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A new erythrocyte-based biochemical approach to predict the antiproliferative effects of heterocyclic scaffolds: The case of indolone

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ABSTRACT

Background: The indole core is a key structural feature of many natural products and biomolecules with broad spectrum chemotherapeutic properties. Some of us have recently synthesized a library of biologically promising indolone-based compounds. The present study focuses on the effects of one of them, namely DPIT, on human erythrocytes.
Methods: We have examined the influence of DPIT on band 3 protein, intracellular ATP concentration and transport, caspase 3 activation, metabolic adaptation and membrane stability.
Results: Our study elucidates that DPIT, intercalated into the phospholipid bilayer, decreases the anion transport, the intracellular ATP concentration and the cytosolic pH, inducing a direct activation of caspase 3.
Conclusions: Starting from the metabolic similarity between erythrocytes and cancer cells, we investigate how the metabolic derangements and membrane alterations induced by selected heterocycles could be related to the antiproliferative effects.
General significance: Our work aims to propose a new model of study to predict the antiproliferative effects of heterocyclic scaffolds, pointing out that only one of the listed conditions would be unfavorable to the life cycle of neoplastic cells.

1. Introduction

Indoledione derivatives are reported to exhibit pronounced biological effects, i.e. antiproliferative and tubulin polymerization inhibitory activities [1], antiproliferative effects in vascular smooth muscle cells resulting in the prevention of atherosclerosis [2,3], antioxidant properties [4], as well as antifungal [5], and antibacterial activities [6].

In the framework of our studies dealing with the design of polyfunctionalized heterocycles [7–14], we have recently reported the synthesis and the antiviral properties of an unprecedented class of indole-3,4-diones [15] and of their aldol-type derivatives [16] as promising lead compounds for antiproliferative agent development. We have hypothesized that these compounds exert an anti-proliferative effect on cellular growth involving cell cycle elements, such as cyclin-dependent kinases (CDKs), which downstream could inhibit the viral replication. In fact, our indoledione scaffold shares structural features with some cyclin-dependent kinase inhibitors (CDKIs) currently evaluated in different phases of preclinical and clinical trials for therapeutic use against cancer, neurodegenerative disorders, and viral infections [17]. Furthermore, our indolone-based molecular architecture, containing an Y-enaminodicarbonyl moiety able of undergoing prototropic changes, shows semipolarity and consequent good solubility in organic solvent as well as water. Therefore this novel class of compounds displays "drug like properties" including favorable physicochemical characteristics for potential in vivo therapeutic purposes. Altogether these findings encouraged us to further expand our studies on the biological usefulness of our indoledione derivatives, focusing on the most promising compound DPIT (Fig. 1A) as a "model compound" starting from which useful and more effective derivatives could be obtained by appropriate chemical modifications and decorations of the indolone scaffold. The present study is aimed at investigating the effects of DPIT on selected parameters of the human red blood cells (RBCs).

Among other cells the RBCs are potentially attractive because they possess unique features such as: they are easy to get, they have a long life in the circulatory system and they travel ~250 km through the cardiovascular system as natural carriers of oxygen. We propose another intriguing reason to further investigate the effect of DPIT on RBCs: their metabolic similarity with cancer cells. What characterizes cancer cells is their ability to acquire various adaptive characteristics. In fact, even in the presence of ample oxygen, cancer cells prefer to metabolize glucose by glycolysis (Embden–Meyerhof pathway — EMP), a seeming paradox as EMP is a less efficient pathway for producing ATP, but it is advantageous because it restricts oxygen-reactive species (ROS)

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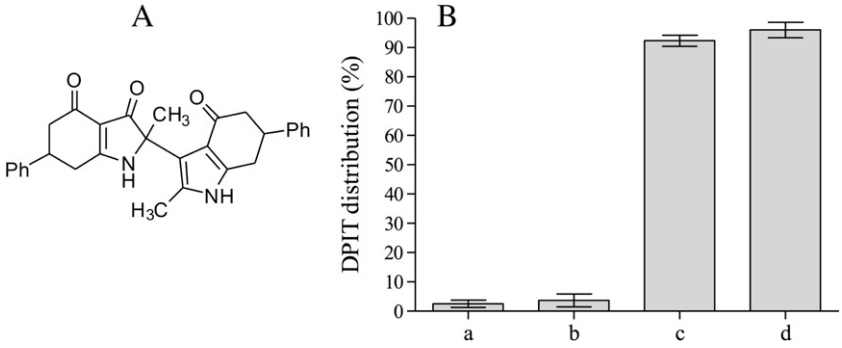

Fig. 1. (A) Chemical structure of 2,2′-dimethyl-6,6′-diphenyl-6,6′,7,7′-tetrahydro-1H,1′H-2,3′-biindole-3,4,4′(2H,5H,5′H)-trione (DPIT). (B) HPLC determination: DPIT distribution inside (a), outside (b) and in the RBC membrane (c), compared to the control (DPIT 100 μM standard solution, d).

generation compared to oxidative phosphorylation. Similarly, despite the high O_2 pressure which RBCs undergo during their life cycle, they having lost the mitochondria are forced to use only a part of the oxidative metabolism of glucose (anaerobic EMP). In fact, in RBCs, glucose 6 phosphate (G6P) is metabolized via two pathways: the EMP and the pentose phosphate pathway (PPP). Although the EMP generates ATP to drive ion pumps, NADH, and 2,3-diphosphoglycerate, the PPP is the only source of NADPH for the RBCs. G6P pathways are regulated by the oxygen pressure through the influence of hemoglobin (Hb) conformational changes on protein complex assembled on the cytoplasmic domain of the Band 3 membrane protein (cdB3). Specifically, competition exists between deoxyHb and key glycolytic enzymes (GE) for binding to cdB3. As the binding to cdB3 inactivates the GE, the EMP flux is monitored as a function of Hb oxygen saturation [18]. This oxygen-dependent interaction underlies a sophisticated regulatory mechanism of anion exchange and metabolism thereby playing a significant role in maintaining the structural and functional integrity and the redox state of the RBCs [19–24]. Within this framework it should be considered that caspase 3 activation induced by oxidative stress leads to the proteolytic cleavage of cdB3 and that this event cancels out the oxygen-dependent modulation of RBC metabolism.

2. Materials and methods

2.1. Reagents and compounds

All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA). Citrate fresh human blood was obtained from informed healthy donors who declared that they had abstained from all drug treatment for at least one week prior to sample collection, in accordance with the principles outlined in the Declaration of Helsinki. Concentrated stock solution of DPIT was prepared by dissolving the drug in dimethyl sulfoxide (DMSO). DPIT was synthesized as previously described [16].

2.2. Preparation of erythrocytes

Citrate blood samples were washed three times with an isosmotic NaCl solution and treated as previously reported [25].

2.3. Kinetic measurements

Cells were incubated in the incubation buffer at 25 °C, in the presence and absence of DPIT 100 μM and treated as previously reported [22].

2.4. HPLC determinations

Washed RBCs were incubated at 37 °C for 2 h with 100 μM DPIT in the above incubation buffer. Samples were washed and the packed cells were lysed with 10% ethanol. Lysates were centrifuged for 10 min at 4000 ×*g* (4 °C) and the supernatant was filtered with 0.45 mm filter. Free DPIT was analyzed by High Performance Liquid Chromatography (HPLC) with a Shimadzu system, consisting of an LC-10AD pump system and an SPDM10A diode array detector, a Rheodyne 7725i injector with a 20 ml sample loop and a reverse-phase Supelco C18 column (5 mm, 250 × 4.6 mm). The mobile phase consisted of a linear gradient of acetonitrile in H_2O as follows: 5–20% (0–2 min), 20–30% (2–4 min), 30–100% (4–7 min) and 100% (7–10 min). The flow rate was 1.0 ml/min at 25 °C. DPIT was detected at 286 nm and determined by comparison of peak areas with a standard solution of 100 μM DPIT. To establish the amount of DPIT in the membrane bilayer we induce erythrocyte hemolysis with hypotonic shock and centrifuge the samples at 12,000 rpm for 15 min at 4 °C. The supernatant was removed and the packed membrane structures were washed and centrifuged, as above described, three times with 0.15 M of NaCl solution to eliminate the unbounded compound. After the packed membranes were treated with DMSO for 2 h and analyzed by HPLC to identify and quantify DPIT.

2.5. Measurement of intra-extracellular ATP

ATP was measured by the luciferin–luciferase technique [26], with minor modifications as previously reported [27].

2.6. Flow cytometry

RBC suspensions after incubation for 2 and 24 h with or without DPIT were diluted to approximately 106 cells/ml and analyzed using a Becton–Dickinson, FACS CANTO II flow cytometer, with simultaneous separate detection at low angle (FSC) and right angle (SSC). The light scattered near the forward direction (low angle) is expected to be proportional to the size (volume) of the particle and is independent of the cell refractive index and shape, whereas scattering at the right angle depends on the cell shape and internal properties of the scattering particles [28]. FSC/SSC is a dual parameter contour plot histogram proportional to total cell diversity. FSC-A histograms represent the light scattered near the forward direction (proportional to the volume of the particles). Each measurement was done for 30,000 cells. Data were analyzed with DIVA Becton–Dickinson. Expression of CD59 was measured incubating RBCs (0.1% suspension in Hepes buffer) with 20 μl of anti-CD59 resuspended in FACS buffer.

2.7. Effects on superoxide anion generation

Superoxide anions were generated using the Nishimiki method [29], with minor modifications [30].

2.8. Caspase 3 assay

Citrate blood samples were washed three times with an isosmotic NaCl solution and treated as previously reported [30], using DPIT 50 μM and t-BHT 100 μM.

2.9. Lipid peroxidation assay

Isolated erythrocytes were incubated for 2 h in the absence or in the presence of DPIT (25, 50, 100 μM), t-BHT (100 μM) or in the presence of both compounds. After incubation, the samples were washed three times with 10 volumes of 0.9% NaCl and centrifuged at 2500 rpm for 5 min. During the last washing, the packed cells were resuspended in 30 volumes with ice-cold hypotonic medium containing 5 mM Tris and 5 mM KCl to yield hemolysate, and then centrifuged for 10 min at 12,000 rpm. This operation was repeated three times. Last, the hemolysate was resuspended in 0.9% of NaCl and used for lipid peroxidation assay by the thiobarbituric acid reactive substances (TBARS) methods [31].

2.10. Hydroxyl radical scavenging

Hydroxyl radical was assayed as described by Halliwell [32], with slight modifications. This assay is based on quantification of the degradation product of 2-deoxyribose by condensation with thiobarbituric acid (TBA). The reaction mixture contains 2.8 mM 2-deoxy-2-ribose, 10 mM phosphate buffer pH 7.4, 25 μM $FeCl_3$, 100 μM EDTA, 2.8 mM H_2O_2, 100 μM ascorbic acid and different concentrations of DPIT (0–100 μM) in a final volume of 1 ml. The sample was incubated for 1 h at 37 ± 0.5 °C in a water bath. Then 1.0 ml of 1% (w/v) TBA was added to each mixture followed by the addition of 1 ml of 2.8% (w/v) TCA. The solutions were heated in a water bath at 100 °C for 15 min to develop the pink colored malondialdehyde–thiobarbituric acid adduct. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed three times. Trolox was used as a positive control.

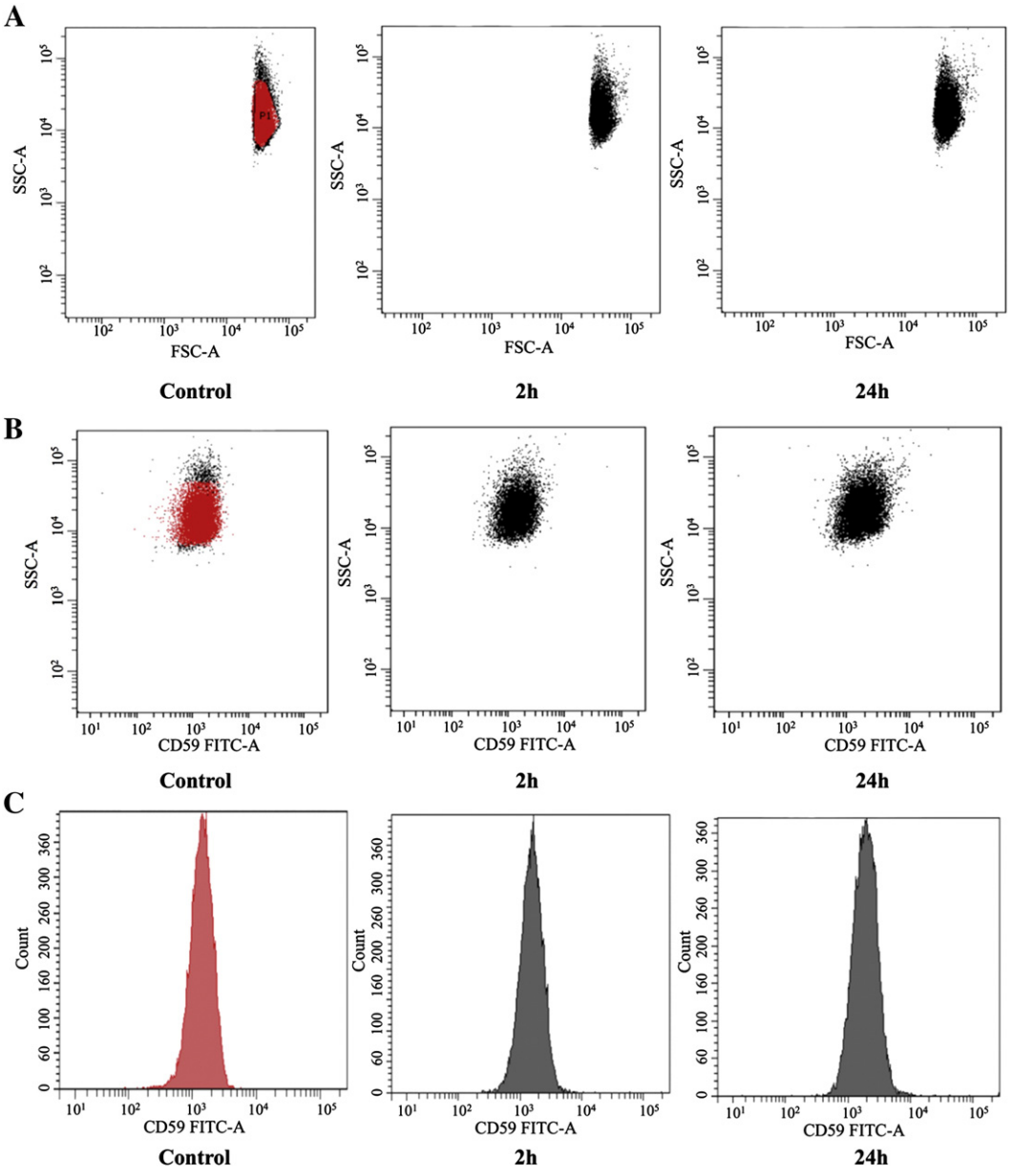

Fig. 2. Flow cytometry analysis of DPIT-induced changes in RBC morphology. Scattering diagrams of controls and RBCs incubated for 2 and 24 h with DPIT 100 μM (A), with DPIT 100 μM and anti-CD59 (B; C).

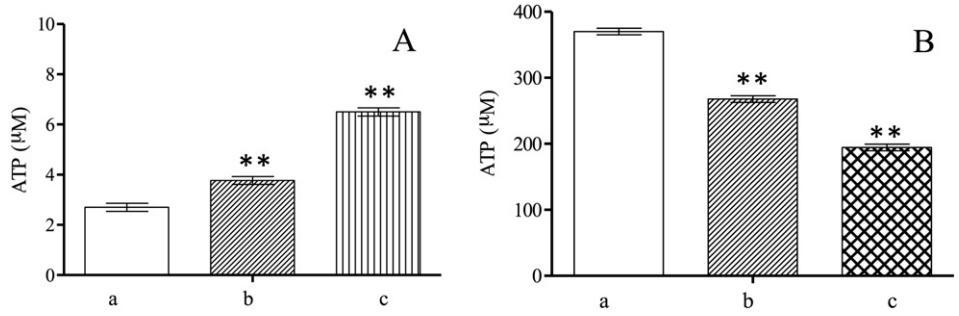

Fig. 3. Effects of DPIT and Mas 7 on extracellular (A) and intracellular (B) ATP levels in RBCs. [ATP] was measured at the end of the incubation time without (a) and with 100 μM DPIT (b), or 4 μM Mas 7 (c). Results are from four independent experiments ± standard deviation. Asterisks indicate significant differences at $P < 0.05$ vs. control.

The inhibition (%) was calculated using the following formula:

$$\text{Inhibition } (\%) = \frac{A_O - A_S}{A_O} \times 100.$$

2.11. Statistical analysis

Data are presented as mean of four different experiments ± standard deviation (SD). The data were analyzed by one-way analysis of variance. The significance of the differences in relation to the respective controls for each experimental test condition was calculated by Student's t-test for each paired experiment. A P value of <0.05 vs. control was regarded as significant difference and indicated with asterisks in the figures.

3. Results and discussion

3.1. The influence of DPIT on cell membrane

In order to investigate the influence of DPIT on RBCs, cells incubated with the drug (100 μM) for 2 h at 37 °C were analyzed by HPLC (Fig. 1B), evidencing that DPIT strongly partitions into the bilayer. The ratio analysis of the DPIT concentrations in and out the RBCs, with the first one obtained after extraction of DPIT from the membrane, indicated that the drug is almost completely intercalated in the phospholipid bilayer, likely because of the semipolarity of our indolone scaffold [15,16].

Literature data [33,34] have recently reported that compounds bearing functional groups suitable for the anchorage on the membrane proteins of RBCs are able to influence the functionality and metabolism of erythrocytes, by interaction and binding on the protein domains. In this context, we can assume that DPIT, containing an Y-enaminodicarbonyl moiety able of undergoing prototropic changes, could expose appropriate anchoring groups, i.e. hydroxyl groups, for the attachment to RBC membrane components, such as band 3 protein (B3) and other cytoskeletal proteins.

To better analyze the influence of DPIT on membrane stability, the morphological changes of RBCs induced by the drug were investigated by both flow cytometry analysis and microscopic observations. RBCs pre-incubated in the absence and in the presence of DPIT (100 μM) for 2 and 24 h did not reveal significant changes in morphology and size (Fig. 2A). These results are in perfect accordance with microscopic observations of RBCs exposed to DPIT in the same conditions (data not shown). The structural integrity of the membrane was further tested through the expression of the CD59, one of the major complement protective proteins in RBC membranes (CD59 expression $1–4 \times 10^4$ copies per cell) anchored by glycosyl-phosphatidyl-inositol on the cell surface. The expression of CD59 is maintained after 2 and 24 h of RBC incubation with DPIT (Fig. 2B–C), supporting the integrity of the membrane.

To highlight any early alterations of the membrane, the influence of DPIT on the activity of acetyl cholinesterase (AChE), a known marker of cell membrane integrity and functionality [35], was analyzed at different concentrations (0–100 μM), resulting that the drug did not significantly modify the enzyme functionality (data not shown). The results on the integrity of the membrane obtained up to now are in good agreement with the hemolysis percentage calculated in the presence of DPIT (100 μM) at different incubation times (2, 12, 24 h). DPIT induced only a weak influence in the RBC hemolysis even lower than the DMSO (data not shown).

Although DPIT does not cross the RBC membrane resulting intercalated in the phospholipid bilayer, its presence destabilizes very much the RBC functionality and metabolism. The first easily deductible effect of the drug is the alteration of inter-membrane exchanges, such as the ATP release and the B3 ion channel functionality.

To find evidences related to mechanical stimulation induced by the drug on the RBCs, its influence on ATP release from erythrocytes was evaluated comparing the effect to Mastoparan 7 (Mas 7), a direct activator of the heterotrimeric G protein. The results reported in Fig. 3A clearly indicate that RBCs pretreated with DPIT released significantly more ATP than RBCs in normal conditions. The increased release of ATP from RBCs in the presence of DPIT seems to affect the intracellular ATP levels, as shown in Fig. 3B. In fact, RBCs pretreated with DPIT (100 μM) evidenced a decreased intracellular [ATP] with respect to the control and Mas 7.

Furthermore, since B3 is one of the most representative proteins of the RBC membrane, the effect of the drug on the B3 anion transporter was tested, to assess whether its intra-membrane localization could somehow affect trade between inside and outside of the lipid bilayer. DPIT remarkably influences B3 anion flux (Fig. 4), decreasing the rate constant from 0.012 to 0.0066 min^{-1} (~50%) for the erythrocytes incubated in the absence and in the presence of the drug (100 μM), respectively.

The direct and strong slowing down of the anion transporter affects the functionality of both the Na^+/H^+ antiporter and the transporter of monocarboxylic acids, the latter greatly contributing to the cytosolic acidification [36].

The strong decreased anion transport leads the cell to an abnormal detoxification of CO_2, while the strong release of ATP from the RBCs affects the intracellular concentration of nucleotide. Both transports are, however, closely related to normal cellular activity and life, since the lack of a fast removal of CO_2 and the decrease in the transport of

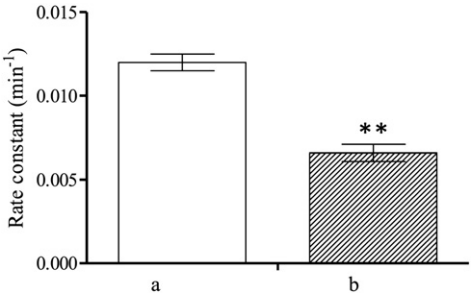

Fig. 4. B3 anion flux measurements. Rates of sulfate transport in RBCs without (a) and with DPIT 100 μM (b). Results are from four independent experiments ± standard deviation. Asterisks indicate significant differences at $P < 0.05$ vs. control.

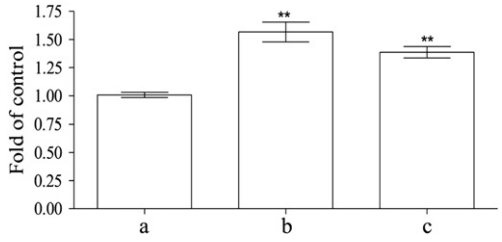

Fig. 5. Caspase 3 activity in RBCs in the absence (a) and in the presence of DPIT 50 μM (b) or t-BHT 100 μM (c). Results are from four independent experiments ± standard deviation. Asterisks indicate significant differences at $P < 0.05$ vs. control.

peroxynitrite $ONOO^-$ may lead to the generation of harmful secondary radicals (carbonate and nitrogen dioxide) and this situation is further exacerbated by the [ATP] intra/extra-cellular values [37]. Under normal conditions, the increased ATP release into the lumen of the vessel is a positive event because stimulating the purinergic receptors promotes the release of NO, vasodilatation and thus the peripheral circulation [38]. In this case, however, ATP is released from the RBCs at the expense of intracellular energy which significantly decreases in the presence of the drug. The low intracellular [ATP] leads to a series of harmful consequences for the cell:

- hyper-activation of EMP (ATP is a negative modulator of EMP) at the expense of NADPH required for the protection against oxidative stress [39];
- severe slowdown of Na^+/K^+-ATPase, H^+-ATPase and Na^+/H^+ pump functionality [40,41] which promotes the cytosolic acidification and destabilizes the normal ion exchange, as confirmed by the observed imbalance of B3 anion flux; and
- increase of intracellular [Ca^{2+}] which in turn causes caspase 3 activation, essential step of the apoptotic process promoted by the cytosolic acidification [42,43].

3.2. The influence of DPIT on caspase 3 activity

To confirm the above, the caspase 3 activity has been measured by incubating RBCs in the absence and in the presence of DPIT 50 μM or tert-butyl-hydroperoxide (t-BHT) 100 μM, a well known oxidant agent [44]. The analysis suggests that DPIT induces a remarkable activation of caspase 3, superior to t-BHT (Fig. 5), even if DPIT does not enter into the RBCs.

The strong enzyme activation, cleaving cdB3, depletes the cell of its oxygen-dependent modulation [25,30]. In fact, cdB3 is the main target of the competitive binding of both deoxygenated Hb and GE. CdB3 cleavage, avoiding this competition, leads to the loss of one of the major modulators of EMP, then this pathway is hyper-activated at the expense of the PPP. This metabolic situation is further aggravated by the low intracellular [ATP] which contributes to hyper-activation of the EMP. Under these conditions, RBCs are deprived of their main defense against oxidative stress, such as the production of NADPH, required for the antioxidant activity of glutathione peroxidase and met-hemoglobin reductase.

3.3. The influence of DPIT on RBC oxidative state

To verify whether the observed caspase 3 activation could be somehow caused by the ROS increase, the effects of DPIT (0–100 μM) on superoxide anion generation were analyzed. The results in Fig. 6A show that DPIT (100 μM) caused an increased generation of superoxide radical of ~1.5 fold with respect to the control. Furthermore, superoxide radical is a potential generating of H_2O_2 which in the presence of Fe^{2+} may produce the harmful hydroxyl radical. In this context, the capacity of DPIT to chelate ferrous ions was tested, but the drug did not show this ability (data not shown). Based on these experimental evidences we further investigated the influences of increasing DPIT concentrations (0–100 μM) on hydroxyl radical elimination (Fig. 6B). The radical was generated with the Fenton reaction in the presence of DPIT. Curiously, the drug shows a double behavior, at low concentrations (up to 40 μM) it acts as a pro-oxidant, increasing the damage due to the generation of hydroxyl radical, while at high concentrations it decreases the damage, albeit in low degree. This double nature has been already described for compounds able to interact with radical species and in many circumstances it is strictly dependent to the tested concentrations [45].

3.4. The influence of DPIT on RBC metabolism

Both the cellular imbalance of the ions and alteration of metabolic modulation (Fig. 7) would orient the RBCs to significant changes of intracellular pH (pH_i), characterized by an acidification of the RBC cytoplasm, in agreement with literature data [36,43,46–49]. It is known that Cl^-/HCO_3^- exchanger activity occurs in conjugation with another transport system, the Na^+/H^+ antiporter which is degraded by caspase 3 [48]. Reduced Na^+/H^+ antiporter activity would acidify cells.

Dysregulated pH is recently emerging as a hallmark of cancer [50]. In particular, cancer cells have a reversed pH gradient compared with normal cells, including a constitutively higher pH_i and a lower extracellular pH (pH_e). In this context, DPIT that we have recently proposed as an antiproliferative agent by CDK inhibition [16], promoting acidosis, could severely restrict the ability of cancer cells to extrude lactic acid and carbonic acid. Such a strategy would block the export of metabolic acids and thus would poison cancer cells with their own acid. Furthermore, this approach would also decrease metastatic formation and sensitize the cells to hyperthermia therapy [51].

In addition, the location of the drug in the phospholipid bilayer, together with the stability of the membrane and the absence of lipid peroxidation, suggests a local scavenger function of DPIT against ROS and make the cell not suitable to necrosis, but instead adapt to the apoptotic process (favored by the low pH_i) via caspase activation induced by DPIT. There is currently a great interest in enhancing mitochondrial-dependent apoptosis as a therapeutic strategy to limit cancer progression and it is well known that the high pH_i in cancer cells confers resistance to apoptosis [43].

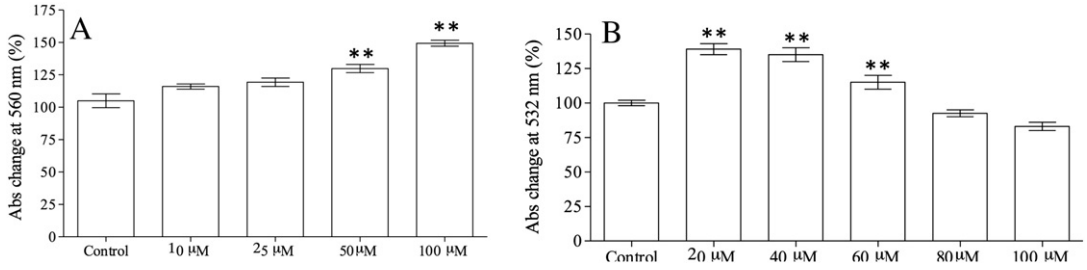

Fig. 6. Effects of DPIT (0–100 μM) on superoxide anion radical generation (A) and on deoxyribose degradation assay (B). Results are from four independent experiments ± standard deviation. Asterisks indicate significant differences at $P < 0.05$ vs. control.

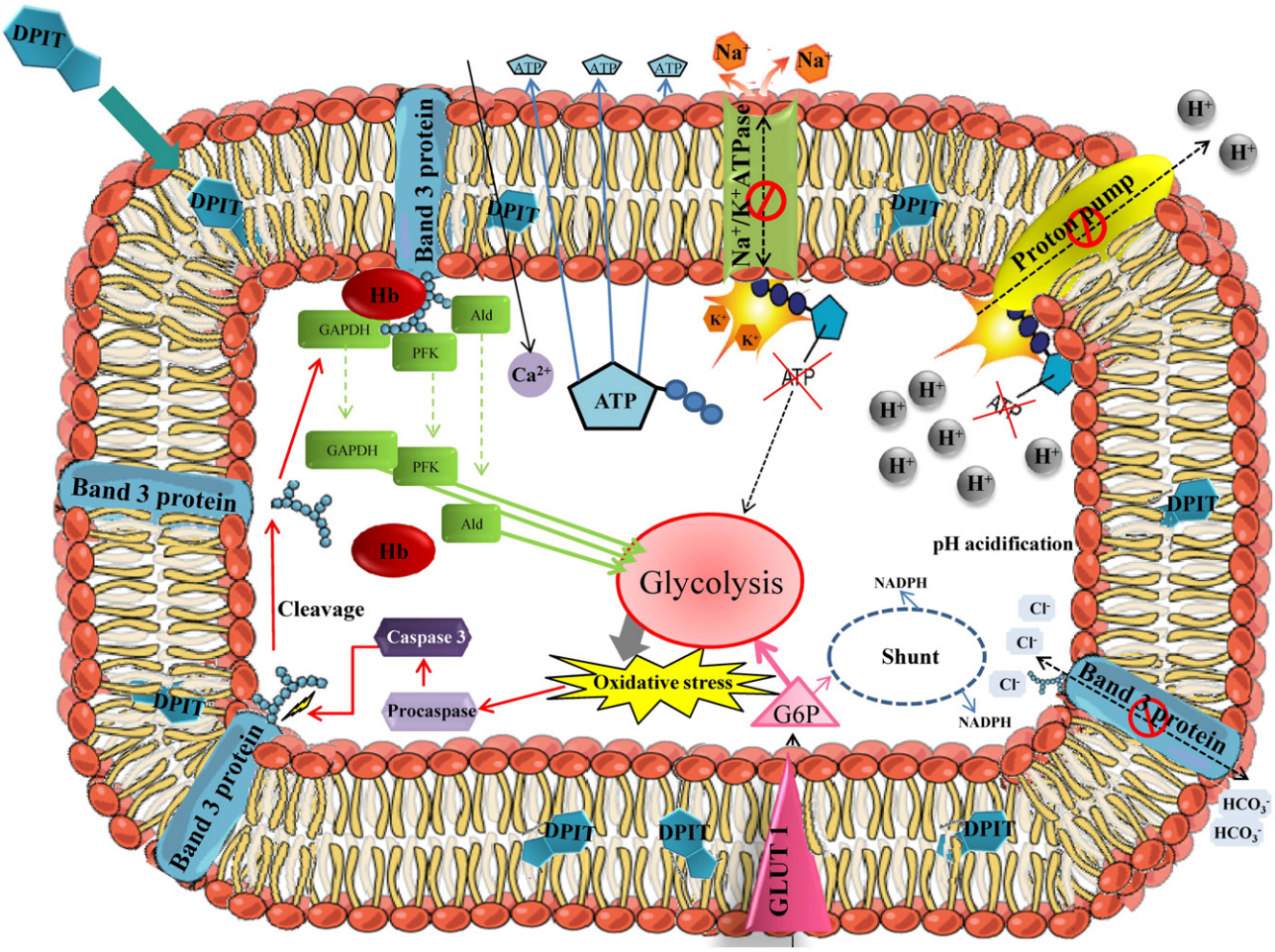

Fig. 7. Summary of the effects of DPIT on RBCs.

Moreover, the strong caspase 3 activation induced by DPIT in a cytochrome c-independent way observed in RBCs may from the one hand strengthen the apoptosis and on the other hand anticipate its action in the cancer cells in which the electron transport chain is strongly suppressed.

Last but not least important, both the increased H^+ concentration and availability of fructose 1,6 bisphosphate (due to the glycolysis enhancement) induced by DPIT represent two conditions positively modulating the pyruvate kinase M2 isoform (PKM2) expressed in cancer cells with low activity. DPIT could therefore be a suitable candidate for a positive therapeutic response by increasing the catalytic efficiency of PKM2, as suggested by an elegant study of Anastasiou [52].

4. Conclusions

In conclusion, by a singular parallelism between RBCs and cancer cells, our study elucidates how the indolone scaffold is able to induce metabolic derangements in human erythrocytes, pointing out that only one of the listed conditions would be certainly unfavorable to the life cycle of neoplastic cells. Thus DPIT could be considered as a very promising molecular architecture that may be exploited to design novel and more effective antiproliferative agents.

Furthermore, our results confirm that a new model of study based on the use of isolated human erythrocytes can be successfully applied to predict the antiproliferative effects of selected heterocyclic scaffolds, opening new perspectives in anticancer drug discovery.

Acknowledgements

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