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Design, synthesis and biological characterization of novel mitochondria targeted dichloroacetate-loaded compounds with antileukemic activity

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ABSTRACT

The mitochondrial kinase inhibitor dichloroacetate (DCA) has recently received attention in oncology due to its ability to target glycolysis. However, DCA molecule exhibits poor bioavailability and cellular uptake with limited ability to reach its target mitochondria. To overcome these biases, we have synthesized novel DCA-loaded compounds. The selection of the most promising therapeutic molecule was evaluated by combining *in vitro* assays, to test the anti-tumoral potential on leukemic cells, and a preliminary characterization of the molecule stability *in vivo*, in mice. Among the newly synthetized compounds, we have selected the multiple DCA-loaded compound **10**, characterized by a tertiary amine scaffold because it exhibited enhanced (>30 fold) *in vitro* anti-tumor activity with respect to DCA and increased *in vivo* stability. Based on these results we believe that compound **10** should be considered for further preclinical evaluations for the treatment of cancers and/or other diseases characterized by altered metabolic origin.

INTRODUCTION

Dichloroacetic acid and in particular dichloroacetate (DCA) is an inhibitor of mitochondrial pyruvate dehydrogenase kinase (PDK) that down-regulates the activity of pyruvate dehydrogenase (PDH) controlling the switch between oxidative phosphorylation and glycolysis. Beside the use for the chronic treatment of lactic acidosis,[1] in more recent years DCA has gained increased attention for the treatment of cancers for its ability to target altered metabolism that makes cancer cells resistant to death by normal apoptotic processes.[2-5] In this field, several works have demonstrated DCA activity against tumour cells of different origin in preclinical settings, using mono or combinational treatments.[6,7] The data reported in literature addressed the activity of DCA against solid tumors, in particular human breast- and colorectal- carcinoma and neuroblastoma,[8-10] and against haematological neoplasia,[11-13] suggesting DCA as a promising molecule to halt tumour development and/or, when used in combination, to overcome resistance to conventional chemo/radiotherapy.[14,15]

Nevertheless, there are major issues associated to clinical use of DCA for cancer treatment which are principally related to its low stability in the biological environment and to the low permeability of the cellular membrane to DCA molecule, that translates into high dosages required to reach therapeutic efficacy and clinical results.[6,7,15,16] As a matter of fact, DCA is unable to cross the cell membrane through passive diffusion due to its anionic charge. Moreover, the classical mitochondrial pyruvate carrier (MPC)[17] doesn't work for dissociated acid but only for the carboxylic form and, in addition, the plasma protein SLC5A8, that can mediate DCA entry into cells, is down-regulated in

several cancer types.[18] Based on these reasons, there is increasing interest for the develop of new strategies aimed to engineer DCA analogues able to cross the cellular membranes, thus facilitating the cellular uptake and entry of DCA into mitochondria of cancer cells, characterized by a $\Delta\psi m$ higher than normal cells.

To overcome the drawbacks (e.g. problems of solubility, cellular uptake, biological availability and stability) of DCA and/or the DCA-analogues recently described,[19] which hamper the translation of this molecule to clinic, in this work we envisioned the design and synthesis of novel compounds backboned with ammonium salts and/or tertiary amine scaffolds. The ability of these scaffolds to carry DCA into mitochondrial matrix, and thus to access to the PDK protein, has been assessed *in vitro* on leukemic cell models as well as in *in vivo* settings, with the final intent to select a new molecule with enhanced anti-tumoral efficacy and improved potential clinical applications with respect to primitive DCA.

RESULTS

Design and synthesis of the mitochondria backbone carriers

Different strategies have been previously applied for the design and synthesis of mitochondria targeting molecules, such as phosphonium salt,[20] guanidine moiety[21,22] or polyamine structures.[23] In this work, beside the phosphonium salt,[19] we adopted a classical ammonium salt and tertiary amine with the aim to generate soluble, non-cytotoxic and stable carriers specifically designed for the targeted delivery of DCA to the mitochondria. As depicted in **Scheme 1**, commercially available 6-bromo-hexanoic acid **1** was reacted with triphenylphosphine in acetonitrile at reflux to obtain the corresponding phosphonium salt, compound **2**. The latter was reacted with 2-amino-2-(hydroxymethyl)propane-1,3-diol **3** in presence of EEDQ in ethanol at 50°C to yield amide compound **4** in 60% yield. Compound **4** was acetylated using acetyl chloride (AcCl) in DCM to obtain the first targeting mitochondria compound **5** in quantitative yield. The second targeting mitochondria carrier was obtained by protection of primary amine functions of Tris(2-aminoethyl)amine compound **6** with di-*tert*-butyl carbonate in a mixture of water, dioxane and sodium hydroxide to obtain the fully protected derivative compound **7** in quantitative yield. Compound **7** was methylated in acetonitrile using methyl iodide as an acylating agent to obtain the third targeting compound **8** in quantitative yield.

As first comparative analysis, we tested the cytotoxicity of the backbone carriers, compounds **5**, **7** and **8**, by using a panel of leukemic cell line cultures (JVM-2, MAVER, MEC-1, MEC-2 and HL-60) exposed to the different compounds, used in a range of

concentration from 3 to 300 μM. As shown in **Figure 1**, after 48 hours of treatment, while a significant reduction in cell viability was observed with the carrier **5** starting at the concentration of 30 μM, no significant toxicity was observed for both carriers **7** and **8** at any of the concentrations assessed. Similar results were observed in all the leukemic cell lines employed.

Conjugation of carriers with dichloroacetate molecules

Once validated the chemical strategy to obtain the carriers moieties, the backbone compounds were conjugated with DCA to obtain the corresponding DCA analogues. For this purpose, as represented in **Scheme 2**, dichloro acetyl chloride was reacted with compound **4** in DCM at room temperature to obtain the corresponding acylated product **9** in high yield (92%) and purity (>95%) in a single step, corresponding to the Mito-DCA molecule.[19] In parallel, compound **10** was obtained by reacting compound **6** with dichloro acetyl chloride in heterogeneous phase in a mixture of DCM and NaOH 2N solution to obtain the conjugated compound **10** (20% yield). The same procedure was adopted for the synthesis of the corresponding tertiary amine derivative compound starting from compound **8** after its deprotection with TFA in DCM, leading to the conjugated compound **11** in 56% of yield. Of note, the relative easy synthesis of conjugated molecules allowed multi-gram scale preparative for the *in vitro* and *in vivo* experiments.

For the next biological characterization of the DCA-loaded compounds **9**, **10** and **11**, we have used as controls the corresponding backbone carriers **5**, **7** and **8** respectively. The choice for the backbone molecules were made taking in account not only the possible

metabolic products coming from the DCA-compounds, but also considering the physicochemical properties of the carriers (id, cell permeability).

Biological validation of the DCA-loaded compounds

Because the aim of our study was to develop new DCA-analogues for anti-tumor applications, we next assessed the activity of the novel mitochondria targeted DCA-loaded compounds, in comparison with DCA, in *in vitro* cell models. On the bases of recent evidences of the anti-leukemic activity of DCA,[12,13] we analyzed the biologic activity of the new compounds using a panel of leukemic cell lines as well as primary cell cultured derived from B-CLL patients. For this purpose, the different cell cultures were exposed to compounds **9**, **10** and **11** used in the range of concentrations of 10 to 300 μM and cell viability was assessed at 24 and 48 hours of treatment. The analysis of cell viability in response to DCA-loaded compounds was assessed with respect to the corresponding carriers (**5**, **7** and **8**). We found of potential interest the compounds **10** and **11** because of the lack of cell toxicity of their backbones (compounds **7** and **8**, respectively; **Figure 1**), which allows to discriminate the specific action of the DCA-loaded molecules (**Supplementary Figure 1**). Anyhow, while no significant effects on cell viability were observed when the cell cultures were treated with the compound **11** (**Supplementary Figure 1**), we observed a dose-dependent cytotoxicity when cell cultures were treated with the compound **10 (Figure 2A)**. In particular, the IC_{50} values for compound **10** are reported in **Table 1** and document an anti-leukemic activity on all cell lines, irrespectively of the p53 status, which was validated also on B-CLL patients' cell cultures. On the other hand, PBMC obtained from healthy blood donors were less

susceptible to compound **10**, with IC_{50} mean values approximately 3 folds higher (1.26±0.19), suggesting that normal PBMC were substantially resistant to this molecule in the range of concentrations effective against leukemic cells. Of particular relevance, the comparative analysis of the anti-leukemic activity revealed that the compound **10** was able to exhibit the anti-leukemic effect of DCA at significantly lower concentrations (> 1 log; **Figure 2A**), as indicated also by the IC_{50} values (**Table 1** and **Supplementary Figure 2**), both acting through the same mechanisms. Indeed, in-depth analysis of the cytotoxicity induced by compound **10** confirmed that its effect on cell viability was the cumulative result of induction of cell cycle arrest (**Figure 2B**) and induction of apoptosis (**Figure 2C-D**), coupled to induction of the p21 molecular mediator (**Figure 2E**), exhibiting results comparable to those induced by treatment with DCA used at 30-100 fold higher concentrations than compound **10**.

In parallel, the new synthetized compounds were assessed for their ability to deliver DCA to the mitochondria by using the most advanced assay, based on the XF technology, which is able to measure the oxygen consumption rate (OCR) as an indicator of mitochondrial respiratory capacity (**Figure 3** and **Supplementary Figure 3**). Results showed that a pre-treatment with the mitochondria targeted DCA-loaded compound **10** (**Figure 3**), but not **11** (**Supplementary Figure 3**), significantly reduced the basal respiration (OCR values before oligomycin addition) as well as the maximal respiration (OCR values after FCCP) of the leukemic cells, while the coupling efficiency was less affected (not reaching statistical significance) as well as the spare respiratory capacity, indicating a decrease of oxidative phosphorylation levels with a preservation of a residual mitochondrial activity. These results are in line with recent observations indicating that

CLL cells are characterized by high oxidative phosphorilation (due to intrinsic oxidative stress) and that targeting the mitochondrial respiratory chain in these cells brakes the redox balance and thereby induces cell death. [24]

Finally, it is important to underline that, in all the *in vitro* assays (**Figure 2** and **Figure 3**) the cell cultures treated with the compound **7** were substantially indistinguishable from the untreated cultures, confirming that the backbone carrier **7** did not account for any of the biological-molecular effects observed upon treatment with the DCA-loaded compound **10**.

***In vivo* analysis of compound 10 stability**

Having observed that compound **10** exhibits anti-leukemic activity *in vitro*, at significant lower concentrations with respect to DCA, we next preliminarily evaluated its potential bio-availability and stability *in vivo* in mice. For this purpose, a single dose of compound **10** (57 mg/kg) was given by subcutaneous inoculation (s.c.) and blood was collected at different time-points after injection. Plasma samples were then analyzed for the presence of the compound **10** with Agilent 6520 LC-MS instrument equipped with nano-HPLC Chip cube separative system. The unambiguously identification of the compound **10** was possible by the comparison between the calculated exact mass (that is absolutely typical due to the presence of 6 chlorine atom) with the found value, leading to the classification of a specific molecule profile (**Figure 4**). Results showed that compound **10** was present in the mice blood circulation for more than five hours after injection (**Figure 4**; **Supporting information S1-S3**) and at the same time no metabolites

were found, proving that the amide bond between the carrier and the dichloroacetic moiety was stable.

DISCUSSION AND CONCLUSION

Different carrier moieties, such as phosphonium salts, polyamine and guanidine derivatives, have been proposed for targeting different molecules, such as ROS inhibitors or anticancer agents, into the mitochondria.[20,25] In the present study, in order to enhance the potential of DCA for clinical applications, such as to target the tumor metabolic alterations,[6,7,15,16] we explored the ability of a tertiary amine scaffold and the corresponding quaternary ammonium salt to carry DCA. As a first step, the results of assays performed *in vitro* on different cell models revealed that these scaffolds had significant lower cell toxicity compared with the corresponding phosphonium salts, as well as a good solubility in physiological medium. On this base, the amine scaffolds were used for the synthesis of compounds **10** and **11**, delivering multiple DCA molecules, with the aim to reach relevant therapeutic concentrations of the drug into tumor cells. In fact, in analogy with other molecules bringing positively charged atoms, such as guanidine and polyamine,[10-25] the compounds **10** and **11** were expected to pass more efficiently than the DCA molecule the inner mitochondrial membrane (IMM), because of the great negative membrane potential ($\Delta\Psi m$) that exists across the IMM, and thus to reach the PDK target in the mitochondrial matrix.

The biological validation of the new synthesized DCA-loaded compounds **10** and **11** was performed assessing their anti-tumour efficiency that revealed a complete different behaviour between the two DCA-compounds. Of interest, the multiple DCA-loaded compound **10** exhibited *in vitro* anti-tumor activity at significant lower (>30 fold) concentrations compared to DCA, when tested both on a panel of leukemic cell lines and

on B-CLL patient derived cell cultures. Of interest, similar results were obtained also on cell models of human solid tumors of epithelial origin (**Supplementary Table 1**). On the opposite, the quaternary salt compound **11** was completely inactive in affecting cell viability in all cell lines employed. Although this result was quite unexpected, due to the strong mitochondrial affinity for positively charged molecules, one possible explanation could be related to the high hydrophilic behaviour of compound **11** that limits the cell membrane crossing.

The *in vitro* characterization of the anti-leukemic activity of compound **10**, supported by its low toxicity on human healthy primary cells, was integrated by a preliminary assessment of its *in vivo* stability in mouse models. The data obtained from mice injected subcutaneously with a relative low dose (single injection of 57 mg/kg) of drug indicated that compound **10** entered the blood circulation from the subcutaneous compartment without being degraded. In fact, HPLC-MS analysis of murine plasma suggests that the molecule was quite stable in the blood for a relatively long period of time (5.5 hours), if compared with the rapid metabolism of DCA described in literature.[26,27] Moreover the lack of detection of metabolites in plasma samples at each time point assessed, suggested that the dichloroacetyl moiety carried by a neutral tertiary amine scaffold was more stable and long lasting when exposed to the biological environment in *in vivo* experiments, compared to dichloroacetyl anion.[26,27]

Considering the increased interest for DCA as a potential metabolic targeting therapy for cancer,[3-7] we believe that our strategy design for DCA-carrying molecules deserves further pre-clinical characterization since it could provide future advancements

for the treatment of cancer, as well as for other diseases (such as proliferative vascular diseases)[28] characterized by an altered metabolic origin.

EXPERIMENTAL SECTION

Chemistry general information

All the NMR spectra were elaborated using Mestre Nova 6.0.2 software and FID data are available on request. Analytical thin layer chromatography (TLC) was performed on silica gel Macherey-Nagel poligram SIL/UV 254 of 0.25 mm, visualization was achieved using UV light (254) and potassium permanganate ($KMnO_4$) 2% in water. Flash column chromatography was undertaken on silica gel Merck 60-200 mesh using Isolera Biotage (Sweden). Products were dried using sodium sulfate anhydrous (Carlo Erba). Proton nuclear magnetic resonance (^{1}H NMR) and carbon nuclear magnetic resonance (^{13}C NMR) were recorded using VARIAN 400 MHz. All spectra were recorded using as solvent $CDCl_3$ otherwise the solvent was specified. Chemical shifts (δ) were quoted in ppm relative to residual solvent and coupling constants (J) were quoted in Hertz (Hz). Multiplicity was reported with the following abbreviations: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet, bs = broad signal. Molecular weights were measured with a mass spectrometer ESI MICROMASS ZMD 2000 (Waters, UK) and high resolution spectra with an Agilent ESI-Q-TOF LC/MS 6520 System (Agilent Technologies, USA). Solvents and chemicals used for TLC, chromatographic purification, crystallizations and reactions were reported with the following abbreviations: Et_2O for diethyl ether, THF for tetrahydrofuran, AcOEt for ethyl acetate, DCM for methylene chloride, CH_3CN for acetonitrile, TFA for trifluoro acetic acid.

Synthesis of (5-carboxypentyl)triphenylphosphonium bromide (2)

In a round bottom flask, 6-bromohexanoic acid (4000 mg, 20.50 mmol) and triphenylphosphine (5600 mg, 21.49 mmol) were solved in 80mL of acetonitrile and heated at reflux for 24 hours. The title compound was obtained in quantitative yield as white sticky oil. MS (ESI) of compound **1**: $[M+H]^+$ = 377.32

Synthesis of (6-((1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl)amino)-6-oxohexyl)triphenylphosphonium bromide (4)

Compound **2** (1000 mg, 2.18 mmol), 2-amino-2-(hydroxymethyl)propane-1,3-diol (292 mg, 2.40 mmol) and N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (650 mg, 2.62 mmol) were solved in 50mL of EtOH and heated at 50 °C for 12 hours. The reaction mixture was concentrated in vacuo, dissolved in 30mL of AcOEt and washed twice with 20mL of brine. The organic phase was dried to yield compound **4** as sticky yellow oil. MS (ESI) of compound **4**: $[M+H]^+$ = 480.45. ^{1}H-NMR (400 MHz, Chloroform-d), δ 8.92, (bs, 1H, N*H*), 7.82-7.38 (15 H*, CHar*), 3.72-3.65 (m, 9H,-CH_2-*OH*), 3.51-3.44 (m, 2H, CH_2-CONH*),* 2.37 (t, 2H, J=7.2Hz, CH_2-CH_2-CH_2-C=O), 1.65-1.79, (m, 6H, -CH_2-CH_2-CH_2). ^{13}C NMR (101 MHz, CDCl3) δ 175.75, 150.52, 136.22, 135.37, 133.70, 133.60, 130.79, 130.67, 129.59, 127.92, 126.67, 121.20, 117.68, 64.13, 62.46, 36.42, 29.46, 25.05, 23.10, 22.60, 21.89, 18.55.

Synthesis of (6-((1,3-diacetoxy-2-(acetoxymethyl)propan-2-yl)amino)-6-oxohexyl)triphenylphosphonium bromide (5)

To a stirred solution of compound **4** (460 mg, 0.82 mmol) dissolved in 50mL of

DCM, 0.192 mL of acetyl chloride (212 mg, 2.71 mmol) were added, the reaction mixture was allowed to stay at room temperature for 4h. After this time the reaction was completed (checked by ESI mass spectrometry), the DCM vas evaporated in vacuum to yield compound **5** in quantitative yield. ^{1}H-NMR (400 MHz, Chloroform-d), δ 8.92, (bs, 1H, N*H*), 8.20-8.12 (m, 2H*, CHar*), 7.85-7.41 (m, 13H, *CHar*), 4.44 (s, 6H, *CH*$_2$-OAc*),* 2.34 (m, 2H, CH$_2$-*C*H$_2$-C*H*$_2$-C=O), 2.05 (s, 9H, O-C=O-*CH*$_3$) 1.69, (m, 6H, -*CH*$_2$-*CH*$_2$-*CH*$_2$). ^{13}C NMR (101 MHz, CDCl3) δ 174.32, 170.79, 150.25, 136.59, 135.19, 130.69, 130.64, 129.81, 129.28, 127.95, 126.83, 121.22, 118.84, 117.99, 66.18, 62.66, 57.71, 36.49, 29.72, 29.56, 24.69, 22.93, 22.43, 21.89.

Synthesis of (6-((1,3-bis(2,2-dichloroacetoxy)-2-((2,dichloroacetoxy)methyl)propan-2-yl)amino)-6-oxohexyl)triphenylphosphonium (9)

A stirred solution of compound **4** and 2,2-dichloroacetyl chloride (627 mg, 4.25 mmol, 410 µl) in DCM (30mL) was reacted at room temperature for 2 hours. The reaction mixture was checked by ESI-mass spectrometry and when the product was observed the solvent was removed under vacuum. The title compound was obtained as white off oil with 92% yield. HRMS (ESI) of compound **9**: $[M+H]^+$ = 812.028640. ^{1}H-NMR (400 MHz, Chloroform-d), δ 7.72-7.80 (m, 15 H*, CHar*), 5.90 (s, 3H, C*H*-Cl$_2$), 4.45 (m, 2H, C*H*$_2$-PPh$_3$), 3.86 (m, 4H, -*CH*$_2$-*CH*$_2$-*CH*$_2$), 3.44 (bs, 2H, C*H*$_2$-CONH*),* 2.43 (m, 2H, -*C<u>H</u>*$_2$-*CO*), 1.64(6H, -*CH*$_2$-*CH*$_2$-*CH*$_2$). ^{13}C-NMR (400 MHz, Chloroform-d), δ: 177.7, 174.0, 166.0, 146.4, 141.1, 138.1, 135.0, 133.6, 130.7, 129.8, 128.7, 121.5, 118.4, 117.9, 66.4, 64.3, 62.1, 61.5, 59.7, 50.3, 34.1, 29.5, 23.9, 22.7, 22.2.

Synthesis of tri-tert-butyl (nitrilotris(ethane-2,1-diyl))tricarbamate (7)

In a round-bottom flask the amine (1g, 6.83 mmol, 1.02 ml) was solved in a mixture of water and dioxane (10mL/20mL). NaOH (2.5g, 61.47 mmol) and $(Boc)_2O$ were added at 0 C° and the solution was stirred overnight at room temperature. The reaction mixture was extracted with 40mL of AcOEt, dried over sodium sulphate anhydrous, filtered and the solvent was removed under vacuum. The title compound **7** was obtained as white solid in a quantitative yield. MS (ESI) of compound **7**: $[M+H]^+$ = 447.36.

Synthesis of 2-((tert-butoxycarbonyl)amino)-N,N-bis((2-((tert-butoxycarbonyl)amino)ehtyl)-N-methylthanaminium iodide (8)

To a stirred solution of compound **7** (1.8 g, 4.21 mmol) dissolved in 50mL of CH_3CN, methyl iodide (33.69 mmol, 2.1 mL) was added and the reaction mixture was heated at 60 C° for 24 hours. After one day, the reaction mixture was concentrated in vacuo, dissolved in 50 mL of AcOEt and washed twice with water (20mL). The organic layers were dryed to achieve a yellow solid salt in a quantitative yield. m.p. = 140-145 °C. MS (ESI) of compound **8**: $[M+H]^+$ = 461.63. ^{1}H-NMR (400 MHz, Chloroform-d), δ: 3.56 (s, 12H, CH_2-CH_2), 3.27 (s, 3H, N-CH_3), 1.44 (m, 27H, C(CH_3)$_3$). ^{13}C-NMR (400 MHz, chloroform-d), δ: 160.56, 83.51, 64.75, 52.95, 38.79, 37.91, 31.39.

Synthesis of N,N',N''-(nitrilotris(ethane-2,1-diyl))tris(2,2-dichloroacetamide) (10)

Schotten–Baumann reaction involves the amine (500 mg, 3.42 mmol, 512.29 µl) solved in DCM (10 ml), and NaOH 2N solution (5.13 ml) to have an heterogeneous

system by adding 2,2-dichloroacetyl chloride (1515 mg, 10.26 mmol, 990 µl) at room temperature overnight. The reaction mixture was washed with water (10mL), and the organic layers were dried, filtered and concentrated under vacuum. The crude was purified by flash chromatography (AcOEt/ Petroleum ether 5:1) to give the title compound **10** with 20 % yield and used for the tests as hydrochloric salt. m.p. = 134-137 °C. HRMS (ESI) of compound **10**: $[M+H]^+$ = 476.95839. ^{1}H-NMR (400 MHz, Methanol-d), δ: 6.34 (s, 3H, C*H*-Cl_2) 3.31 (t, 6H, J=6.2Hz, N-CH_2-CH_2), 2.67 (t, 6H, J=6.2Hz, N-CH_2-CH_2). ^{13}C-NMR (400 MHz, Methanol-d), δ: 39.50, 54.39, 67.72, 166.97.

Synthesis of 2-(2,2-dichloroacetamido)-N,N-bis(2-(2,2-dichloroacetamido)ethyl)-N-methylethanaminium iodide (11)

Compound **8** (1,8 g, 6.5 mmol), earlier treated with 10 mL of TFA in 50 mL of DCM to remove Boc protecting group, was solved in DCM (40mL), NaOH 2N solution (20mL) and 2,2-dichloroacetyl chloride (39 mmol, 3 ml) were added. The reaction mixture worked overnight at room temperature, after this time, the reaction mixture was extracted in AcOEt (50mL) and the product was purified by flash chromatography (AcOEt) to give a white solid in 50% yield. HRMS (ESI) of compound **11**: $[M+H]^+$ = 490.974115. ^{1}H-NMR (400 MHz, Methanol-d), δ: 6.34 (s, 3H, C*H*Cl_2), 3.81-3.78 (t, 6H, J=6.2Hz, -N-CH_2-CH_2), 3.61-3.59 (t, 6H, J=6.2Hz, -N-CH_2-CH_2), 3.28 (s, 3H, N-CH_3). ^{13}C-NMR (400 MHz, Methanol-d), δ: 167.45, 60.85, 34.75.

Further details on compound characterization (spectra analysis and purity grade ≥95% for each compound) are present in **Supporting Information S4-S23**.

Cell cultures and treatments

Primary peripheral blood samples were collected in heparin-coated tubes from B-CLL patients (n=8) and healthy blood donors (n=4) after informed consent provided for research purposes, in accordance with the Declaration of Helsinki and in agreement with institutional guidelines (University-Hospital of Ferrara). All patients had been without prior therapy at least for three weeks before blood collection. Peripheral blood mononuclear cells (PBMC) were isolated as previously described.[29] The $p53^{wild\text{-}type}$ JVM-2, the $p53^{mutated}$ MAVER, MEC-1, MEC-2 and the $p53^{null}$ HL-60 leukemic cell lines were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Primary cells were cultured in RPMI-1640 medium containing 10% FBS, L-glutamine and Penicillin/streptomycin (all from Gibco, Grand Island, NY). The leukemic JVM-2, MAVER and HL-60 cell lines were routinely cultured in RPMI-1640, whereas MEC-1 and MEC-2 cell lines were maintained in IMDM, all supplemented with 10% FBS, L-glutamine and Penicillin/streptomycin (all from Gibco).

For the *in vitro* assays, leukemic cells and normal PBMC were seeded at a density of $1x10^6$ cells/mL and treated with DCA (Sigma-Aldrich, St Louis, MO; used in the range of 1-30 mM) and DCA-loaded compounds and carries (used in the range of 3-300 μM).

Assessment of cell viability, apoptosis and cell cycle profile

At different time points after treatments, cell viability was evaluated both by Trypan blue dye exclusion and MTT (3-(4,5-dimethilthiazol- 2yl)-2,5-diphenyl

tetrazolium bromide) colorimetric assay (Roche Diagnostics Corporation, Indianapolis, IN), as previously reported.[30] In order to investigate the concentration required to induce death in 50% of cells relative to control, IC_{50} values were calculated after 48 hours of culture for DCA (range of 0.1-30 mM) and compound **10** (range of 3-1000 μM). The cell cycle profile was analyzed by flow-cytometry after 5-bromodeoxyuridine (BrdU) incorporation as previously described.[31] Levels of apoptosis were quantified by Annexin V-FITC/PI staining (Immunotech, Marseille, France) and analyzed by using a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA).[32]

Analysis of p21 expression

Total RNA was extracted from cells using the QIAGEN RNeasy Plus mini kit (Qiagen, Hilden, Germany) accordingly to the supplier's instructions and as previously described.[33] Total RNA was transcribed into cDNA and amplified using the Express One-Step Superscript qRT-PCR Kit (Invitrogen, Carlsbad, CA). Analysis of *CDKN1A* (p21) gene expression was carried out with validated TaqMan Gene Expression Assays specific PCR primers sets (Invitrogen). Expression values were normalized to the housekeeping gene *POLR2A* amplified in the same sample.[12] For p21 protein analysis, cells were lysed and processed for Western blot, as previously described,[34] by using the anti-p21 (C-19) antibody, purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Bioenergetics assays

Bioenergetics assays were performed by using the XF^{e}96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA) and the Seahorse XF96 cell Mito

Stress Test Kit (Seahorse Bioscience). After treatments, leukemic cells were seeded in specific tissue culture plates, previously coated with Poly-L-lysine solution (Sigma-Aldrich), in the optimized concentration of $3x10^5$ cells per well. One hour before measurement, cells were incubated at 37°C in a CO_2-free atmosphere. Different parameters of respiration, i.e. basal respiration, coupling efficiency and spare respiratory capacity, were analyzed by using a XF96 mito stress assay kit. For this purpose, after establishing a baseline, basal oxygen consumption rate (OCR), a measure of mitochondrial respiration, was first detected before sequentially injecting the following mitochondrial inhibitors: the ATP synthase inhibitor oligomycin (1.0 μM), the uncoupling agent FCCP (Carbonyl cyanide-ptrifluoromethoxyphenylhydrazone) (1.0 μM) and the combination of Complex III inhibitor antimycin A (1.0 μM) and the Complex I inhibitor rotenone (1.0 μM). OCR values were automatically calculated by the Seahorse XF96 software. All experiments were performed at least in hexaplicates.

Experiments in mice and HPLC-MS analysis of plasma

Female BALB/c mice (8 weeks old) were purchased from Charles River Laboratories (Hollister, CA). All the experimental procedures were approved by the institutional animal ethical care committee (CEASA) of the University of Ferrara and by the Italian Ministry of Health. Upon arrival, mice were acclimated for one week before starting the study. Mice were housed under pathogen-free conditions in vented cabinet, exposed to a regular light–dark cycle of 12 hour each; and food and water were available ad-libitum, in compliance with the guidelines for the care and use of laboratory animals. Mice (n = 12) were administered via subcutaneous (s.c.) injection with a single dose of

compound **10** solution (57 mg/kg in 200 μL PBS/25% DMSO). Blood samples were collected from the tail vein into micro-vials containing 2 μL of heparinized saline (Epsoclar 25.000 U.I./5 ml, Hospira), at different time points (1, 2.5, 4, 5.5, 7, 16 and 24 hours) after s.c. injection.[35] Plasma samples were obtained after centrifugation of blood samples at 10.000 rpm for 10 min at room temperature and stored immediately at -80°C.

Analysis of plasma samples was performed using Agilent 6520 LC-MS instrument equipped with nano-HPLC Chip cube separative system. For each sample, 100 μL of plasma were diluted with 300 μL of acetonitrile/water/TFA (60%/40%/0.1%) solution and then passed on a 0.22 μm regenerated cellulose filter before the LC-MS injection. The HPLC-MS analysis was performed in a linear gradient from 100% of solvent A (97% water, 3% acetonitrile and 0.1% formic acid) to 100% of solvent B (97% acetonitrile, 3% water and 0.1% formic acid) in 15 minutes using a Zorbax 300SB C-18 (43mm X 75μm) separation column.

Statistical analysis

Descriptive statistics were calculated. For each set of experiments, values were reported as means ± standard deviation (SD). The results were evaluated by using analysis of variance with subsequent comparisons by Student's t-test and with the Mann-Whitney rank-sum test. Statistical significance was defined as $p<0.05$. All statistical analyses were performed with SPSS Statistic 20 software (SPSS Inc., Chicago, IL).

ASSOCIATED CONTENT

Supporting information

Spectra of compounds and HPLC-MS analysis of mouse plasma. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

B-CLL, B-cell chronic lymphocytic leukemia; BrdU, 5-bromodeoxyuridine; $(Boc)_2O$, Di-tert-butyl-dicarbonate; DCA, dichloroacetate; DCM, dichloro methane; EEDQ, N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; FCCP, carbonyl cyanide-ptrifluoromethoxyphenylhydrazone; FITC, fluorescein isothiocyanate; IC_{50}, half maximal inhibitory concentration; IMDM, iscove's modified dulbecco's media; IMM, inner mitochondrial membrane; LC-MS, liquid chromatography–mass spectrometry; MPC, mitochondrial pyruvate carrier; MTT, 3-(4,5-dimethilthiazol- 2yl)-2,5-diphenyl tetrazolium bromide; OCR, oxygen consumption rate; PBMC, peripheral mononuclear cells; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PI, propidium iodide; RPMI, roswell park memorial institute; SLC5A8, solute carrier family 5 member 8; TFA, trifluoro acetic acid; TLC, Thin-Layer Chromatoraphy; $\Delta\psi m$, mitochondrial membrane potential.

REFERENCES

1. Abdelmalak, M.; Lew, A.; Ramezani, R.; Shroads, A.L.; Coats, B.S.; Langaee, T.; Shankar, M.N.; Neiberger, R.E.; Subramony, S.H.; Stacpoole, P.W. Long-term safety of dichloroacetate in congenital lactic acidosis. *Mol. Genet. Metab.* **2013**, *109*, 139-143.

2. Christofk, H.R.; Vander Heiden, M.G.; Harris, M.H.; Ramanathan, A.; Gerszten, R.E.; Wei, R.; Fleming, M.D.; Schreiber, S.L.; Cantley, L.C. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature*. **2008**, *452*, :230-233.

3. Lu, J.; Tan, M.; Cai, Q. The Warburg effect in tumor progression: mitochondrial oxidative metabolism as an anti-metastasis mechanism. *Cancer Lett.* **2015**, *356*, 156-164.

4. Elf, S.E.; Chen, J. Targeting glucose metabolism in patients with cancer. *Cancer.* **2014**, *120*, 774-780.

5. Bonnet, S.; Archer, S.L.; Allalunis-Turner, J.; Haromy, A.; Beaulieu, C.; Thompson, R.; Lee, C.T.; Lopaschuk, G.D.; Puttagunta, L.; Bonnet, S.; Harry, G.; Hashimoto, K.; Porter, C.J.; Andrade, M.A.; Thebaud, B.; Michelakis, E.D. A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. *Cancer Cell.* **2007**, *11*, 37-51.

6. Bhat, T.A.; Kumar, S.; Chaudhary, A.K.; Yadav, N.; Chandra, D. Restoration of mitochondria function as a target for cancer therapy. *Drug Discov. Today.* **2015**, *20*, 635-643.

7. Michelakis, E.D.; Webster, L.; Mackey, J.R. Dichloroacetate (DCA) as a potential metabolic-targeting therapy for cancer. *Br. J. Cancer*. **2008**, *99*, 989-94.

8. Robey, I.F.; Martin, N.K. Bicarbonate and dichloroacetate: evaluating pH altering therapies in a mouse model for metastatic breast cancer. *BMC Cancer*. **2011**, *11*, 235.

9. Lin, G.; Hill, D.K.; Andrejeva, G.; Boult, J.K.; Troy, H.; Fong, A.C.; Orton, M.R.; Panek, R.; Parkes, H.G.; Jafar, M.; Koh, D.M.; Robinson, S.P.; Judson, I.R.; Griffiths, J.R.; Leach, M.O.; Eykyn, T.R.; Chung, Y.L. Dichloroacetate induces autophagy in colorectal cancer cells and tumours. *Br. J. Cancer*. **2014**, *111*, 375-385.

10. Niewisch, M.R.; Kuçi, Z.; Wolburg, H.; Sautter, M.; Krampen, L.; Deubzer, B.; Handgretinger, R.; Bruchelt, G. Influence of dichloroacetate (DCA) on lactate production and oxygen consumption in neuroblastoma cells: is DCA a suitable drug for neuroblastoma therapy? *Cell. Physiol. Biochem.* **2012**, *29*, 373-380.

11. Fujiwara, S.; Kawano, Y.; Yuki, H.; Okuno, Y.; Nosaka, K.; Mitsuya, H.; Hata, H. PDK1 inhibition is a novel therapeutic target in multiple myeloma. *Br. J. Cancer.* **2013**, *108*, 170-178.

12. Agnoletto, C.; Melloni, E.; Casciano, F.; Rigolin, G.M.; Rimondi, E.; Celeghini, C.; Brunelli, L.; Cuneo, A.; Secchiero, P.; Zauli, G. Sodium dichloroacetate exhibits anti-leukemic activity in B-chronic lymphocytic leukemia (B-CLL) and synergizes with the p53 activator Nutlin-3. *Oncotarget*. **2014**, *5*, 4347-4360.

13. Agnoletto, C.; Brunelli, L.; Melloni, E.; Pastorelli, R.; Casciano, F.; Rimondi, E.; Rigolin, G.M.; Cuneo, A.; Secchiero, P.; Zauli, G. The anti-leukemic activity of sodium dichloroacetate in p53mutated/null cells is mediated by a p53-independent ILF3/p21 pathway. *Oncotarget*. **2015**, *6*, 2385-2396.

14. Kankotia, S.; Stacpoole, P.W. Dichloroacetate and cancer: new home for an orphan drug? *Biochim. Biophys. Acta*. **2014**, *1846*, 617-629.

15. Garon, E.B.; Christofk, H.R.; Hosmer, W.; Britten, C.D.; Bahng, A.; Crabtree, M.J.; Hong, C.S.; Kamranpour, N.; Pitts, S.; Kabbinavar, F.; Patel, C.; von Euw, E.; Black, A.; Michelakis, E.D.; Dubinett, S.M.; Slamon, D.J. Dichloroacetate should be considered with platinum-based chemotherapy in hypoxic tumors rather than as a single agent in advanced non-small cell lung cancer. *J. Cancer Res. Clin. Oncol*. **2014**, *140*, 443-452.

16. Dunbar, E.M.; Coats, B.S.; Shroads, A.L.; Langaee, T.; Lew, A.; Forder, J.R.; Shuster, J.J.; Wagner, D.A.; Stacpoole, P.W. Phase 1 trial of dichloroacetate (DCA) in adults with recurrent malignant brain tumors. *Invest. New Drugs*. **2014**, *32*, 452-464.

17. Jackson, V.N.; Halestrap, A.P. The kinetics, substrate, and inhibitor specificity of the monocarboxylate (lactate) transporter of rat liver cells determined using the fluorescent intracellular pH indicator, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein. *J. Biol. Chem.* **1996**, *271*, 861-868.

18. Babu, E.; Ramachandran, S.; CoothanKandaswamy, V.; Elangovan, S.; Prasad, P.D. Ganapathy, V.; Thangaraju, M. Role of SLC5A8, a plasma membrane transporter and a tumor suppressor, in the antitumor activity of dichloroacetate. *Oncogene*. **2011**, *30*, 4026-4037.

19. Pathak, R.K.; Marrache, S.; Harn, D.A.; Dhar, S. Mito-DCA: a mitochondria targeted molecular scaffold for efficacious delivery of metabolic modulator dichloroacetate. *ACS Chem. Biol*. **2014**, *9*, 1178-1187.

20. Biasutto, L.; Mattarei, A.; Marotta, E.; Bradaschia, A.; Sassi, N.; Garbisa, S.; Zoratti, M.; Paradisi, C. Development of mitochondria-targeted derivatives of resveratrol. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5594-5597.

21. Zatelli, M.C.; Gagliano, T.; Pelà, M.; Bianco, S.; Bertolasi, V.; Tagliati, F.; Guerrini, R.; degli Uberti, E.; Salvadori, S.; Trapella, C. N-carbamidoyl-4-((3-ethyl-2,4,4-trimethylcyclohexyl)methyl)benzamide enhances staurosporine cytotoxic effects likely inhibiting the protective action of Magmas toward cell apoptosis. *J. Med. Chem.* **2014**, *57*, 4606-4614.

22. Jubinsky, P.T; Short, M.K.; Ghanem, M.; Das, B.C. Design, synthesis, and biological activity of novel Magmas inhibitors. *Bioorg. Med. Chem. Lett*. **2011**, *21*, 3479-3482.

23. Simoni, E.; Bergamini, C.; Fato, R.; Tarozzi, A.; Bains, S.; Motterlini, R.; Cavalli, A.; Bolognesi, M.L.; Minarini, A.; Hrelia, P.; Lenaz, G.; Rosini, M.; Melchiorre, C. Polyamine conjugation of curcumin analogues toward the discovery of mitochondria-directed neuroprotective agents. *J. Med. Chem.* **2010**, *53*, 7264-7268.

24. Jitschin, R.; Hofmann, A.D.; Bruns, H.; Giessl, A.; Bricks, J.; Berger, J.; Saul, D.; Eckart, M.J.; Mackensen, A.; Mougiakakos, D. Mitochondrial metabolism contributes to oxidative stress and reveals therapeutic targets in chronic lymphocytic leukemia. *Blood*. **2014** , *123*, 2663-2672.

25. Biasutto, L.; Dong, L.F.; Zoratti, M.; Neuzil, J. Mitochondrially targeted anti-cancer agents. *Mitochondrion.* **2010**, *10*, 670-681.

26. Saghir, S.A.; Schultz, I.R. Low-dose pharmacokinetics and oral bioavailability of dichloroacetate in naive and GST-zeta-depleted rats. *Environ. Health Perspect.* **2002,** *110*, 757-763.

27. Schultz, I.R.; Shangraw, R.E. Effect of short-term drinking water exposure to dichloroacetate on its pharmacokinetics and oral bioavailability in human volunteers: a stable isotope study. *Toxicol. Sci*. **2006**, *92*, 42-50.

28. Deuse, T.; Hua, X.; Wang, D.; Maegdefessel, L.; Heeren, J.; Scheja, L.; Bolaños, J.P.; Rakovic, A.; Spin, J.M.; Stubbendorff, M.; Ikeno, F.; Länger, F.; Zeller, T.; Schulte-Uentrop, L.; Stoehr, A.; Itagaki, R.; Haddad, F.; Eschenhagen, T.; Blankenberg, S.; Kiefmann, R.; Reichenspurner, H.; Velden, J.; Klein, C.; Yeung, A.; Robbins, R.C.; Tsao, P.S.; Schrepfer, S. Dichloroacetate prevents restenosis in preclinical animal models of vessel injury. *Nature*. **2014**, *509*, 641-644.

29. Zauli, G.; Celeghini, C.; Melloni, E.; Voltan, R.; Ongari, M.; Tiribelli, M.; di Iasio, M.G.; Lanza, F.; Secchiero, P. The sorafenib plus nutlin-3 combination promotes synergistic cytotoxicity in acute myeloid leukemic cells irrespectively of FLT3 and p53 status. *Haematologica.* **2012**, *97*, 1722-1730.

30. Voltan, R.; di Iasio, M.G.; Bosco, R.; Valeri, N.; Pekarski, Y.; Tiribelli, M.; Secchiero, P.; Zauli, G. Nutlin-3 downregulates the expression of the oncogene TCL1 in primary B chronic lymphocytic leukemic cells. *Clin. Cancer Res.* **2011**, *17*, 5649-5655.

31. Celeghini, C.; Voltan, R.; Rimondi, E.; Gattei, V.; Zauli, G. Perifosine selectively induces cell cycle block and modulates retinoblastoma and E2F1 protein levels in p53 mutated leukemic cell lines. *Invest. New Drugs.* **2011**, *29*, 392-395

32. Secchiero, P.; Melloni, E.; di Iasio, M.G.; Tiribelli, M.; Rimondi, E.; Corallini, F.; Gattei, V.; Zauli, G. Nutlin-3 up-regulates the expression of Notch1 in both myeloid and lymphoid leukemic cells, as part of a negative feedback antiapoptotic mechanism. *Blood.* **2009,** *113*, 4300-4308.

33. Secchiero, P.; Rimondi, E.; di Iasio, M.G.; Agnoletto, C.; Melloni, E.; Volpi, I.; Zauli, G. C-Reactive protein downregulates TRAIL expression in human peripheral monocytes via an Egr-1-dependent pathway. *Clin. Cancer Res.* **2013**, *19*, 1949-1959.

34. Zauli, G.; Voltan, R.; Bosco, R.; Melloni, E.; Marmiroli, S.; Rigolin, G.M.; Cuneo, A.; Secchiero, P. Dasatinib plus Nutlin-3 shows synergistic antileukemic activity in both p53 wild-type and p53 mutated B chronic lymphocytic leukemias by inhibiting the Akt pathway. *Clin. Cancer Res.* **2011**, *17*, 762-770.

35. Tisato, V.; Garrovo, C.; Biffi, S.; Petrera, F.; Voltan, R.; Casciano, F.; Meroni, G.; Agnoletto, C.; Zauli, G.; Secchiero, P. Intranasal administration of recombinant TRAIL down-regulates CXCL-1/KC in an ovalbumin-induced airway inflammation murine model. *PLoS One*. **2014**, *9*, e115387.

Table 1. IC_{50} values for DCA and compound **10** in leukemic cell lines and in primary B-CLL patient derived cells

	IC_{50} (mM)	
Cells	*DCA*	*compound **10***
Leukemic cell lines:		
JVM-2	11.47±1.07	0.41±0.03
MAVER	11.83±0.96	0.55±0.06
MEC-1	8.29±0.70	0.40±0.02
MEC-2	12.19±1.11	0.37±0.03
HL-60	13.87±1.29	0.34±0.02
Primary B-CLL cells:		
B-CLL Pt.#1	29.28±2.75	0.40±0.04
B-CLL Pt.#2	25.65±1.98	0.27±0.02
B-CLL Pt.#3	4.92±0.35	0.14±0.01
B-CLL Pt.#4	1.54±0.09	0.12±0.01
B-CLL Pt.#5	13.34±0.99	0.53±0.03
B-CLL Pt.#6	3.02±0.26	0.34±0.02
B-CLL Pt.#7	3.77±0.29	0.61±0.04
B-CLL Pt.#8	47.70±3.63	0.56±0.06

Values are mean±standard deviation calculated after 48 hours of culture treatment of experiments carried out at least in triplicate for each cell culture.

FIGURE LEGENDS

Figure 1. Assessment of the potential cytotoxicity of backbone carriers. Different leukemic cell lines (n=5) were exposed at growing concentrations (range: 3-300 μM) for 48 hour to the indicated scaffold molecules before analysis of cell viability. Cell viability was calculated as percentage with respect to untreated cultures set to 100%. Data are reported as the means±SD of results from three independent experiments performed in all cell lines. The asterisk indicates $p<0.05$ with respect to the untreated cultures.

Figure 2. Characterization of the anti-leukemic effect of the DCA-loaded compound 10. In **A**, a panel of leukemic cell lines and of B-CLL patient cell samples (as reported in Table 1) were exposed to serial doses of compound **10** (10-300 μM) or DCA (1-10 mM). Cell viability was analyzed after 48 hours of treatment and calculated as percentage with respect to untreated cultures set to 100%. Data are graphed as scatter plots of at least six independent experiments, for leukemic cell lines and B-CLL samples respectively. In **B-E**, leukemic cells were treated for 24 or 48 hours with 300 μM of either compound **10** or control scaffold compound **7**. In most experiments, scaffold-treated cultures are not shown since they were highly comparable to the untreated cultures. For comparison, in some experiments, results obtained by treating the same cultures with a 100-fold higher dose of DCA (30mM) are also shown. In **B**, cell distribution in the different phases of cell cycle was calculated from the flow-cytometry dot plots after BrdU/PI staining and expressed as percentage of the total population. In the right panel, representative cell cycle profiles of cultures, either left untreated or treated with the indicated compounds,

analyzed by flow-cytometry are shown. For each cytofluorimetric analysis, the rectangles represent the cells in G0/G1, S, G2/M phases of the cell cycle. In **C**, the percentage of leukemic apoptotic cells was determined by flow-cytometry after Annexin V/PI staining. In the right panel, a representative flow-cytometry analysis of apoptosis is shown. In **D**, induction of apoptosis was calculated as percentage of Annexin V/PI cells in leukemic cells from a primary B-CLL sample. In the right panel, a representative flow-cytometry analysis of apoptosis is shown. In **E**, levels of p21 mRNA were analyzed in primary B-CLL samples by quantitative RT-PCR and data are expressed as fold of modulation with respect to the control untreated cultures set at 1. A representative western blot result documenting the induction of p21 protein in a B-CLL patient sample is shown in the insert. Data are reported as the means±SD of results from at least three independent experiments. In **C** and **D**, the asterisk indicates $p<0.05$ with respect to the untreated or to compound **7** treated cultures.

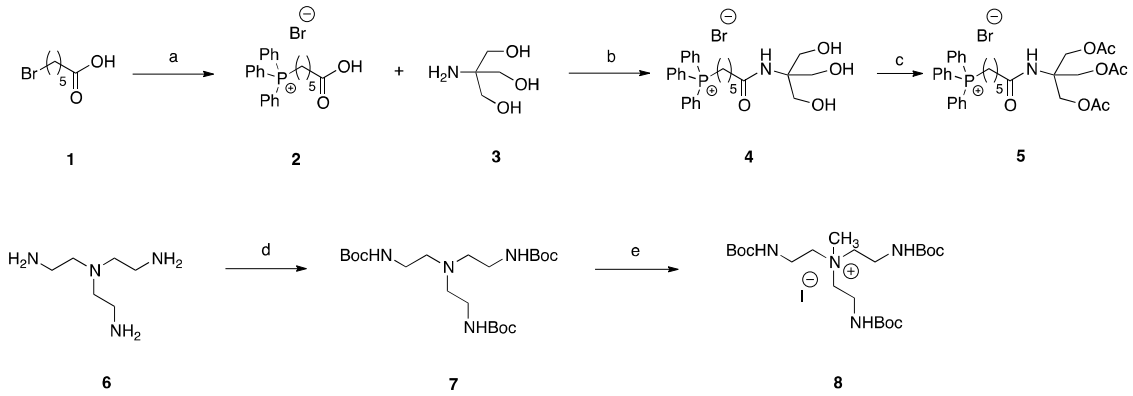
Figure 3. Characterization of the effects of the DCA-loaded compound 10 on cellular bioenergetics functions. Leukemic cells were left untreated or treated for 3h with 300 μM of compound **10**, or compound **7** (control carrier) or with DCA and run on the Seahorse analyzer. Oxygen consumption rate (OCR) measurements were recorded after the sequential addition of oligomycin, FCCP (Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) and the combination of antimycin A and rotenone. OCR traces are expressed as pMoles O_2 per minute and normalized to cell number. Each data point represents means±SD of six independent experiments. The graphs reports the different parameters of cellular respiration analyzed. The basal respiration corresponds to

the OCR measurement before oligomycin injection; the maximal respiration corresponds to the OCR measurement after FCCP addition; the coupling efficiency and the spare respiratory capacity were calculated by subtracting the minimum Mito Inhibitor (i.e. antimycin A + rotenone) responses, that account for non-mitochondrial respiration, to the values registered before and after FCCP addition respectively, and results are referred above the basal respiration. Scaffold (compound **7**)-treated cultures are not shown since they were highly comparable to the untreated cultures. The asterisk indicates $p<0.05$ with respect to the untreated cultures.

Figure 4. *In vivo* analysis of compound 10 stability by HPLC-MS analysis of mice plasma. A representative HPLC-MS analysis of a plasma sample collected from mice 4 hours after a subcutaneous (s.c.) injection of a single dose (57 mg/kg) of compound **10** is reported. In **A**, the Total Ion Current chromatogram (TIC) and the peack corresponding to compound **10** (^{t}R 0.455 minutes). In **B**, the total MS spectra for the selected TIC indicates the 478.95558u $[M+H]^+$ peak ascribing at the presence of compound **10** and the peak at 500.93822u $[M+Na]^+$. In **C**, the enlargement of MS region of interest showing the presence of $[M+H]^+$ peak at 476.95839u corresponding to compound **10**.

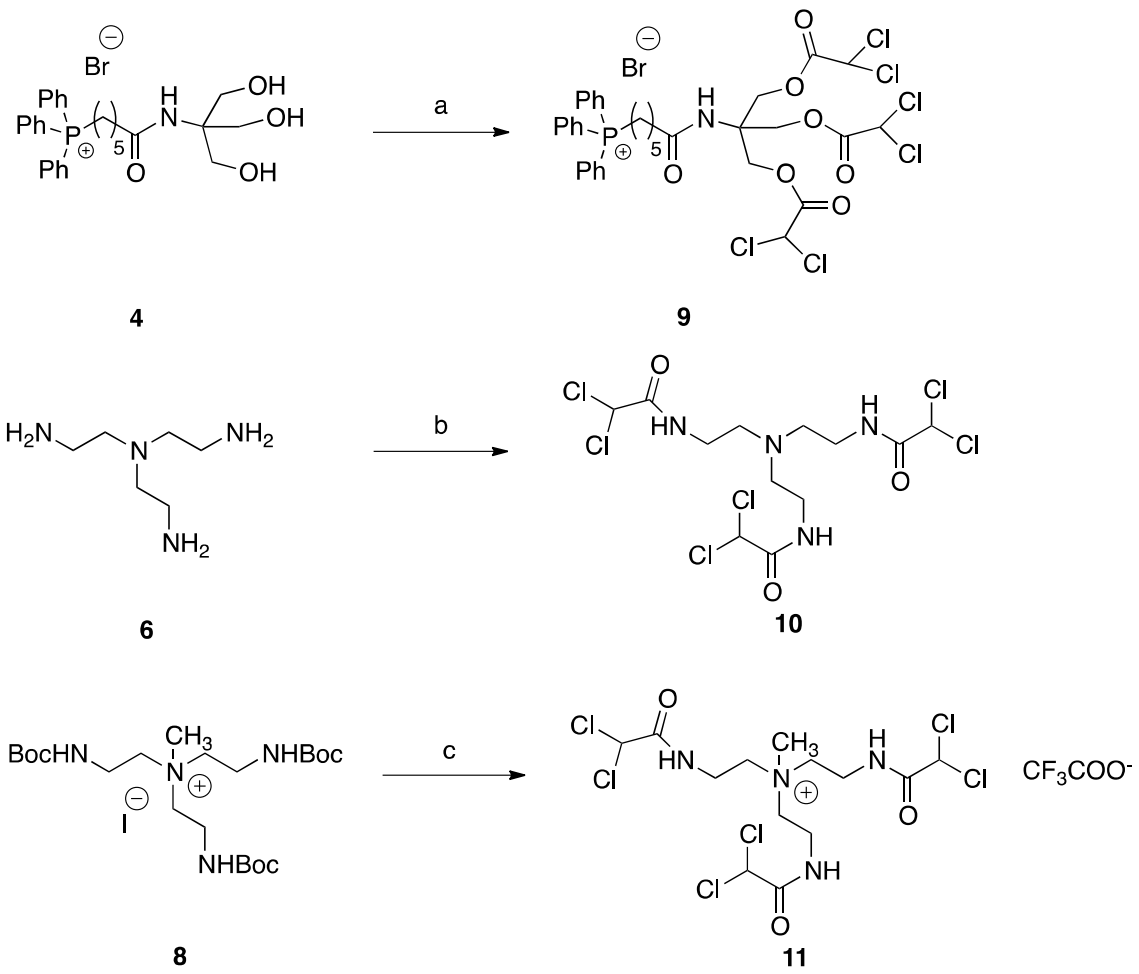
SCHEMES

Scheme 1



Conditions: a) CH_3CN, TPP, reflux, 24h, Y= quant.; b) EtOH, EEDQ, 50°C, 12h, Y=60%; c) DCM, AcCl, r.t., 4h, Y= quant.; d) Water/Dioxane 1/2, NaOH, BOC_2O, r.t, 12h, Y= 96%; e) CH_3CN, CH_3I, 60°C, 12h, Y= quant.

Scheme 2



Conditions: a) DCM, $Cl_2CHC{=}OCl$, r.t., 2h, Y=92%; b) DCM/NaOH 2N,$Cl_2CHC{=}OCl$, r.t., 2h, Y=20%; c) 1)DCM, TFA, r.t, 12h; 2)DCM/NaOH 2N,$Cl_2CHC{=}OCl$, r.t., o.n., Y=50%

FIGURES

Figure 1

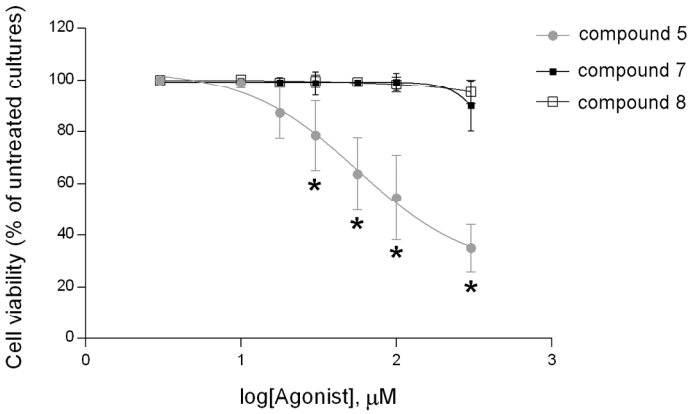


Figure 2

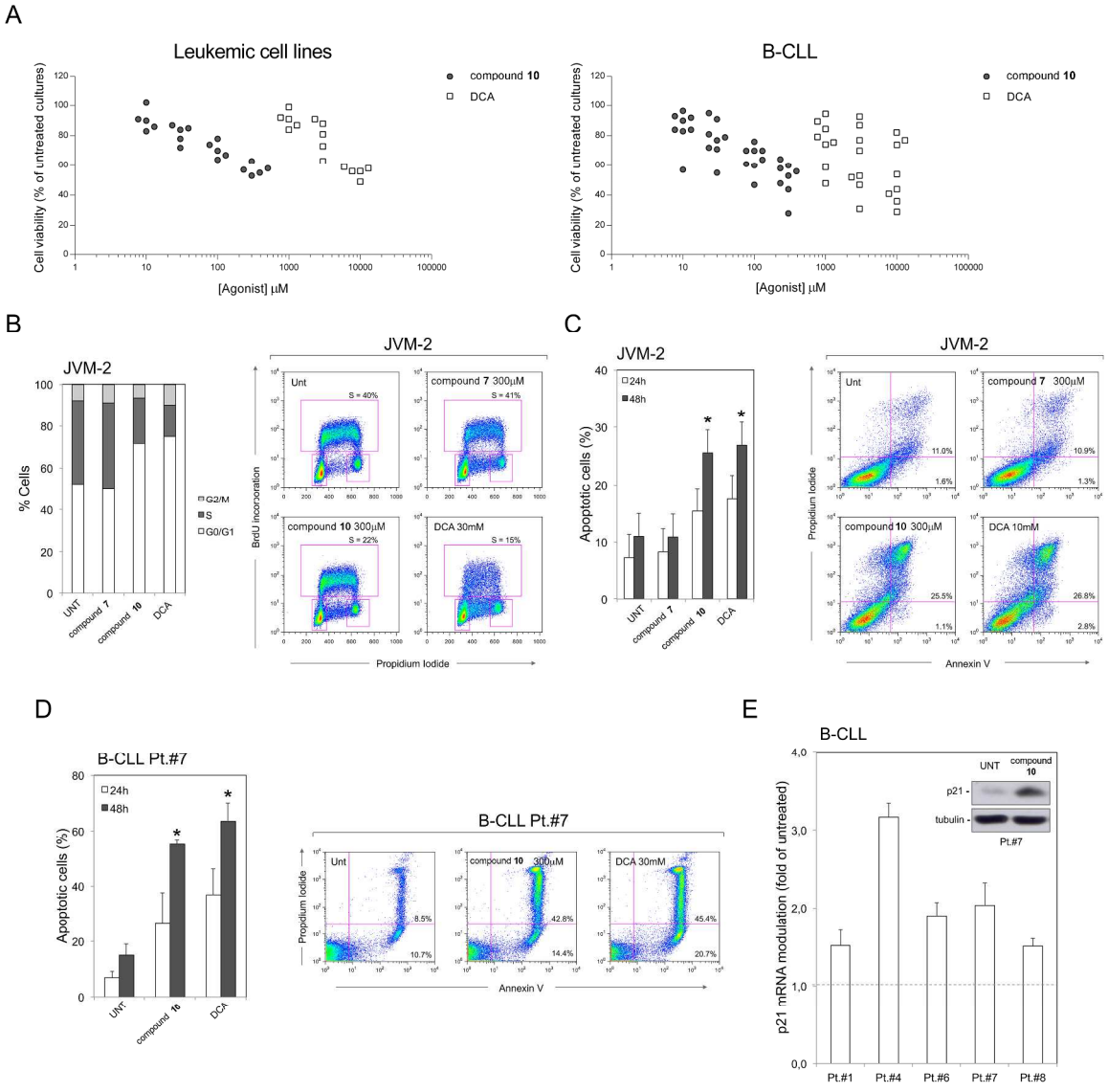

A
Leukemic cell lines
B-CLL
compound 10
DCA
Cell viability (% of untreated cultures)
[Agonist] µM
B
JVM-2
% Cells
G2/M
S
G0/G1
UNT
compound 7
compound 10
DCA
BrdU incorporation
Unt
S = 40%
compound 7 300µM
S = 41%
compound 10 300µM
S = 22%
DCA 30mM
S = 15%
Propidium Iodide
C
JVM-2
24h
48h
Apoptotic cells (%)
Unt
compound 7 300µM
11.0%
1.6%
10.9%
1.3%
compound 10 300µM
25.5%
1.1%
DCA 10mM
26.8%
2.8%
Annexin V
D
B-CLL Pt.#7
24h
48h
Apoptotic cells (%)
UNT
compound 16
DCA
Unt
8.5%
10.7%
compound 10 300µM
42.8%
14.4%
DCA 30mM
45.4%
20.7%
E
B-CLL
UNT
compound 10
p21
tubulin
Pt.#7
p21 mRNA modulation (fold of untreated)
Pt.#1
Pt.#4
Pt.#6
Pt.#7
Pt.#8

Figure 3

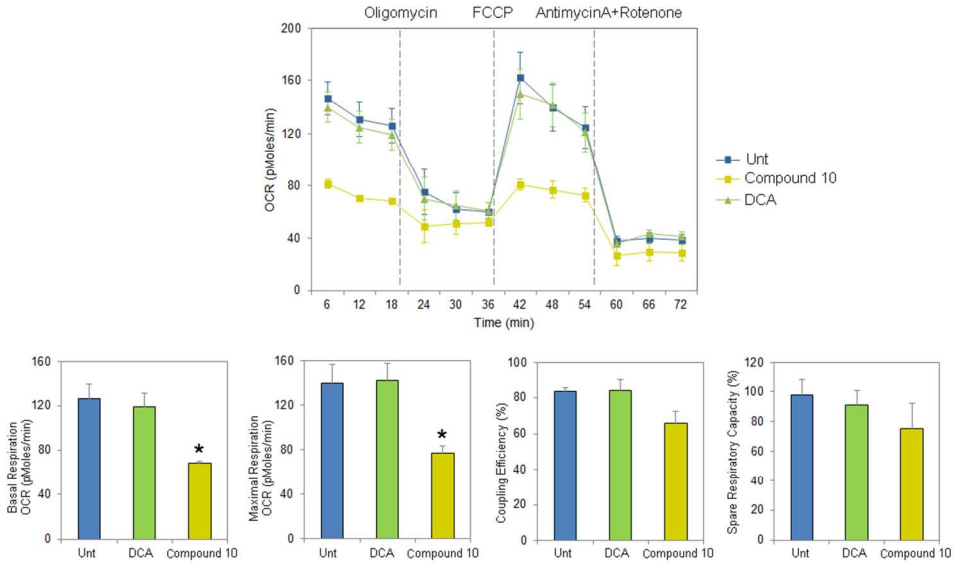
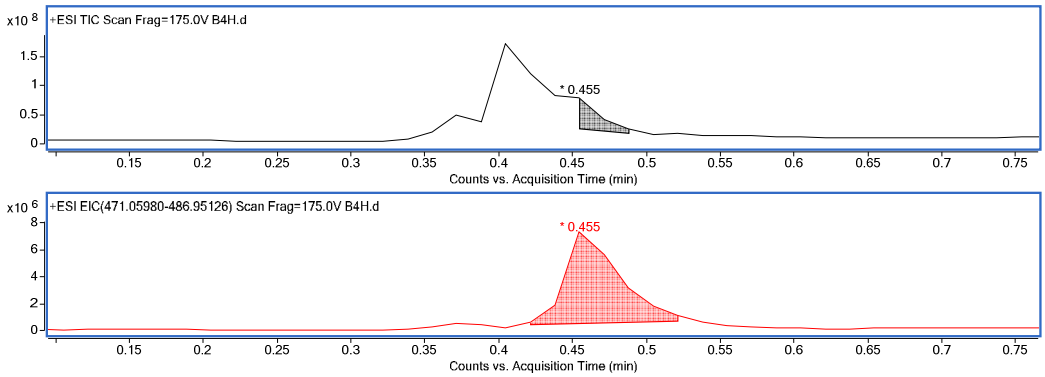
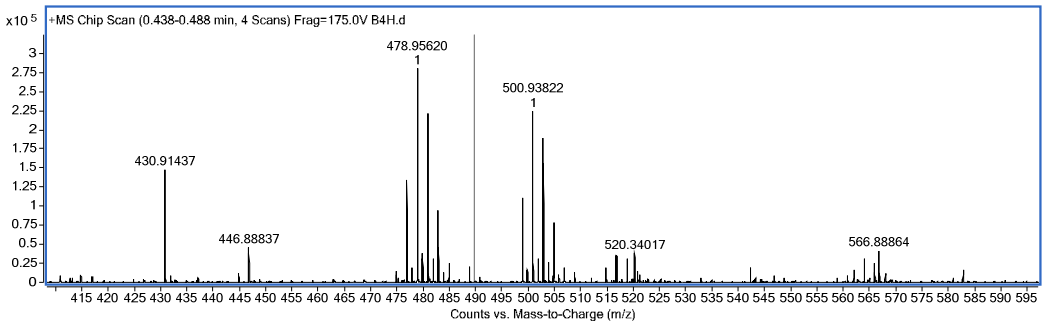

Oligomycin
FCCP
AntimycinA+Rotenone
OCR (pMoles/min)
Time (min)
Unt
Compound 10
DCA
Basal Respiration OCR (pMoles/min)
Maximal Respiration OCR (pMoles/min)
Coupling Efficiency (%)
Spare Respiratory Capacity (%)
Unt
DCA
Compound 10

Figure 4

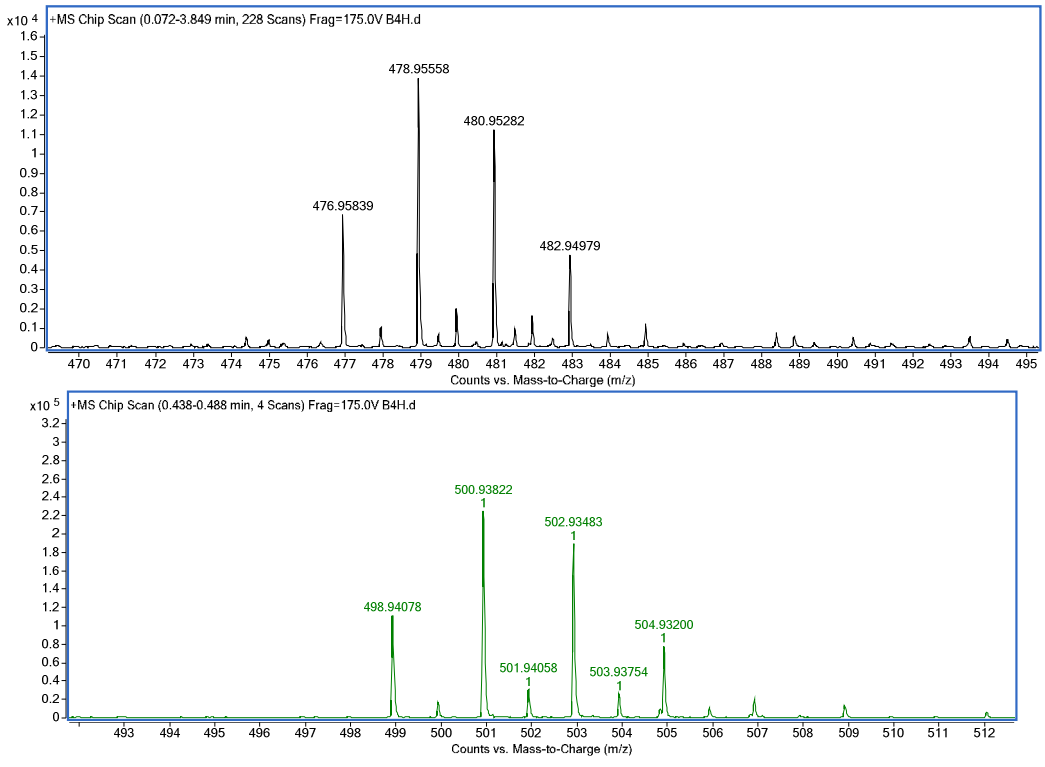
A


+ESI TIC Scan Frag=175.0V B4H.d
* 0.455
Counts vs. Acquisition Time (min)
+ESI EIC(471.05980-486.95126) Scan Frag=175.0V B4H.d
* 0.455
Counts vs. Acquisition Time (min)

B


+MS Chip Scan (0.438-0.488 min, 4 Scans) Frag=175.0V B4H.d
478.95620
500.93822
430.91437
446.88837
520.34017
566.88864
Counts vs. Mass-to-Charge (m/z)

C


+MS Chip Scan (0.072-3.849 min, 228 Scans) Frag=175.0V B4H.d
478.95558
480.95282
476.95839
482.94979
Counts vs. Mass-to-Charge (m/z)
+MS Chip Scan (0.438-0.488 min, 4 Scans) Frag=175.0V B4H.d
500.93822
502.93483
498.94078
504.93200
501.94058
503.93754
Counts vs. Mass-to-Charge (m/z)

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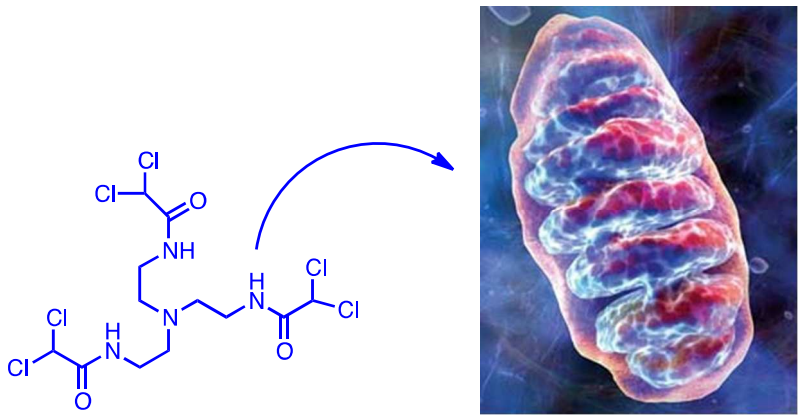

PDK Inhibition
Tumours cell death