



Article

Exploiting the Metabolic Consequences of PTEN Loss and Akt/Hexokinase 2 Hyperactivation in Prostate Cancer: A New Role for δ -Tocotrienol

Fabrizio Fontana *, Martina Anselmi and Patrizia Limonta

Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, 20133 Milan, Italy; martina.anselmi@unimi.it (M.A.); patrizia.limonta@unimi.it (P.L.)

* Correspondence: fabrizio.fontana@unimi.it

Abstract: The Warburg effect is commonly recognized as a hallmark of nearly all tumors. In prostate cancer (PCa), it has been shown to be driven by PTEN loss- and Akt hyperactivation-associated upregulation of hexokinase 2 (HK2). δ -Tocotrienol (δ -TT) is an extensively studied antitumor compound; however, its role in affecting PCa glycolysis is still unclear. Herein, we demonstrated that δ -TT inhibits glucose uptake and lactate production in PTEN-deficient LNCaP and PC3 PCa cells, by specifically decreasing HK2 expression. Notably, this was accompanied by the inhibition of the Akt pathway. Moreover, the nutraceutical could synergize with the well-known hypoglycemic agent metformin in inducing PCa cell death, highlighting the crucial role of the above metabolic phenotype in δ -TT-mediated cytotoxicity. Collectively, these results unravel novel inhibitory effects of δ -TT on glycolytic reprogramming in PCa, thus providing new perspectives into the mechanisms of its antitumor activity and into its use in combination therapy.

Keywords: prostate cancer; tocotrienols; Warburg effect; glycolysis; hexokinase 2; Akt; PTEN; metformin



Citation: Fontana, F.; Anselmi, M.; Limonta, P. Exploiting the Metabolic Consequences of PTEN Loss and Akt/Hexokinase 2 Hyperactivation in Prostate Cancer: A New Role for δ -Tocotrienol. *Int. J. Mol. Sci.* **2022**, *23*, 5269. <https://doi.org/10.3390/ijms23095269>

Academic Editor: Niall M. Corcoran

Received: 19 April 2022

Accepted: 6 May 2022

Published: 9 May 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Prostate cancer (PCa) is the third leading cause of cancer mortality among men in Western countries [1]. Despite advances in early detection and standard androgen-deprivation treatment, 30–40% of PCas progress towards an aggressive phenotype, often characterized by therapy resistance and poor prognosis [2–4]. Therefore, there is an urgent demand for the identification of novel pharmacological targets as well as of new anti-PCa agents that can complement current therapeutic strategies.

To increase macromolecule biosynthesis and sustain unlimited proliferation, most tumor cells preferentially utilize glycolysis as their major energy source, even in the presence of oxygen. This phenomenon, named the Warburg effect, is an emerging hallmark of cancer [5,6]. Hexokinase (HK), which catalyzes the conversion of glucose to glucose-6-phosphate, is the first rate-limiting enzyme in glycolysis. Four different HK isoforms have been found in mammals; however, only HK2 is responsible for the elevated aerobic glycolytic rate in tumor cells [7,8]. Overexpression of this protein has been reported in PCa, where it correlates with PTEN loss and Akt activation [9]. This evidence suggests that inhibition of the Akt/HK2-mediated Warburg effect might improve the efficacy of PCa therapy [10,11].

Tocotrienols (TTs) are vitamin E derivatives displaying potent antitumor properties [12–15]. We previously demonstrated that the δ isomer could induce mitochondrial functional and structural impairment in PCa cells [16,17]. However, the effects of δ -TT on Akt/HK2-regulated glycolysis in PTEN-deficient PCa have not been investigated yet.

In the present study, we further dissected the molecular mechanisms underlying the antiproliferative activity of δ -TT in human PCa cells, with a focus on its ability to specifically target the Akt/HK2 signaling-associated metabolic phenotype.

2. Results

2.1. δ -TT Treatment Affects LNCaP PCa Cell Viability and Proliferation

We have previously reported that δ -TT can inhibit castration-resistant PCa cell growth [16]. Here, we showed that it can also dose- and time-dependently reduce LNCaP androgen-sensitive PCa cell viability and proliferation (δ -TT 5–20 $\mu\text{g}/\text{mL}$, 24–48 h), as evidenced by MTT and Trypan blue exclusion assays (Figure 1a,b). In particular, its IC₅₀ at 24 h was found to be 3.54×10^{-5} M; thus, δ -TT 15 $\mu\text{g}/\text{mL}$, a cytotoxic dose close to this value, was selected as an appropriate concentration for further experiments.

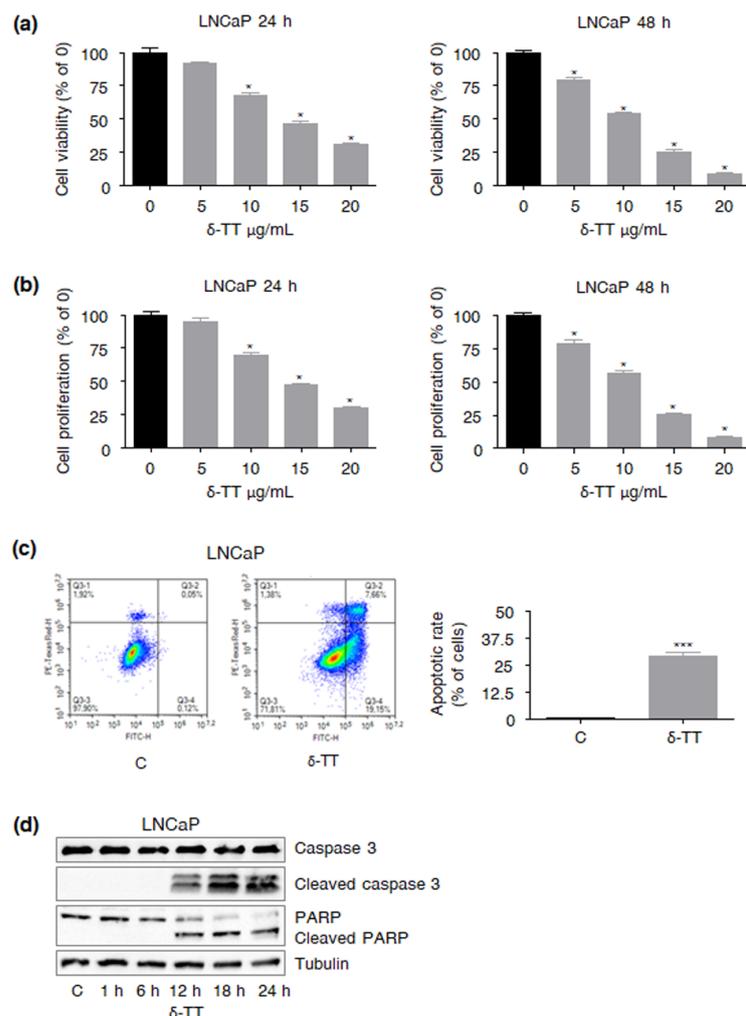


Figure 1. δ -TT treatment affects LNCaP PCa cell viability and proliferation, leading to apoptosis.

(a) LNCaP cells were treated with δ -TT (5–20 $\mu\text{g}/\text{mL}$) for 24 and 48 h. Cell viability was then evaluated by MTT assay. Each experiment was repeated three times. Data represent mean values \pm SEM and were analyzed by Dunnett's test after one-way analysis of variance. * $p < 0.05$ vs. C, controls (vehicle). (b) LNCaP cells were treated with δ -TT (5–20 $\mu\text{g}/\text{mL}$) for 24 and 48 h. Cell proliferation was then evaluated by Trypan Blue exclusion assay. Each experiment was repeated three times. Data represent mean values \pm SEM and were analyzed by Dunnett's test after one-way analysis of variance. * $p < 0.05$ vs. C, controls (vehicle). (c) LNCaP cells were treated with δ -TT (15 $\mu\text{g}/\text{mL}$, 24 h); apoptotic rate was then evaluated by cytofluorimetric analysis after staining with eBioscience™ Annexin V-FITC Apoptosis Detection Kit (according to the manufacturer's protocol). Each experiment was repeated three times. Data represent mean values \pm SEM and were analyzed by *t*-test. *** $p < 0.001$ vs. C, controls (vehicle). (d) After δ -TT treatment (15 $\mu\text{g}/\text{mL}$, 1–24 h), Western blot analysis was performed to investigate the expression levels of cleaved caspase 3 and PARP. Tubulin expression was evaluated as a loading control. One representative of three experiments performed is shown.

2.2. δ -TT Induces Apoptosis in LNCaP PCa Cells

The specific molecular mechanisms by which δ -TT (15 μ g/mL) could induce LNCaP cell death were next investigated. Interestingly, the compound was found to trigger apoptosis, with mortality rates being around 30% (24 h, Figure 1c). This hypothesis was also confirmed by the parallel cleavage of caspase 3 and PARP (24 h, Figure 1d). Overall, these results demonstrated the involvement of the apoptotic pathway in the anti-PCa activity of the compound, which appears to be effective regardless of the specific tumor stage.

2.3. δ -TT Cytotoxicity Correlates with Suppression of the HK2-Driven Warburg Effect in LNCaP PCa Cells

The LNCaP cell line has been recently proposed as a model of HK2-overexpressing PCa [9]. After confirming that these cells display this specific molecular signature (compared with RWPE-1 normal prostate epithelial cells) (Figure 2a), we investigated whether δ -TT could target the associated Warburg effect. Figure 2b shows that in this cell line, the compound (15 μ g/mL, 24 h) can induce a time-dependent decrease in HK2 protein levels, without affecting the expression of other key glycolytic proteins, such as glucose transporter 1 (GLUT1), pyruvate kinase M2 (PKM2) and lactate dehydrogenase A (LDH-A) (Figure 2c). As expected, HK2 downregulation was followed by inhibition (at 18 h) of glucose consumption (Figure 2d) and lactate production (Figure 2e). Collectively, these data highlight the ability of δ -TT to severely alter HK2-regulated aerobic glycolysis in LNCaP cells.

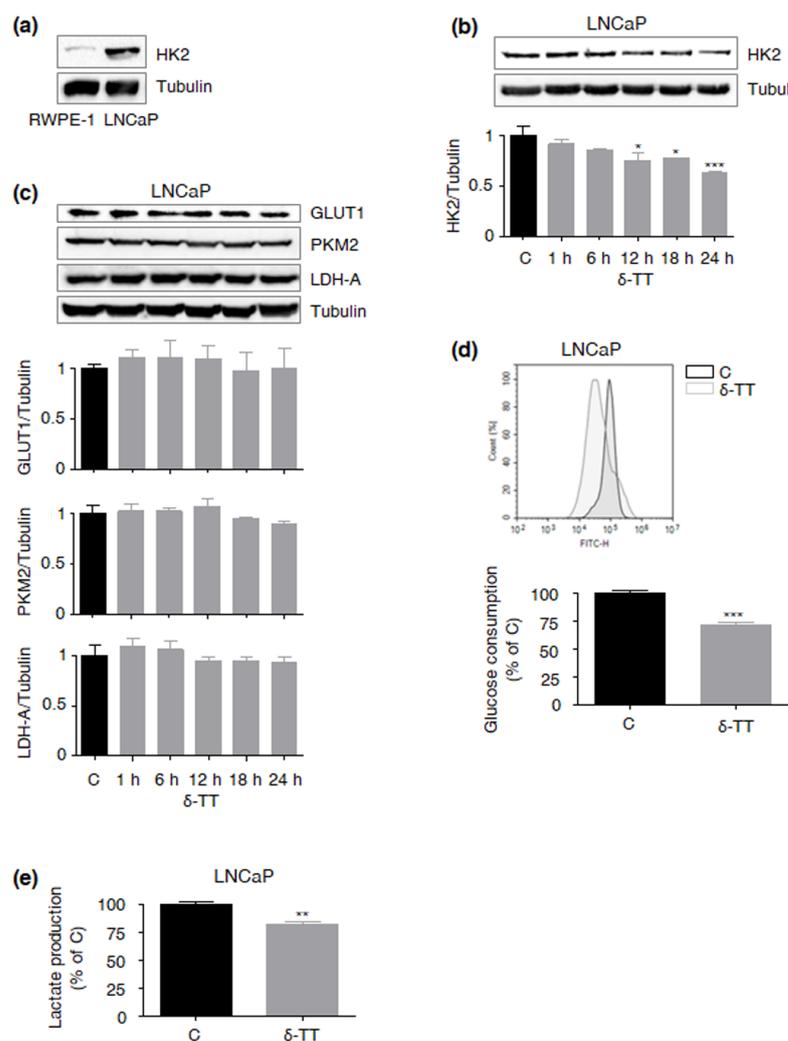


Figure 2. δ -TT cytotoxicity correlates with the suppression of the HK2-driven Warburg effect in LNCaP PCa cells. (a) HK2 expression was evaluated in RWPE-1 and LNCaP cells by Western blot analysis. Tubulin

expression was evaluated as a loading control. One representative of three experiments performed is shown. (b) After δ -TT treatment (15 μ g/mL, 1–24 h), Western blot analysis was performed to investigate the expression levels of HK2 in LNCaP cells. Tubulin expression was evaluated as a loading control. Data represent mean values \pm SEM and were analyzed by Dunnett's test after one-way analysis of variance. * $p < 0.05$ vs. C, controls (vehicle); *** $p < 0.001$ vs. C, controls (vehicle). (c) After δ -TT treatment (15 μ g/mL, 1–24 h), Western blot analysis was performed to investigate the expression levels of GLUT1, PKM2 and LDH-A in LNCaP cells. Tubulin expression was evaluated as a loading control. Data represent mean values \pm SEM and were analyzed by Dunnett's test after one-way analysis of variance. No significant changes in protein expression were observed. (d) LNCaP cells were treated with δ -TT (15 μ g/mL, 18 h); glucose consumption was then evaluated by cytofluorimetric analysis after staining with 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) 100 μ M for 30 min. Each experiment was repeated three times. Data represent mean values \pm SEM and were analyzed by *t*-test. *** $p < 0.001$ vs. C, controls (vehicle). (e) LNCaP cells were treated with δ -TT (15 μ g/mL, 18 h); lactate production was then evaluated by colorimetric assay. Each experiment was repeated three times. Data represent mean values \pm SEM and were analyzed by *t*-test. ** $p < 0.01$ vs. C, controls (vehicle).

2.4. δ -TT Downregulates HK2 via Specific Inhibition of Akt in PTEN-Null PCa Cells

In PCa cells, including LNCaP cell line, HK2 upregulation has been associated with PTEN deficiency and consequent Akt hyperactivation [9]. After validating our model in terms of PTEN and Akt expression (Figure 3a,b), we demonstrated that δ -TT (15 μ g/mL, 24 h) can time-dependently inhibit the latter (Figure 3c). In particular, upon δ -TT treatment, decreased HK2 expression coincided with reduced p-Akt levels, suggesting that the compound can downregulate this enzyme by inactivating the Akt signaling. To test this hypothesis, we also examined the anti-glycolytic activity of δ -TT (15 μ g/mL, 24 h) in PC3 castration-resistant PCa cells, known to be PTEN-deficient and to undergo Akt dephosphorylation-related apoptosis after exposure to the nutraceutical [9,11,17]. As expected, specific downregulation of HK2, followed by reduced glucose uptake and lactate release (at 18 h), was observed in this cell line (Figure 4a–d). Contrarily, no significant changes in the glycolytic rate were found in DU145 androgen-unresponsive PCa cells (data not shown), previously reported to be PTEN-wild type and Akt/HK2-null [9,17]. Altogether, these findings emphasize the vulnerability of PCas characterized by a glucose-dependent metabolic phenotype to δ -TT anti-tumor effects.

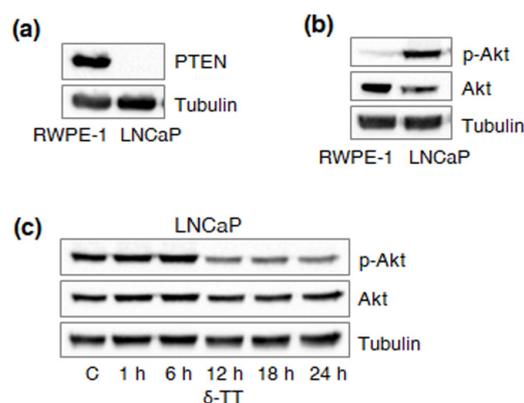


Figure 3. δ -TT downregulates HK2 in LNCaP PCa cells via inhibition of Akt. (a) PTEN expression was evaluated in RWPE-1 and LNCaP cells by Western blot analysis. Tubulin expression was evaluated as a loading control. One representative of three experiments performed is shown. (b) Akt hyperactivation was evaluated in RWPE-1 and LNCaP cells by Western blot analysis. Tubulin expression was evaluated as a loading control. One representative of three experiments performed is shown. (c) After δ -TT treatment (15 μ g/mL, 1–24 h), Western blot analysis was performed to investigate the expression levels of p-Akt in LNCaP cells. Tubulin expression was evaluated as a loading control. One representative of three experiments performed is shown.

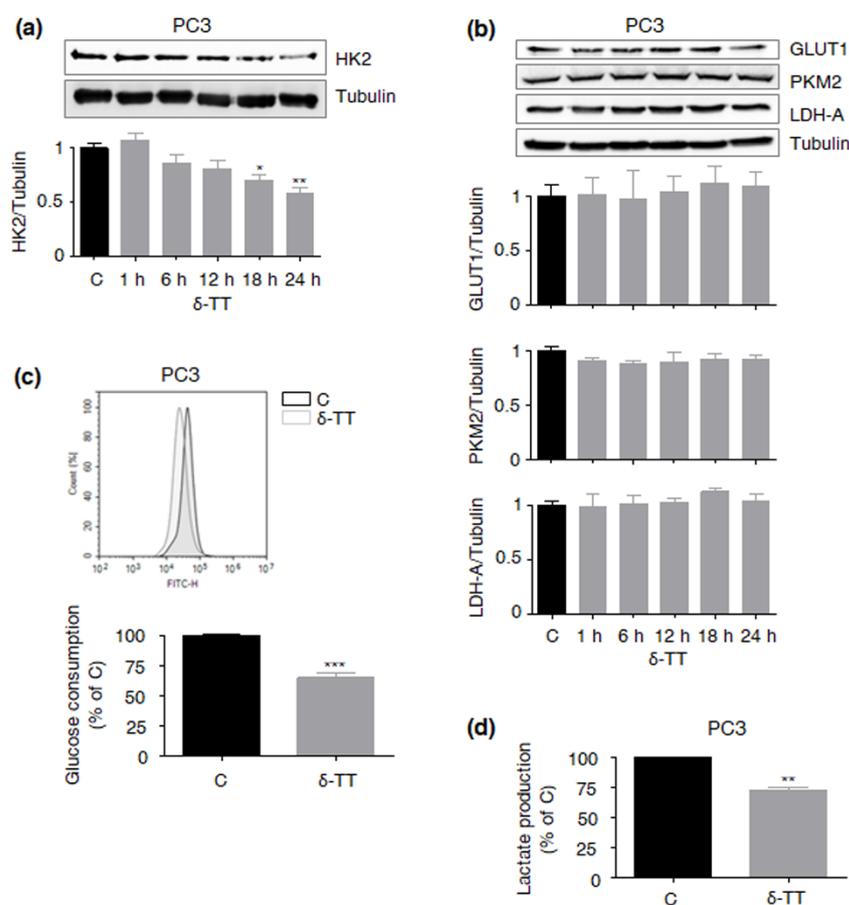


Figure 4. δ -TT targets the Akt/HK2-driven Warburg effect also in PC3 PCa cells. (a) After δ -TT treatment (15 μ g/mL, 1–24 h), Western blot analysis was performed to investigate the expression levels of HK2 in PC3 cells. Tubulin expression was evaluated as a loading control. Data represent mean values \pm SEM and were analyzed by Dunnett’s test after one-way analysis of variance. * $p < 0.05$ vs. C, controls (vehicle); ** $p < 0.01$ vs. C, controls (vehicle). (b) After δ -TT treatment (15 μ g/mL, 1–24 h), Western blot analysis was performed to investigate the expression levels of GLUT1, PKM2 and LDH-A in PC3 cells. Tubulin expression was evaluated as a loading control. Data represent mean values \pm SEM and were analyzed by Dunnett’s test after one-way analysis of variance. No significant changes in protein expression were observed. (c) PC3 cells were treated with δ -TT (15 μ g/mL, 18 h); glucose consumption was then evaluated by cytofluorimetric analysis after staining with 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) 100 μ M for 30 min. Each experiment was repeated three times. Data represent mean values \pm SEM and were analyzed by *t*-test. *** $p < 0.001$ vs. C, controls (vehicle). (d) PC3 cells were treated with δ -TT (15 μ g/mL, 18 h); lactate production was then evaluated by colorimetric assay. Each experiment was repeated three times. Data represent mean values \pm SEM and were analyzed by *t*-test. ** $p < 0.01$ vs. C, controls (vehicle).

2.5. δ -TT Synergizes with Metformin in Reducing PTEN-Null and Akt/HK2-Overexpressing PCa Cell Viability

Based on these observations, we finally verified if δ -TT could enhance the cytotoxicity of the hypoglycemic drug metformin, currently under study for PCa management [18–20]. As shown in Figure 5a,b, the nutraceutical was able to synergize with the anti-diabetic therapeutic in inducing LNCaP and PC3 cell death, demonstrating great potential in both mono- and combination therapy when used to treat PTEN-null and Akt/HK2-overexpressing PCa.

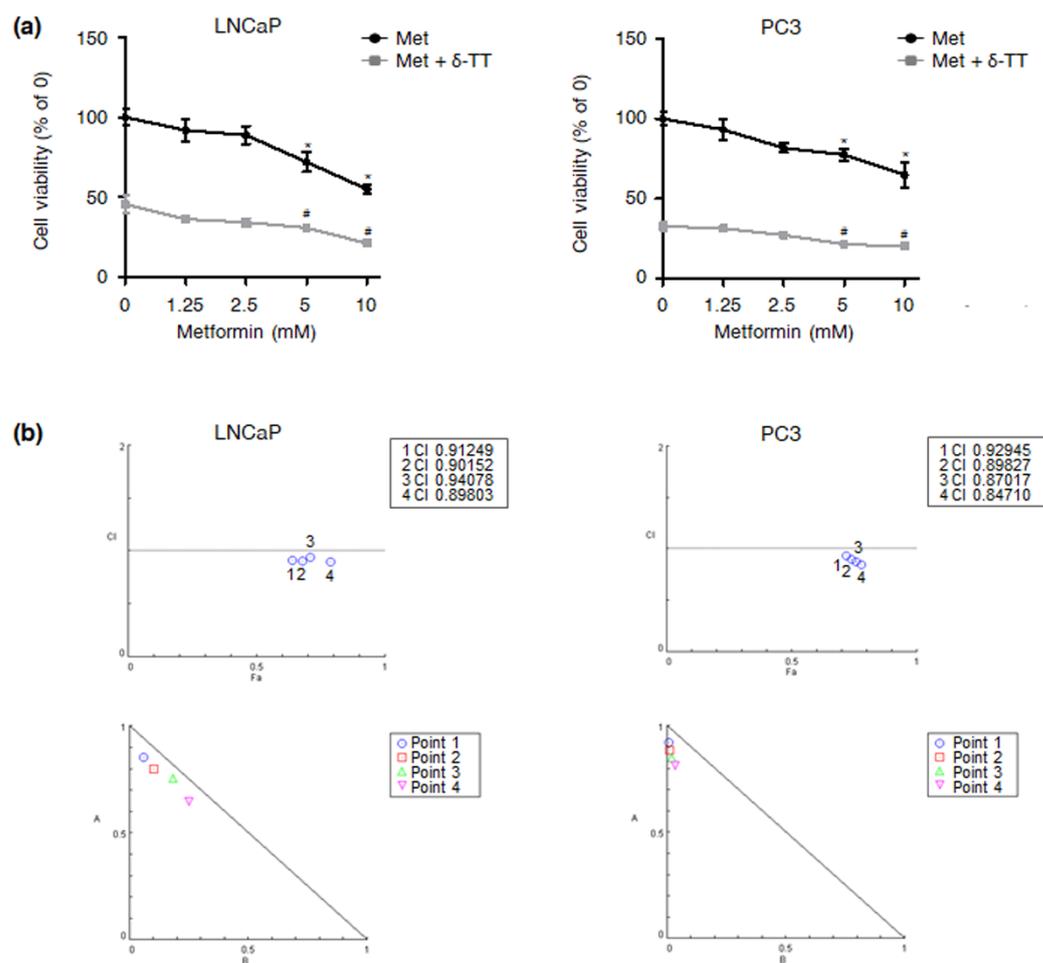


Figure 5. δ -TT synergizes with metformin in reducing PTEN-null and Akt/HK2-overexpressing PCa cell viability. (a) LNCaP and PC3 cells were treated with metformin (1.25, 2.5, 5, 10 mM), alone or in combination with δ -TT (15 μ g/mL) for 24 h. Cell viability was then evaluated by MTT assay. Each experiment was repeated three times. Data represent mean values \pm SEM and were analyzed by Dunnett's test after one-way analysis of variance. * $p < 0.05$ vs. C, controls (vehicle). # $p < 0.05$ vs. δ -TT-treated cells. (b) Data from Figure 5a were converted to Fraction Affected (FA) and plotted against Combination Index (CI). Straight line on the graph designates a CI equal to 1. Combination Index interpretation was as follows: CI value of 1 indicates additivity; CI < 1 indicates synergism; and CI > 1 indicates antagonism. Points 1, 2, 3 and 4 are the combination of δ -TT 15 μ g/mL and metformin 1.25, 2.5, 5 or 10 mM, respectively.

3. Discussion

In recent years, cancer metabolic reprogramming has gained increasing attention due to its immense potential as a crucial pharmacological target. In particular, metabolic enzymes might represent vital therapeutic hotspots, based on their role as key regulators of tumor cell proliferation [21]. A large body of evidence suggests that HK2 is fundamental in modulating cancer glycolysis, and several compounds have shown promise in suppressing cancer growth by specifically inhibiting this protein [22,23]. In PCa, HK2 upregulation correlates with PTEN deficiency and subsequent Akt hyperactivation [9], suggesting that targeting the Akt/HK2 axis could represent an effective antitumor strategy [10,11].

In the present study, we investigated whether δ -TT, a well-known antitumor nutraceutical, was able to specifically target the Akt/HK2-driven Warburg effect in PCa cells.

First, we demonstrated that δ -TT can exert significant antitumor effects, not only in androgen-independent but also in androgen-sensitive PCa cells, by decreasing both cell viability and proliferation regardless of the tumor stage. Specifically, we observed that

the compound can trigger apoptosis, paralleled by caspase 3 and PARP cleavage. This is in agreement with recent findings evidencing the proapoptotic effects elicited by TTs in various malignancies, such as skin, pancreatic, lung, liver, gastric, colon, ovarian and breast cancer [12,13,24–27].

Next, we found that δ -TT treatment leads to a specific decrease in HK2 protein levels in PTEN-null PCa cells, without altering the expression of other key glycolytic enzymes, including GLUT1, PKM2 and LDH-A; as expected, these changes were followed by a significant reduction in glucose consumption and lactate production. Interestingly, HK2 downregulation coincided with the suppression of the Akt pathway, highlighting the ability of δ -TT to selectively modulate the Akt/HK2 signaling in PTEN-deficient PCa. These data are consistent with previous reports describing the crucial role of the Akt/HK2 cascade in the cytotoxicity induced by different natural products, such as daphnetin, deguelin, jolkinolide B, licochalcone A, limonin, piperlongumine, sinomenine and xanthohumol, in several malignancies [28–35]. Regarding TTs, the γ isoform has been recently shown to suppress the Warburg effect in breast cancer [36]; our results demonstrate for the first time that the δ component is also endowed with anti-glycolytic properties, being able to selectively target the aberrant glucose metabolism of PCa.

A large body of evidence suggests that direct targeting of specific metabolic traits can successfully lead to cancer eradication [37,38]. In this regard, metformin, a well-established anti-diabetic molecule, has recently emerged as a promising anti-PCa agent [18–20]. Based on these observations, we conducted a combination study with this drug and δ -TT, evidencing that the phytochemical could sensitize PCa cells characterized by the Warburg effect to hypoglycemic therapy. To our knowledge, this is the first study highlighting the synergism between δ -TT and metformin in PCa.

In conclusion, these results provide a deeper understanding of the anticancer properties of δ -TT, demonstrating that it exerts potent antitumor activity in PTEN-null and Akt/HK2-overexpressing PCa cells by targeting their specific metabolic features and that it might serve as a promising therapeutic strategy when given in combination with anti-glycolytic agents.

4. Materials and Methods

4.1. Chemicals

δ -TT was purified from a commercial extract of Annatto seeds (*Bixa orellana* L.) (kindly provided by American River Nutrition Inc., Hadley, MA, USA), as previously described [25].

The primary antibodies caspase 3 (9656), cleaved caspase 3 (9664), PARP (9542), PTEN (9188), HK2 (2867), Akt (2938) and p-Akt (9271), GLUT1 (12,939) and PKM2 (4053) were from Cell Signaling Technology Inc. (Danvers, MA, USA), LDH-A (sc-13,7243) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and α -tubulin (T6199) was from Sigma-Aldrich (Milano, Italy). All of the antibodies were used at the concentration 1:1000.

Horseshoe-peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents were from Cyanagen (Bologna, Italy).

4.2. Cell Lines and Cell Culture

PTEN-deficient LNCaP and PC3 PCa cells and PTEN-wild type DU145 PCa cells were from American Type Culture Collection (ATCC, Manassas, VA, USA), and they were cultured in RPMI medium supplemented with 7.5% FBS, glutamine and antibiotics, in a humidified atmosphere of 5% CO₂/95% air at 37 °C. Normal prostate epithelial RWPE-1 cell line was also from ATCC and was grown in keratinocyte-SFM medium supplemented with bovine pituitary extracts and EGF (2.5 μ M) (Thermo Fisher Scientific, Waltham, MA, USA). Original stocks of cells were stored frozen in liquid nitrogen; after resuscitation, cells were kept in culture for no more than 10–12 weeks. Cells were detached through trypsin-EDTA solution and passaged once/week.

4.3. MTT Viability Assay

Cells were seeded at a density of 3×10^4 cells/well in 24-well plates for 48 h and then exposed to the specific compounds. The medium was then changed with MTT solution (0.5 mg/mL) in RPMI without phenol red and FBS; cells were incubated at 37 °C for 30 min, and violet precipitate was dissolved with isopropanol. Absorbance at 550 nm was measured through an EnSpire Multimode Plate reader (PerkinElmer, Milano, Italy).

4.4. Trypan Blue Exclusion Assay

Cells were plated (1.5×10^5 cells/dish) in 6 cm dishes. After 48 h, cells were treated with δ -TT (5–20 μ g/mL, 24 h). Adherent (viable) and floating (dead) cells were harvested, stained with Trypan blue 0.4% (1:1 *v/v*) and counted by Luna automated cell counter (Logos Biosystems, Annandale, VA, USA).

4.5. Annexin V/PI Apoptosis Assay

Cells were plated (1.5×10^5 cells/dish) in 6 cm dishes. After 48 h, cells were treated with δ -TT (15 μ g/mL, 24 h). Adherent (viable) and floating (dead) cells were harvested, washed in PBS and incubated with Annexin V and PI, using the eBioscience™ Annexin V-FITC Apoptosis Detection Kit. The flow cytometry analyses were performed with a Novocyte3000 instrument (ACEA Biosciences, San Diego, CA, USA). Data were analyzed with Novoexpress software.

4.6. Measurement of Glucose Consumption

Cells were plated (1.5×10^5 cells/dish) in 6 cm dishes. After 48 h, cells were treated with δ -TT (15 μ g/mL, 18 h). Adherent (viable) and floating (dead) cells were harvested, washed in PBS and incubated with 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (Thermo Fisher Scientific) 100 μ M for 30 min. The flow cytometry analyses were performed with a Novocyte3000 instrument. Data were analyzed with Novoexpress software.

4.7. Measurement of Lactate Production

Lactate production was measured by using a lactate assay kit (Sigma-Aldrich). An EnSpire Multimode Plate reader (PerkinElmer, Milano, Italy) was used to quantify absorbance at 570 nm.

4.8. Western Blot Analysis

Cells were seeded at 5×10^5 cells/dish in 10 cm dishes. After each treatment, cells were lysed in RIPA buffer; protein preparations (25 μ g) were resolved on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with the specific primary antibodies. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Westar Etac Ultra 2.0, XLS075,0100, Cyanagen). α -Tubulin was utilized as a loading control.

4.9. Isobologram Analysis

Cells were treated for 24 h using four different concentrations of metformin and δ -TT 15 μ g/mL. Viable cells were quantitated by MTT assay as described above, and CalcuSyn software was used to generate an isobologram.

4.10. Statistical Analysis

Statistical analysis was performed with a statistic package (GraphPad Prism5, GraphPad Software San Diego, CA, USA). Data are represented as the mean \pm SEM of three or four independent experiments. Differences between groups were assessed by *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's test. A *p* value < 0.05 was considered statistically significant.

Author Contributions: Conceptualization, F.F.; methodology and investigation, F.F. and M.A.; writing—original draft preparation, F.F.; writing—review and editing, M.A. and P.L.; funding acquisition, P.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by PRIN 2015 (grant number 2015B7M39T_004) and by MIUR Progetto di Eccellenza [Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milan, Italy]. F.F. was supported by an AIRC fellowship for Italy.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Fitzmaurice, C.; Allen, C.; Barber, R.M.; Barregard, L.; Bhutta, Z.A.; Brenner, H.; Dicker, D.J.; Chimed-Orchir, O.; Dandona, R.; Dandona, L.; et al. Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-years for 32 Cancer Groups, 1990 to 2015. *JAMA Oncol.* **2017**, *3*, 524. [[PubMed](#)]
2. Fontana, F.; Marzagalli, M.; Montagnani Marelli, M.; Raimondi, M.; Moretti, R.; Limonta, P. Gonadotropin-Releasing Hormone Receptors in Prostate Cancer: Molecular Aspects and Biological Functions. *Int. J. Mol. Sci.* **2020**, *21*, 9511. [[CrossRef](#)]
3. Fontana, F.; Limonta, P. Dissecting the Hormonal Signaling Landscape in Castration-Resistant Prostate Cancer. *Cells* **2021**, *10*, 1133. [[CrossRef](#)] [[PubMed](#)]
4. Fontana, F.; Anselmi, M.; Limonta, P. Molecular mechanisms and genetic alterations in prostate cancer: From diagnosis to targeted therapy. *Cancer Lett.* **2022**, *534*, 215619. [[CrossRef](#)] [[PubMed](#)]
5. Liberti, M.V.; Locasale, J.W. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem. Sci.* **2016**, *41*, 211–218. [[CrossRef](#)] [[PubMed](#)]
6. Schwartz, L.; Supuran, C.T.; Alfarouk, K.O. The Warburg Effect and the Hallmarks of Cancer. *Anticancer Agents Med. Chem.* **2017**, *17*, 164–170. [[CrossRef](#)] [[PubMed](#)]
7. Wilson, J.E. Isozymes of mammalian hexokinase: Structure, subcellular localization and metabolic function. *J. Exp. Biol.* **2003**, *206*, 2049–2057. [[CrossRef](#)]
8. Mathupala, S.P.; Ko, Y.H.; Pedersen, P.L. Hexokinase II: Cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. *Oncogene* **2006**, *25*, 4777–4786. [[CrossRef](#)]
9. Wang, L.; Xiong, H.; Wu, F.; Zhang, Y.; Wang, J.; Zhao, L.; Guo, X.; Chang, L.J.; Zhang, Y.; You, M.J.; et al. Hexokinase 2-Mediated Warburg Effect Is Required for PTEN- and p53-Deficiency-Driven Prostate Cancer Growth. *Cell Rep.* **2014**, *8*, 1461–1474. [[CrossRef](#)]
10. Deng, Y.; Lu, J. Targeting hexokinase 2 in castration-resistant prostate cancer. *Mol. Cell. Oncol.* **2015**, *2*, e974465. [[CrossRef](#)]
11. Wang, L.; Wang, J.; Xiong, H.; Wu, F.; Lan, T.; Zhang, Y.; Guo, X.; Wang, H.; Saleem, M.; Jiang, C.; et al. Co-targeting hexokinase 2-mediated Warburg effect and ULK1-dependent autophagy suppresses tumor growth of PTEN- and TP53-deficiency-driven castration-resistant prostate cancer. *EBioMedicine* **2016**, *7*, 50–61. [[CrossRef](#)] [[PubMed](#)]
12. Fontana, F.; Raimondi, M.; Marzagalli, M.; Moretti, R.M.; Marelli, M.M.; Limonta, P. Tocotrienols and Cancer: From the State of the Art to Promising Novel Patents. *Recent Pat. Anticancer Drug Discov.* **2019**, *14*, 5–18. [[CrossRef](#)] [[PubMed](#)]
13. Montagnani Marelli, M.; Marzagalli, M.; Fontana, F.; Raimondi, M.; Moretti, R.M.; Limonta, P. Anticancer properties of tocotrienols: A review of cellular mechanisms and molecular targets. *J. Cell. Physiol.* **2019**, *234*, 1147–1164. [[CrossRef](#)] [[PubMed](#)]
14. Fontana, F.; Raimondi, M.; Di Domizio, A.; Moretti, R.M.; Montagnani Marelli, M.; Limonta, P. Unraveling the molecular mechanisms and the potential chemopreventive/therapeutic properties of natural compounds in melanoma. *Semin. Cancer Biol.* **2019**, *59*, 266–282. [[CrossRef](#)]
15. Fontana, F.; Raimondi, M.; Marzagalli, M.; Di Domizio, A.; Limonta, P. Natural Compounds in Prostate Cancer Prevention and Treatment: Mechanisms of Action and Molecular Targets. *Cells* **2020**, *9*, 460. [[CrossRef](#)]
16. Fontana, F.; Moretti, R.M.; Raimondi, M.; Marzagalli, M.; Beretta, G.; Procacci, P.; Sartori, P.; Montagnani Marelli, M.; Limonta, P. δ -Tocotrienol induces apoptosis, involving endoplasmic reticulum stress and autophagy, and paraptosis in prostate cancer cells. *Cell Prolif.* **2019**, *52*, e12576. [[CrossRef](#)]
17. Fontana, F.; Raimondi, M.; Marzagalli, M.; Audano, M.; Beretta, G.; Procacci, P.; Sartori, P.; Mitro, N.; Limonta, P. Mitochondrial functional and structural impairment is involved in the antitumor activity of δ -tocotrienol in prostate cancer cells. *Free Radic. Biol. Med.* **2020**, *160*, 376–390. [[CrossRef](#)]
18. Whitburn, J.; Edwards, C.M.; Sooriakumaran, P. Metformin and Prostate Cancer: A New Role for an Old Drug. *Curr. Urol. Rep.* **2017**, *18*, 46. [[CrossRef](#)]
19. He, K.; Hu, H.; Ye, S.; Wang, H.; Cui, R.; Yi, L. The effect of metformin therapy on incidence and prognosis in prostate cancer: A systematic review and meta-analysis. *Sci. Rep.* **2019**, *9*, 2218. [[CrossRef](#)]
20. Zaidi, S.; Gandhi, J.; Joshi, G.; Smith, N.L.; Khan, S.A. The anticancer potential of metformin on prostate cancer. *Prostate Cancer Prostatic Dis.* **2019**, *22*, 351–361. [[CrossRef](#)]
21. Kalyanaraman, B. Teaching the basics of cancer metabolism: Developing antitumor strategies by exploiting the differences between normal and cancer cell metabolism. *Redox Biol.* **2017**, *12*, 833–842. [[CrossRef](#)]

22. Hamanaka, R.B.; Chandel, N.S. Targeting glucose metabolism for cancer therapy. *J. Exp. Med.* **2012**, *209*, 211–215. [[CrossRef](#)]
23. Abdel-Wahab, A.F.; Mahmoud, W.; Al-Harizy, R.M. Targeting glucose metabolism to suppress cancer progression: Prospective of anti-glycolytic cancer therapy. *Pharmacol. Res.* **2019**, *150*, 104511. [[CrossRef](#)] [[PubMed](#)]
24. Marzagalli, M.; Moretti, R.M.; Messi, E.; Marelli, M.M.; Fontana, F.; Anastasia, A.; Bani, M.R.; Beretta, G.; Limonta, P. Targeting melanoma stem cells with the Vitamin E derivative δ -tocotrienol. *Sci. Rep.* **2018**, *8*, 587. [[CrossRef](#)]
25. Beretta, G.; Gelmini, F.; Fontana, F.; Moretti, R.M.; Montagnani Marelli, M.; Limonta, P. Semi-preparative HPLC purification of δ -tocotrienol (δ -T3) from *Elaeis guineensis* Jacq. and *Bixa orellana* L. and evaluation of its in vitro anticancer activity in human A375 melanoma cells. *Nat. Prod. Res.* **2018**, *32*, 1130–1135. [[CrossRef](#)] [[PubMed](#)]
26. Raimondi, M.; Fontana, F.; Marzagalli, M.; Audano, M.; Beretta, G.; Procacci, P.; Sartori, P.; Mitro, N.; Limonta, P. Ca^{2+} overload- and ROS-associated mitochondrial dysfunction contributes to δ -tocotrienol-mediated paraptosis in melanoma cells. *Apoptosis* **2021**, *26*, 277–292. [[CrossRef](#)]
27. Fontana, F.; Marzagalli, M.; Raimondi, M.; Zuco, V.; Zaffaroni, N.; Limonta, P. δ -Tocotrienol sensitizes and re-sensitizes ovarian cancer cells to cisplatin via induction of G1 phase cell cycle arrest and ROS/MAPK-mediated apoptosis. *Cell Prolif.* **2021**, *54*, e13111. [[CrossRef](#)] [[PubMed](#)]
28. He, Z.; Dong, W.; Yao, K.; Qin, C.; Duan, B. Daphnetin inhibits proliferation and glycolysis in colorectal cancer cells by regulating the PI3K/Akt signaling pathway. *RSC Adv.* **2018**, *8*, 34483–34490. [[CrossRef](#)]
29. Li, W.; Gao, F.; Ma, X.; Wang, R.; Dong, X.; Wang, W. Deguelin inhibits non-small cell lung cancer via down-regulating Hexokinases II-mediated glycolysis. *Oncotarget* **2017**, *8*, 32586–32599. [[CrossRef](#)]
30. Gao, X.; Han, H. Jolkinolide B inhibits glycolysis by downregulating hexokinase 2 expression through inactivating the Akt/mTOR pathway in non-small cell lung cancer cells. *J. Cell. Biochem.* **2018**, *119*, 4967–4974. [[CrossRef](#)]
31. Wu, J.; Zhang, X.; Wang, Y.; Sun, Q.; Chen, M.; Liu, S.; Zou, X. Licochalcone A suppresses hexokinase 2-mediated tumor glycolysis in gastric cancer via downregulation of the Akt signaling pathway. *Oncol. Rep.* **2018**, *39*, 1181–1190. [[CrossRef](#)] [[PubMed](#)]
32. Yao, J.; Liu, J.; Zhao, W. By blocking hexokinase-2 phosphorylation, limonin suppresses tumor glycolysis and induces cell apoptosis in hepatocellular carcinoma. *Oncotargets Ther.* **2018**, *11*, 3793–3803. [[CrossRef](#)]
33. Zhou, L.; Li, M.; Yu, X.; Gao, F.; Li, W. Repression of hexokinases II-mediated glycolysis contributes to piperlongumine-induced tumor suppression in non-small cell lung cancer cells. *Int. J. Biol. Sci.* **2019**, *15*, 826–837. [[CrossRef](#)] [[PubMed](#)]
34. Liu, W.; Yu, X.; Zhou, L.; Li, J.; Li, M.; Li, W.; Gao, F. Sinomenine inhibits non-small cell lung cancer via downregulation of hexokinases II-mediated aerobic glycolysis. *Oncotargets Ther.* **2020**, *13*, 3209–3221. [[CrossRef](#)] [[PubMed](#)]
35. Liu, W.; Li, W.; Liu, H.; Yu, X. Xanthohumol inhibits colorectal cancer cells via downregulation of Hexokinases II-mediated glycolysis. *Int. J. Biol. Sci.* **2019**, *15*, 2497–2508. [[CrossRef](#)] [[PubMed](#)]
36. Dronamraju, V.; Ibrahim, B.A.; Briski, K.P.; Sylvester, P.W. γ -Tocotrienol Suppression of the Warburg Effect Is Mediated by AMPK Activation in Human Breast Cancer Cells. *Nutr. Cancer* **2019**, *71*, 1214–1228. [[CrossRef](#)]
37. Luengo, A.; Gui, D.Y.; Vander Heiden, M.G. Targeting Metabolism for Cancer Therapy. *Cell Chem. Biol.* **2017**, *24*, 1161–1180. [[CrossRef](#)]
38. Vander Heiden, M.G. Targeting cancer metabolism: A therapeutic window opens. *Nat. Rev. Drug Discov.* **2011**, *10*, 671–684. [[CrossRef](#)]