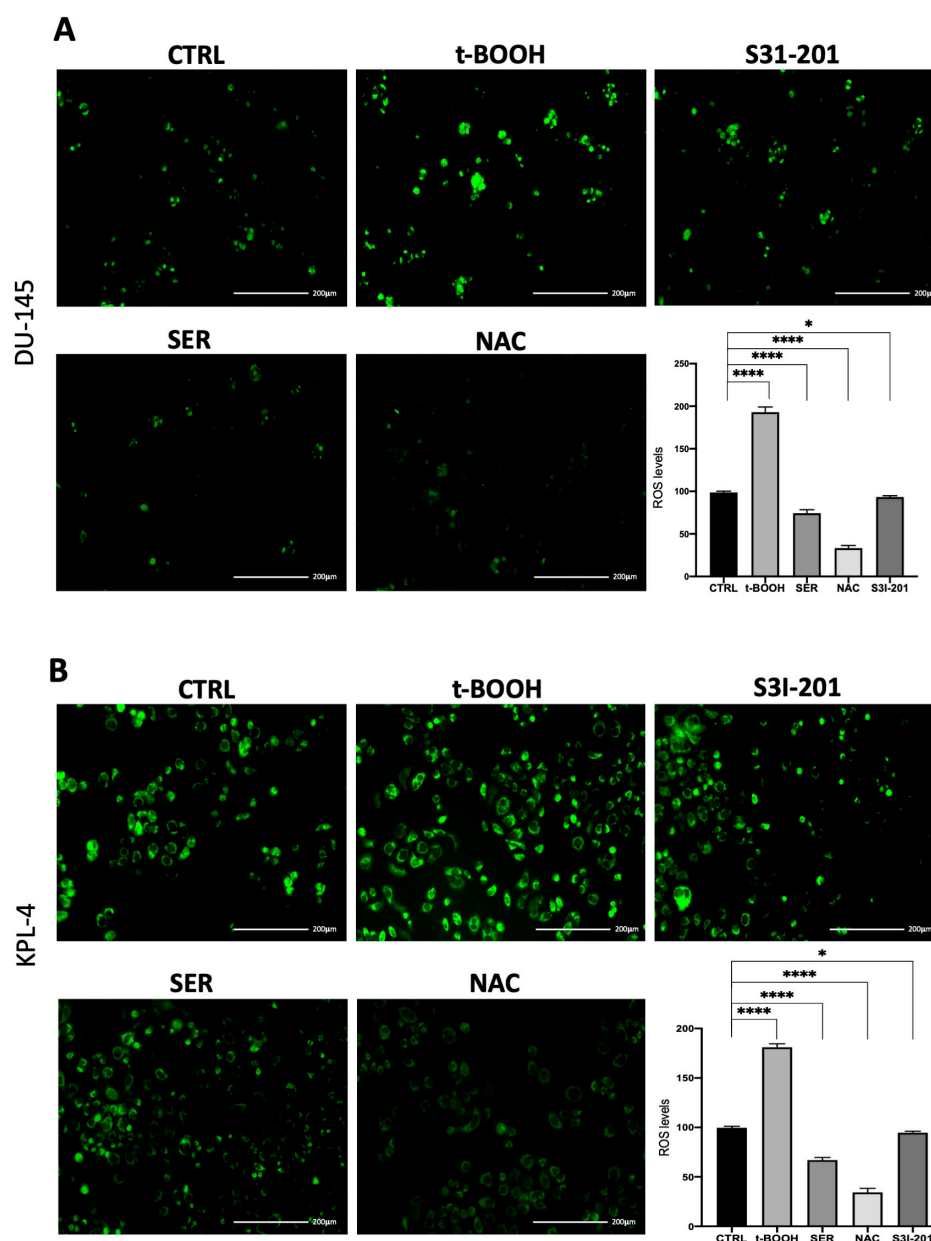


Figure 5. Serine and NAC reduce ROS production in DU-145 (A) and in KPL-4 (B) cell lines. The images and the relative fluorescence quantification reported in the histogram clearly show a decrease in ROS levels upon treatment with 1 mM of N-acetylcysteine (NAC) and 1 mM of serine (SER) for 48 h, compared to the control (CTRL); 100 μ M of S3I-201 for 24 h does not exert an effect. A total of 75 μ M tBOOH for 1 h was used as a positive control. Fluorescence intensity was quantified by averaging across the CTCF (Corrected Total Cell Fluorescence) calculated with ImageJ version 1.54p. Images were captured under the same acquisition parameters and are representative of three independent experiments. Results are presented as the mean and standard deviation. Statistical analysis was performed with GraphPad Prisma software version 8.2.1 using ANOVA followed by Tukey's post hoc test. Statistically significant differences (* $p < 0.05$, *** $p < 0.0001$) are marked with asterisks and are referred to the control. Scale bar 200 μ m.

2.7. Detection of Ki67

In previous experiments, we demonstrated that it is possible to negatively modulate the loop by using specific inhibitors targeting its individual components and the ROS that act as its driving force, forming a functional tandem partnership.

The results obtained indicate a molecular-level effect, showing inhibition of activation mechanisms such as protein phosphorylation and subcellular translocation of the molecules

involved. However, it is crucial to determine whether these molecular events impact the tumor phenotype. As a first step, we investigated whether the treatments applied could reduce the high aggressiveness and proliferative capacity typically observed in triple-negative breast cancer and androgen-independent prostate cancer cells. The marker for these characteristics, as previously mentioned, is the Ki67 protein, a well-established indicator of tumor cell proliferation [70]. In the treated samples, following the same experimental protocol (with the only variation being a 72 h treatment), total protein extracts were prepared and analyzed by immunofluorescence. The images shown in Figure 6 qualitatively reveal a decrease in Ki67 fluorescence in all treated samples, which is more pronounced in those treated with NAC and S3I-201. These findings provide initial evidence that the negative modulation of the loop and its ROS-related tandem interaction can remodel tumor phenotypic features, shifting them toward a less aggressive profile.

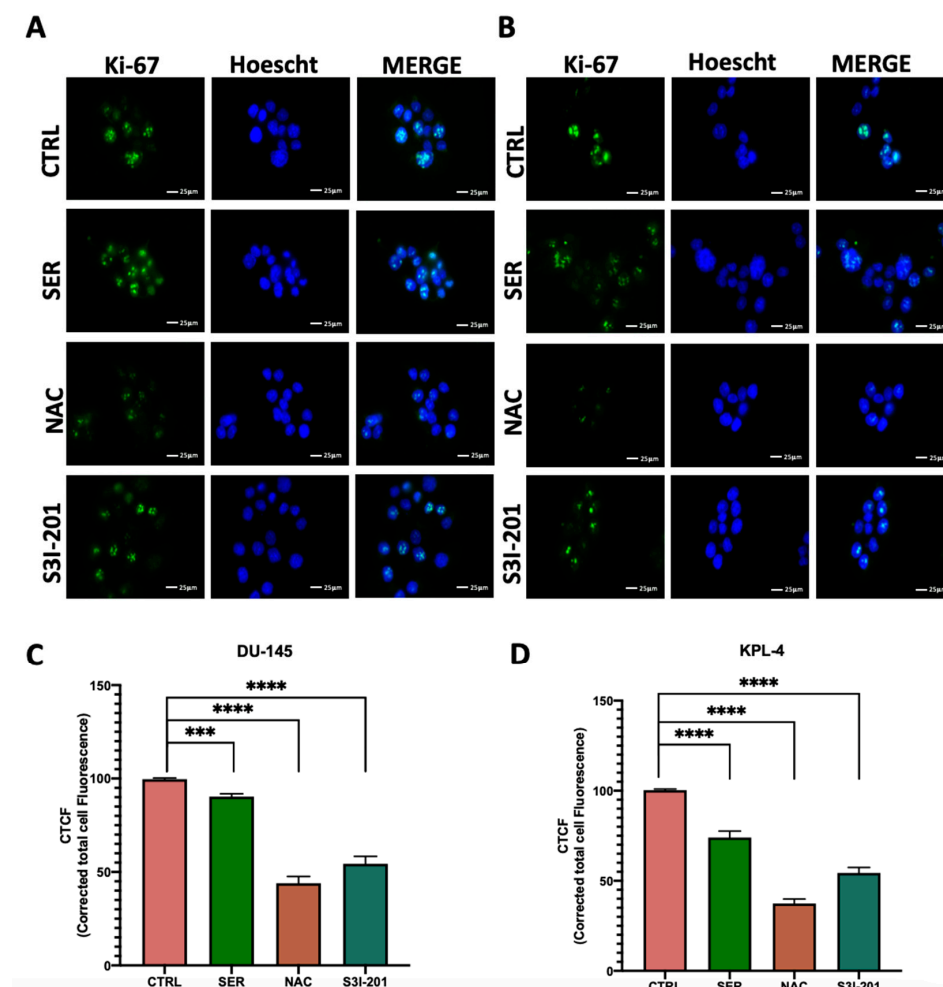


Figure 6. Cellular distribution of Ki-67 analyzed by immunofluorescence in DU-145 (A) and KPL-4 (B) cells treated for 48 h with 1 mM of serine (SER), 1 mM of N-acetylcysteine (NAC), and 100 μ M of S3I-20 for 24 h. Images clearly show a reduction in Ki-67 nuclear localization in cells treated with NAC, serine and S3I-201 with respect to control (CTRL). Fluorescence intensity (C,D) was then quantified by averaging across the CTCF (Corrected Total Cell Fluorescence) calculated with ImageJ on the same number of cells for both control and treated samples from different images. Images were captured under the same acquisition parameters and were background subtracted before analysis; the selected images are representative of three independent experiments. Results are presented as the mean and standard deviation. Statistical analysis was performed with GraphPad Prisma software version 8.2.1 using ANOVA followed by Tukey's post hoc test. Statistically significant differences (** $p < 0.001$, **** $p < 0.0001$) are marked with asterisks and are referred to the control.

2.8. Modulation of the Pyruvate-to-Lactate Ratio in DU145 and KPL-4 Cells

Triple-negative breast cancer and androgen-independent prostate cancer are characterized by a predominantly fermentative metabolic phenotype, commonly referred to as the Warburg effect [71,72]. Previous studies have established a strong association between the STAT3–PKM2–HIF-1 α loop and its functional partner, the ROS level, with this metabolic reprogramming [61]. Accordingly, and based on our molecular data, inhibition of the loop and ROS level is expected to restore oxidative metabolism at the expense of the fermentative one. To test this hypothesis, culture media from DU145 and KPL-4 cell lines, treated under the same experimental conditions described above for 48 h, were analyzed by gas chromatography–mass spectrometry (GC–MS) to quantify pyruvate and lactate levels. The histograms shown in Figure 7 highlight that the pyruvate-to-lactate ratio increases under all three treatment conditions compared to the control, with a markedly significant rise observed in NAC-treated samples. This finding can be explained by the fact that ROS activate all three proteins within the loop and exert a direct influence on cellular energy metabolism. Therefore, their reduction results in a more pronounced metabolic shift compared to the other treatments. A difference was also observed between S3I-201 and serine treatments, with S3I-201 producing a stronger effect. This outcome is consistent with the mechanism of S3I-201, which inhibits STAT3 and thus impacts multiple components of both the loop and energy metabolism, whereas serine primarily targets PKM2, leading to a comparatively weaker effect.

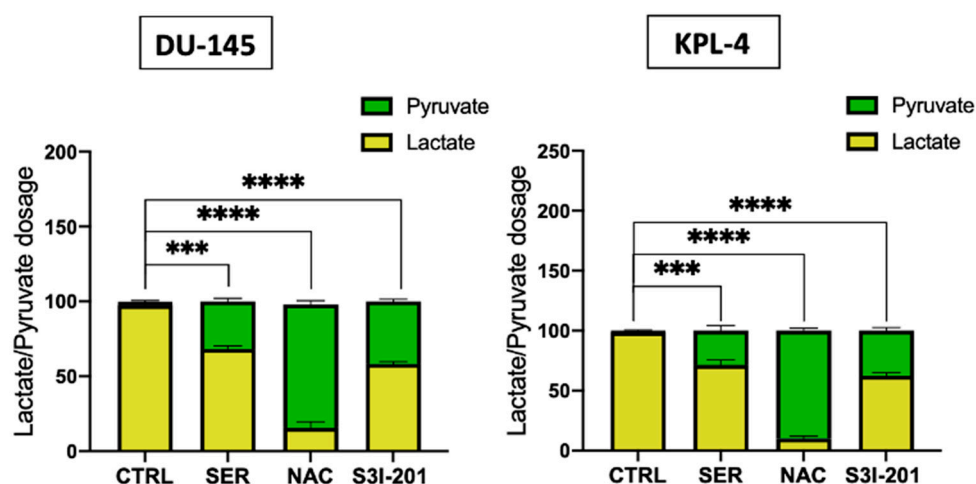


Figure 7. Effect of serine (SER), N-acetylcysteine (NAC), and S3I-201 on the lactate-to-pyruvate ratio in the culture medium of DU-145 and KPL-4 cells. Lactate and pyruvate levels were measured by gas chromatography–mass spectrometry (GC–MS), and the ratio was calculated to assess changes in cellular redox state and glycolytic activity under each treatment compared to control (CTRL). Statistical analysis was performed with GraphPad Prisma software version 8.2.1 using ANOVA followed by Tukey’s post hoc test. Statistically significant differences (***) $p < 0.001$; **** $p < 0.0001$ are marked with asterisks and are referred to the control.

2.9. Clonogenic Assay

The clonogenic assay, an *in vitro* method used to evaluate the reproductive capacity of a single cell to form a colony, was performed. This assay represents a standard approach to assessing the effects of natural or synthetic compounds on cell survival and proliferation and allows the evaluation of how treatments influence cellular growth and division. In both cell lines, treatments were carried out for two months under the same experimental conditions applied in the previous experiments. The images of the colonies (Figure 8A) and the quantitative analysis counts (Figure 8B) indicate that all three compounds reduced the clonogenic capacity of both cell lines. In agreement with the pyruvate/lactate assay, the

most pronounced effect was observed following NAC treatment, followed by the STAT3 inhibitor and, lastly, serine. This differential response to the three compounds can be attributed, as previously described, to the distinct roles of their respective targets within the loop.

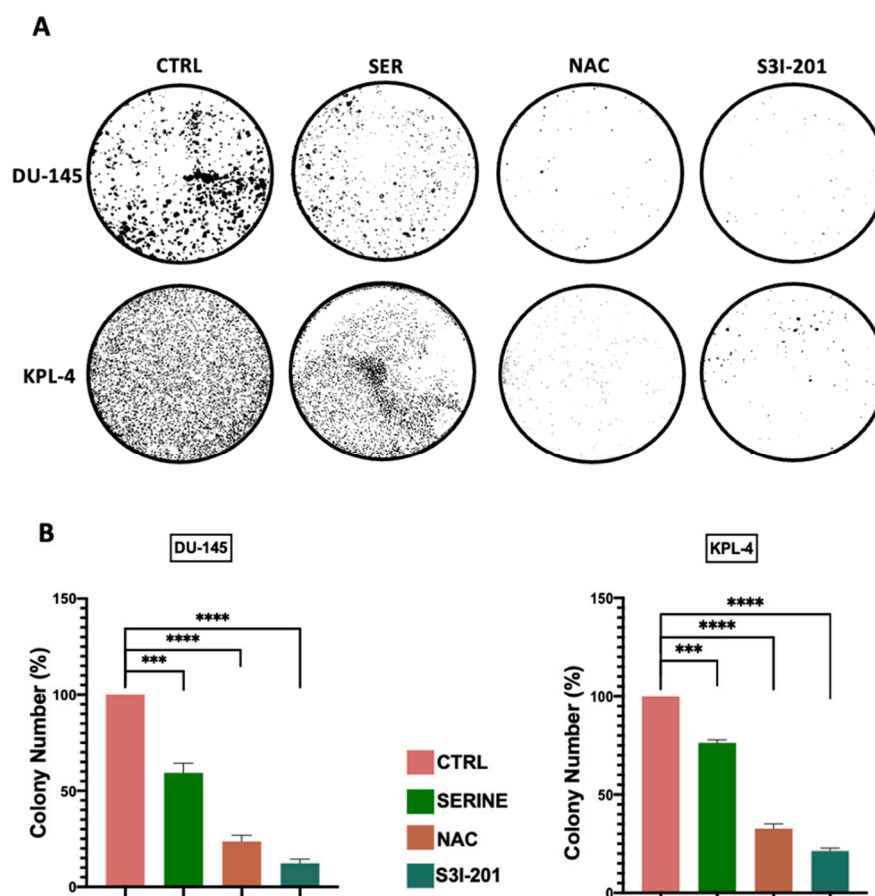


Figure 8. Clonogenic assay conducted on Du-145 and KPL-4 cell lines. Serine, NAC, and S3I-201 reduce colony formation ability. The cells were pretreated with 1 mM of serine and 1 mM of NAC in flasks for 7 days and then seeded at a density of 500 cells/mL in 6-well plates and treated for 5 days with S3I-201 100 μ M. After treatments, the colonies formed were evidenced using crystal violet dye (A) and counted, and the total areas of colonies (expressed as percentages with respect to the control and SD) are shown in the histogram (B). These images are representative of three independent experiments with similar results. Statistical analysis was performed with the GraphPad Prisma software version 8.2.1 using ANOVA followed by Tukey's post hoc test. Statistically significant differences (** $p < 0.001$ and **** $p < 0.0001$) are marked with asterisks and are referred to the control.

3. Discussion

Breast and prostate cancers represent the most prevalent hormone-dependent malignancies in women and men, respectively, and, therefore, constitute major global public health concerns [73,74]. A hallmark of hormone-dependent tumors is their ability to activate alternative or compensatory growth and survival pathways, leading to the emergence of hormone resistance [75]. This condition reflects the transition toward a more aggressive tumor phenotype and is frequently associated with therapeutic resistance [76].

The molecular mechanisms underlying hormone resistance—such as mutations or downregulation of steroid hormone receptors and the activation of non-hormonal signaling pathways in prostate cancer (PCa) and breast cancer (BCa)—are well documented [77]. However, accumulating experimental evidence indicates that hormone resistance is also driven by dysregulation of redox signaling and cellular energy metabolism. These alter-

ations promote global transcriptional reprogramming, transient cell-cycle arrest, and the maintenance of tumor stemness [78,79]. Indeed, hormone-resistant cancer cells rely on several adaptive strategies—activated individually or concurrently—including metabolic reprogramming, phenotypic plasticity, remodeling of the tumor microenvironment, and uncontrolled proliferation [80].

Given the central role of redox homeostasis and energy metabolism in carcinogenesis [5], particularly in supporting tumor cell survival under adverse conditions such as nutrient deprivation, hypoxia, ROS accumulation, or therapy-induced cellular stress, we hypothesized that PCa and BCa may share common cellular mechanisms despite their biological heterogeneity. This hypothesis is supported by the fact that both malignancies exhibit early dependence on sex hormones, molecular alterations in steroid receptors conferring treatment resistance, and convergent signaling pathways, particularly those involved in steroid hormone metabolism and IGF-1 signaling [5].

Metabolic studies indicate that, in the context of hormone resistance, both tumor types display major alterations in metabolic pathways, including glycolysis, glutamine, glycine, and lipid metabolism, and are characterized by profound oxidative stress [81]. We, therefore, posited that hormone-resistant PCa and BCa might share the following: (i) constitutive activation of canonical and non-canonical STAT3 pathways; (ii) a glycolytic metabolic phenotype; and (iii) a ROS-enriched microenvironment. This led us to investigate whether both tumor types also exhibit activation of the STAT3–PKM2–HIF-1 oncogenic loop, supported by ROS. This circuit governs key processes involved in the onset and progression of endocrine resistance and, when reinforced by ROS, promotes the metabolic shift from oxidative phosphorylation to aerobic glycolysis (Warburg effect).

Our findings confirm that this loop is active in both hormone-resistant PCa and BCa cell lines. We hypothesized that inhibition or negative modulation of the loop and its ROS partnership would reorient cellular metabolism toward OxPhos and restore a less oxidized redox state, thereby re-establishing microenvironmental and intracellular conditions closer to physiological homeostasis.

Treatment with S3I-201 effectively inhibited both canonical and non-canonical STAT3 pathways, resulting in decreased HIF-1 expression, reduced ROS production, and increased cytosolic localization of PKM2. Modulation of PKM2 with serine, a metabolite typical of proliferating cells, also diminished STAT3 pathway activation and led to reduced HIF-1 expression and the attenuation of the Warburg phenotype. Finally, treatment with N-acetylcysteine (NAC), an antioxidant that buffers ROS, exerted a combined action on all three proteins of the loop, resulting in complete inhibition of the circuit and restoring a molecular profile characterized by (i) cytosolic, inactive STAT3 and PKM2; (ii) reduced HIF-1 expression (via both transcriptional repression and proteasomal degradation); and (iii) a marked decrease in ROS levels.

Functionally, loop inhibition restored features typical of non-transformed cells, as a decreased lactate production with concomitant restoration of pyruvate levels, a significant reduction in Ki-67 expression, indicating diminished proliferation and tumor aggressiveness, and a sharp decrease in clonogenic capacity in treated versus untreated cells. Moreover, negative modulation of the STAT3–PKM2–HIF-1/ROS loop enhanced the chemosensitivity of DU-145 and KPL4 cells.

The most pronounced responses, both at the molecular and functional levels, were observed following NAC treatment and STAT3 inhibition, highlighting the central role of STAT3 as the core node of the HIF-1 α –PKM2–STAT3 oncogenic loop in partnership with ROS. Through its canonical pathway, STAT3 enhances HIF-1 transcription and indirectly modulates PKM2 expression, whereas through its non-canonical pathway, it promotes chronic oxidative stress by increasing ROS levels, which in turn, reinforce loop activa-

tion [82]. Consequently, an antioxidant such as NAC produces a particularly robust cellular response because it simultaneously targets all components of the loop and ROS partnership, whereas direct STAT3 inhibition specifically suppresses loop activity and secondarily reduces ROS.

Overall, our findings are promising and provide new insights into the metabolic alterations and oxidative stress conditions associated with tumor progression toward more aggressive hormone-resistant phenotypes, as well as changes induced by anticancer treatments. Furthermore, they suggest that integrated investigation of metabolic enzymes, redox status, and the protein pathways regulating these processes could facilitate the identification of novel molecular targets or biomarkers, ultimately enabling the development of personalized therapeutic strategies for patient subgroups with similar clinical and molecular profiles.

4. Materials and Methods

4.1. Cell Culture

Human prostate cancer cell line DU-145 and human breast cancer cell line KPL-4 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown to 80% confluence at 37 °C in 5% CO₂ in the appropriate culture medium, RPMI 1640 (Sigma-Aldrich, Milano, Italy) or DMEM-LG (Sigma-Aldrich), depending on cell line, and supplemented with 1% sodium pyruvate, 10% fetal bovine serum, 2 mM glutamine, 100 µg/mL streptomycin, and 100 U/mL penicillin.

L-serine (Sigma-Aldrich, S4500) was tested on DU-145 and KPL-4 at a final concentration of 1 mM. N-acetyl-L-cysteine (Sigma-Aldrich, A9165) was tested on DU-145 and KPL-4 at a final concentration of 1 mM. The STAT3 inhibitor used was 100 µM S3I-201 (Sigma-Aldrich, SML0330).

4.2. Protein Extraction and Immunoblotting

Protein extraction and immunoblotting analysis were performed essentially according to Rubini et al. [83]. Cells were cultured on 6-well plates at a density of 300,000 cells/well, harvested by centrifugation, and washed in PBS (Sigma-Aldrich, Milano, Italy, cat. D8662). Total protein extracts were obtained using a lysis buffer containing 2% SDS (BioRad, Segrate, Italy, cat. 161030), 20 mM Tris-hydrochloride pH 7.4 (Sigma-Aldrich, cat. T3253), 2 M urea (Sigma-Aldrich, Milano, Italy, cat. U5378), 10% glycerol (Merck, Milano, Italy, cat. GE17-1325-01) added with 2 mM sodium orthovanadate (Sigma-Aldrich, Milano, Italy, cat. S6508), 10 mM DTT (Sigma-Aldrich, Milano, Italy, cat. D9779), and a protease inhibitor cocktail diluted 1:100 (Immunological Sciences, Roma, Italy, cat. IK-96010). Nuclei were obtained from cell pellets using a hypotonic buffer (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT) added with 0.05% Triton-X, 2 mM sodium orthovanadate, and a protease inhibitor cocktail diluted 1:100 (Sigma-Aldrich). Thus, nuclei were harvested by centrifugation and washed in hypotonic buffer, and nuclear protein extracts were obtained as described above for total protein extracts.

Proteins were resolved by SDS-PAGE 10% TGX FastCast™ Acrylamide gel (BioRad, Segrate, Italy, cat. 161-0183) and transferred to PVDF membranes using Trans-Blot® Turbo™ Transfer System (BioRad, Segrate, Italy, cat. 170-4247). The membranes were blocked with 3% BSA (Immunological Sciences, Roma, Italy, cat. ISP6154-100) or 0.2% *w/v* I-block (Thermo Fisher Scientific, Monza, Italy, cat. T2015) in Tris-buffered saline containing 0.05% Tween-20 (Sigma-Aldrich, Milano, Italy, cat. P7949) (TBS-T) and incubated with a specific primary antibody for 1 h. Subsequently, membranes were washed three times in TBS-T and then incubated for an additional hour with phosphatase-conjugated secondary antibody (Sigma-Aldrich, cat. A3687-A3688, dilution 1:5000) or with StarBright Blue 520 or

700 fluorescent secondary antibodies (BioRad, cat 120005867-120004159, dilution 1:2500) according to the manufacturer's instructions. The alkaline phosphatase signal was detected with BCIP/NBT reagents (Carl Roth, Milano, Italy, cat. 6368.1 and 4421.3). The intensity of protein bands was quantified using the ImageLab Software, version 3.0.1. Membrane images were acquired by Molecular Imager[®] ChemiDoc[™] MP System (Bio-Rad, Segrate, Italy), and the intensity of protein bands was quantified using ImageLab Software. The immunoblotting detection was carried out using specific primary antibodies, as reported further in this section.

4.3. Immunofluorescence

Immunofluorescence analysis was performed essentially according to Cocchiola et al. [84]. DU-145, KPL-4, cells were grown on coverslips. Cells were treated for 48 h with serine 1 mM and NAC 1 mM and for 24 h with 100 μ M S3I-201. Cells grown on coverslips were washed with PBS, fixed with 4% formaldehyde for 15 min, and then rinsed with PBS (Sigma-Aldrich, Milano, Italy, cat. D8662). Cells were permeabilized with cold methanol (20 °C) for 5 min. After washing three times with PBS, the cells were blocked overnight with 3% *w/v* BSA (Immunological Sciences, Roma, Italy, cat. ISP6154-100) in PBS. Fixed cells were processed by immunofluorescence staining using specific primary antibodies, as reported further in this section, properly diluted in PBS containing 2% *w/v* BSA for 1 h. Following three washes with PBS added to with 0.05% Triton and 2% *w/v* BSA (PBS-T), cells were incubated for 1 h in the darkness with a FITC-conjugated secondary antibody (Jackson ImmunoResearch, AlexaFluor 488-conjugated, Cambridge, UK, cat. 211-545-109, dilution 1:800). Cell nuclei were counterstained with 100 ng/mL Hoechst (Sigma-Aldrich, Milano, Italy, cat. 94403) for 15 min. After washing with PBS-T, coverslips were mounted on glass microscope slides with Duolink[™] Mounting Medium and examined using a fluorescence microscope (Leica AF6000 Modular System, Leica, Milano, Italy) with 63 oil immersion objectives. Samples were captured under the same acquisition parameters, and background was subtracted before analysis.

4.4. Mitochondria Staining

Mitochondria were stained with MitoTracker Orange CMTMRos (Invitrogen, Monza, Italy, M7510) before fixation. MitoTracker 1 mM (DMSO stock solution) was diluted to a final concentration of 190 nM in serum-free culture media in accordance with the manufacturer's instructions. Cells were incubated for 30 min with the mitochondrial dye and then washed three times in serum-free culture media before fixation and immunofluorescence analysis.

4.5. Reactive Oxygen Species (ROS) Detection

Reactive oxygen species (ROS) were quantified using the CellROX[™] Green Reagent Kit (Thermo Fisher Scientific, Rodano, Italy; C10492). Cells were treated with NAC, serine, and S3I-201 according to the previously described experimental protocol, and oxidative stress was induced in the positive control by incubation with 75 μ M tBHP (tert-butyl hydroperoxide) for 30 min. At the end of the treatments, cells were incubated for 2 h with the CellROX reagent. Fluorescence images were acquired using a Cytation 1 Cell Imaging Multimode Reader (Biotech, Santa Clara, CA, USA). Fluorescence intensity was quantified by calculating the Corrected Total Cell Fluorescence (CTCF) with ImageJ software version 1.54p, averaging the signal across an equivalent number of cells in control and treated samples analyzed in multiple images.

4.6. Colony Formation Assay

The cells were seeded at a density of 200 cells/mL in 6-well plates and treated for one week with 1 mM of serine and NAC and for a further two weeks in the presence or absence of S3I-201 for STAT3/PKM2/HIF-1 α loop experiments. For chemoresistance experiments, cells were treated for one week with 10 μ M of β -HCH and for a further two weeks in the presence or absence of specific TKIs and S3I-201. The medium was removed, and then the cells were rinsed with PBS and fixed with cold MeOH for 30 min at 4 °C. Thereafter, the colonies were stained by incubating the cells with a mixture of 1% crystal violet in 25% MeOH for 1 h at room temperature. After the removal of the staining solution, each well was washed with abundant H₂O and air-dried at room temperature. The colonies were counted using ImageJ software according to Rubini et al. [85].

4.7. Determination of Lactic and Pyruvic Acid

Lactic acid and pyruvic acid were analyzed by GC-MS as methoxime/tertbutyldimethylsilyl derivatives as previously described by Paik et al. [86]. GC-MS analyses were performed with an Agilent 6850A gas chromatograph coupled to a 5973N quadrupole mass selective detector (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separations were carried out with an Agilent HP5ms fused-silica capillary column (30 m $\times$ 0.25 mm i.d.) coated with 5%-phenyl/95%-dimethylpolysiloxane (film thickness 0.25 μ m) as stationary phase, using helium as the carrier gas at a constant flow rate of 1.0 mL/min, splitless injection mode at a temperature of 280 °C, and the following column temperature program: 70 °C (1 min), then to 300 °C at a rate of 20 °C/min, and held for 10 min. The spectra were obtained in the electron impact mode at 70 eV ionization energy (ion source 280 °C and ion source vacuum 10⁻⁵ Torr). MS analysis was performed simultaneously in TIC (mass range scan from m/z 50 to 600 at a rate of 0.42 scans s⁻¹) and SIM mode. GC-SIM-MS analysis was performed selecting the following ions: m/z 174 for pyruvate, m/z 261 for lactate, and m/z 239 for 3,4-dimethoxybenzoic acid (internal standard). Results were normalized on cell number and expressed as fold change relative to control samples.

4.8. Primary Antibodies

Anti-STAT3 (Cell Signaling, Pero, Italy, cat. 124H6), anti-pY705STAT3 (Cell Signaling, cat. D3A7), anti- β -actin (Sigma-Aldrich, cat. A1978 clone AC-15), anti-pS727STAT3 (Cell Signaling, cat. 9134S), anti-PKM2 (Cell Signaling cat.3198S), anti- HIF-1 α (Cell Signaling, cat.36169S), and anti-Lamin A/C (Invitrogen, cat. Mab636).

4.9. Statistical Analysis

The repeatability of results was confirmed by performing all experiments at least three times. The obtained values are presented as mean and standard deviation. Statistical analysis was performed with GraphPad Prism software using Student's *t*-test.

5. Conclusions

Despite the considerable differences between breast and prostate cancers, including their origin in distinct tissues, the identification of shared molecular mechanisms involved in the transition from a hormone-responsive to a hormone-resistant state may help to elucidate universal principles of both hormone-dependent and hormone-independent tumorigenesis. This comparative approach may facilitate the identification of diagnostic and prognostic biomarkers applicable across multiple tumor types by supporting the development of cross-cutting therapeutic strategies and accelerating the design of personalized and potentially more cost-effective treatments.

Comparative analysis of these two malignancies also provides insight into how biologically distinct tumors adopt convergent adaptive strategies to sustain growth under adverse conditions, offering a model of comparative oncology applicable to other cancer types. In this context, activation of the PKM2–STAT3–HIF-1 loop in partnership with ROS, as well as its modulation, represents a shared biological paradigm that could be exploited to develop more effective therapeutic approaches targeting chemoresistant disease.

Furthermore, negative modulation of this circuit may provide a preliminary mechanistic rationale for chemopreventive or tertiary adjuvant approaches aimed at partially restoring a cellular phenotype resembling earlier stages of carcinogenesis. However, its potential translational relevance remains speculative and will require extensive pharmacological and safety evaluation. Furthermore, negative modulation of this circuit may provide a preliminary mechanistic rationale for chemopreventive or tertiary adjuvant approaches aimed at partially restoring a cellular phenotype resembling earlier stages of carcinogenesis. However, its potential translational relevance remains speculative and will require extensive pharmacological and safety evaluation [87].

When combined with standard anticancer therapies, such an approach may enhance therapeutic responses and potentially contribute to a more durable or complete remission in hormone-resistant breast and prostate cancers.

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