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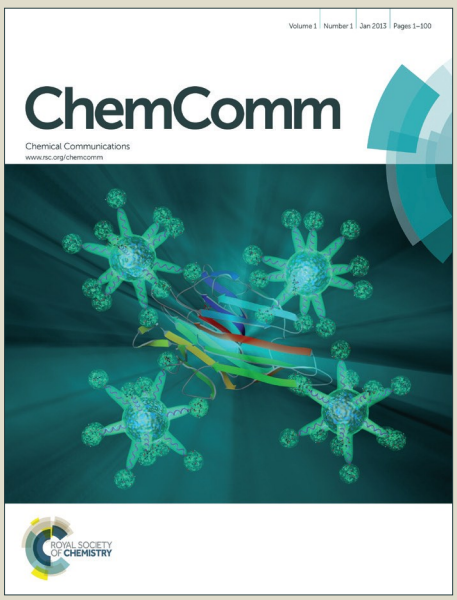

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'Dual hit' metabolic modulator LDCA selectively kills cancer cells by efficient competitive inhibition of LDH-A

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In this perspective, we synthesized and elucidated the potential of a novel 'dual hit' molecule LDCA where it constitutively blocks Lactate Dehydrogenase Isoform-A (LDH-A) to selectively subvert apoptosis and rigorously attenuate breast tumor progression in mice model, comprehensively delineating the therapeutic prospectus of LDCA in the field of cancer metabolics.

The global burden of cancer continues to ascend steeply and so does an unmet need to effectively mitigate it without a manifest in toxicity.[1] Cancer and non-cancer cells differ vitally with respect to their metabolic pathways and interestingly the essential tumor specific hallmarks are intertwined with its intrinsic metabolism either as a consequence or a cause.[2] The phenomenology of consuming elevated levels of glucose to satiate anabolic respiratory reactions is sufficiently prevalent[3] and this major criteria centralizing for the altered behavior of tumors has been conceptualized as the "Warburg effect" that allows a functional redundancy to enhance resistance towards apoptosis.[4] Therefore, targeting tumor metabolism might offer a new therapeutic window as it will help us to visualize and treat multiple deregulated signalling pathways of the cancer cells. LDH-A plays a prominent role in orchestrating aerobic glycolysis within the malignant population and is of particular interest because it not only helps in sustaining the low pH microenvironment through enhanced lactate production, but also responsible for invasive and aggressive tumor outcomes.[5] Since therapies to address grade IV metastatic tumors are limited, development of LDH-A inhibitors would not only revert the bio-energetic flow for anabolic reactions but simultaneously invert the neoplastic phenotype and have a crucial impact in the clinical setting. One of the classic competitive inhibitors of LDH-A is oxamate that is a structural isostere of pyruvate, contains a carboxylic acid group adjacent to the CO-NH backbone and evidence suggests that the vicinal hydroxyl/carbonyl-carboxyl substitution pattern is crucial for inhibitory activity.[6] Equally importantly, fluorinated molecules are being immensely explored in the field of cancer therapeutics and fluorinated congeners are being employed as effective biochemical tools for mechanistic investigations.[7] In this study, we have fabricated a novel compound LDCA (2, 2-dichloro-N-(3-chloro-4-fluorophenyl)acetamide), where an organofluorine moiety has been tactically coupled with -$COCHCl_2$ motif that would present us with a molecule containing fluorine moiety having a CO-NH backbone, hypothesizing that it would competitively inhibit LDH-A to selectively subvert apoptosis in the neoplastic population and be perceived as an important candidate in the arena of therapeutic metabolic modulators. Towards this aim, LDCA was synthesized by a one-step chemical reaction by treating 3-chloro 4-fluoro aniline with dichloroacetylchloride and purified using HPLC (Yield ~92%). Further exploratory structural analysis was confirmed using ESI-HRMS, ^{1}H-NMR (300 MHz), ^{13}C-NMR (300 MHz), elemental analysis and X-Ray single molecule crystallography (Fig. 1A, 1B and S1-S4, ESI†).

The inhibitory effect of LDCA was studied from standard enzyme inhibition kinetics using purified human LDH-A isoform to subsequently determine the type of inhibition against both co-factor NADH and substrate pyruvate. Enzyme activity was determined by measuring the decrease in absorbance per minute at 340 nm, due to the consumption of NADH and its subsequent conversion to NAD+. The apparent

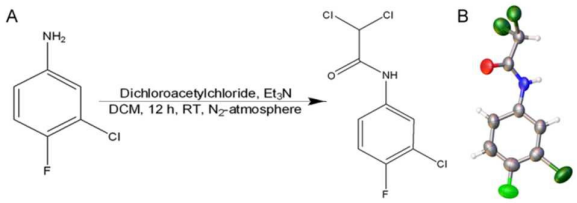

Fig. 1 (A) Synthetic scheme and (B) X-Ray structure of LDCA.

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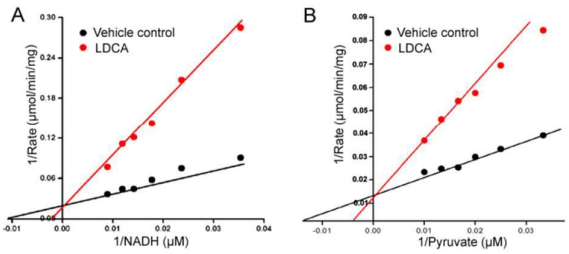

Fig. 2 Lineweaver-Burk plots determined from triplicate experiments with LDCA: competitive experiments with (A) NADH and (B) pyruvate.

Michaelis-Menten constant (K_m) of both NADH and pyruvate was calculated from Lineweaver-Burk plots and subsequently the apparent K_m' was determined in the presence of LDCA (50-200 μM). From the K_m' values obtained, K_i values were determined using double-reciprocal Lineweaver-Burk plots where LDCA proved to be a potent inhibitor of LDH-A with K_i values reaching the low micromolar range (K_i = 39.5 μM versus NADH and 29.6 μM versus pyruvate) (Fig. 2). It is clear from the inhibition kinetics experiment that variations in the concentration of LDCA demonstrated perturbations only in K_m' values, however V_{max} remained constant indicating that LDCA manifest a competitive behavior against both co-factor NADH and substrate pyruvate.

To thoroughly elucidate the selective anticancer efficacy, MTT assay was performed and graph depicts that LDCA mounts a demonstrable tumor killing response sparing the normal cells and thus, surpasses the phenomenon of toxicity (Table S1 and Fig. S5A, ESI†). In support of this premise, flow cytometric analysis and TUNEL assay document that LDCA selectively consequence cancer cell death without affecting fibroblastic viability (Fig. S5B and S5C, ESI†).

To comprehensively reaffirm the selective cytotoxic response of LDCA, a co-culture study of WI-38 and 4T1 cells was employed and visualized under microscope as the cells were easily distinguishable owing to their contrasting morphology. Fig. S5D (ESI†) showcases that LDCA spared the fibroblasts and significantly affected the viability of the cancerous population. Furthermore, we deduced the potential of LDCA by determining its ability to induce chromosomal aberration and micronuclei (MN) formation in primary culture of human lymphocytes. Results suggestively depict that a significantly low percentage of micronuclei formation and chromosomal aberration was observed when compared to that of positive control (Fig. S5E, ESI†). Next, alterations within cell cycle were noted as treatment arrested cells at G1 phase and generated DNA fragments in the sub G0/G1 phase (Fig. S6, ESI†). Taken together, results attribute to the fact that LDCA accentuates selective killing of cancer cells without any toxic manifestations.

Characteristic reliance of the cancer cells on aerobic glycolysis causing profuse lactate production allows them to condition the environment so as to favour sustenance, survival and growth.[2a] Hampering this effect in tumors can be exploited clinically and one way to determine this would be to

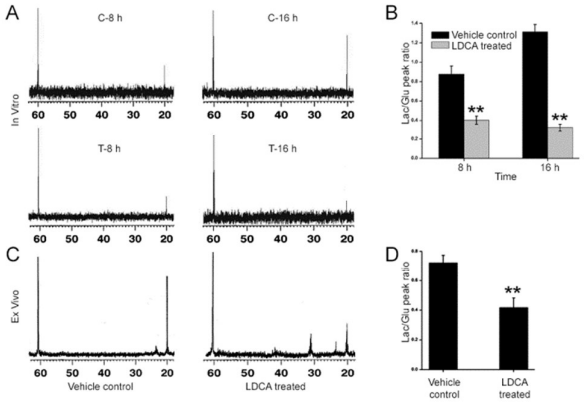

Fig. 3 LDCA attenuates lactate production. (A) Representative NMR peak frequencies depicting formation of 3-^{13}C lactate at mentioned time intervals in vehicle control and 50 μM LDCA treated sets. (B) Graph represents comparative quantification of Lac/Glu peak ratio. **p < 0.01. (C) Freshly excised tissue slices were obtained from vehicle control and the other from 2.5 mg/kg LDCA treated tumors. The tissue slices were incubated with D-[1,6-$^{13}C_2$] glucose and 3-^{13}C lactate was estimated. (D) Quantitation of Lac/Glu peak ratio. **p < 0.01.
Error bars in all panels represent the mean ± SD.

estimate the amount of cellular lactate levels in real time. In this regard, NMR spectroscopic studies were conducted to substantiate the ability of LDCA to bring about a proficient reduction of lactate production. Cultures of 4T1 cells were incubated with D-[1,6-$^{13}C_2$] glucose and the relative production of [3-^{13}C] lactate was determined by scaling the peak area corresponding to the lactate-C3 peak at 20.16 ppm relative to the glucose-C6 peak at 60.76 ppm (Lac/Glu peak ratio).[8, 6b] Quantitative values of peak integrations illuminate that Lac/Glu ratio increased gradually over time in vehicle control whereas treatment concomitantly repressed lactate production (Fig. 3A and B). We further estimated the potential of LDCA to affect lactate production *ex vivo*[9] and completely in

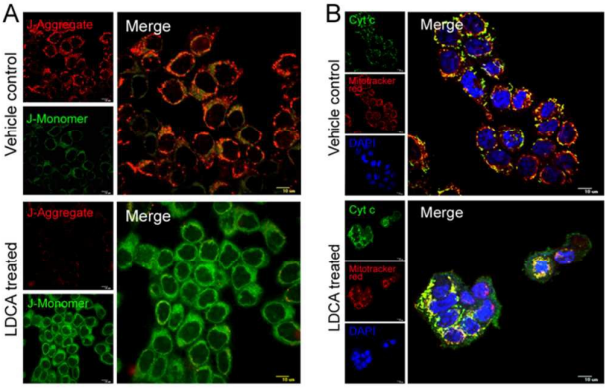

Fig. 4 LDCA alters MMP and promotes Cyt c efflux. (A) Representative images depict JC1 staining pattern in vehicle control and 50 μM LDCA treated sets after 16 h (right panel-enlarged view). (B) CLSM images illustrate Cyt c dumping from mitochondria after 16 h post 50 μM LDCA treatment (right panel- enlarged view).

accordance with the *in vitro* results, treatment appreciably lowered tumor lactate levels post treatment (Fig. 3C and D). Additionally, ELISA assay suggests that LDCA reasonably limits lactate production in a dose dependent manner (Fig. S7, ESI†). It has been evidenced that pharmacological inhibition of LDH-A has a profound impact on mitochondrial membrane potential (MMP) and mitochondrial bioenergetics[9] and any significant alterations in polarization would transform into one of the decisive steps that is exclusively stringed to apoptosis. Thereby, we testified whether LDCA has any impact in modulating the MMP of the cancer cells. MCF-7 and 4T1 cells were treated and subsequently stained with JC-1 dye to detect changes in MMP. A heterogeneous staining pattern in neoplastic cell mitochondria with increased red and decreased green fluorescence was observed in vehicle control whereas, this phenomenon was subsequently reversed post treatment (Fig. 4A). Importantly, flow cytometric analysis depicts that LDCA critically alters mitochondrial hyperpolarization of 4T1 cells, whereas no significant changes was observed in the fibroblastic cell line (Fig. S8A and S8B, ESI†) comprehensively denoting that treatment affects mitochondria factually to a point where tonic inhibitory effect on MMP of the cancer cells is observed. In analogy with the above phenomenon, size and shape of the hampered mitochondria was analyzed using AFM and results show that treatment prompted stress generation as a ~2.6 fold increase in size was observed (Fig. S8C, ESI†). Cancer cell already experience elevated amounts of ROS and thereby further provocation to oxidative offends convey them vulnerable. Supposedly, LDCA exposure accentuated reactive oxygen species (ROS) generation within 4T1 cells in a temporal fashion (Fig. S8D, ESI†).

Since LDCA robustly affects malignant mitochondrial dynamics, we therefore undermined its effect in sequestering mitochondria mediated apoptosis. Cytochrome c (Cyt c) release from the mitochondria has been considered as the "point of no return" and LDCA resulted in Cyt c efflux, as diffuse green cytoplasmic immunofluorescence staining pattern was evidenced that was distinctively different from the punctate staining pattern observed in vehicle control (Fig. 4B). Furthermore, micrographs revealed heightened expression of cleaved caspase-9/3 and PARP levels in treated 4T1 cells (Fig. S8E-S8G, ESI†) conclusively suggesting the role of LDCA to specifically recruit caspase cascade to deliberate apoptosis. Evidences propose that LDH-A expression insists active breast tumor progression and since LDCA competitively inhibits LDH-A we scrutinized whether it could reverse migratory ability and tumorigenic capacity of 4T1 cells. LDCA sufficiently limits cell migration in wound healing assay, and adversely affects anchorage independent growth in soft agar[10] as a staggering regression of colony sizes has been documented (Fig. S9 ESI†).

To distinctively decipher the ability of LDCA to modulate cancer attrition and anti-tumor potential *in vivo*, we adopted syngeneic mouse breast cancer model. 4T1 cells were injected into upper inguinal mammary fat pad of female virgin BALB/c mice and after 7 days mice were randomized to receive intratumorally, either vehicle or 2.5 mg/kg of LDCA according

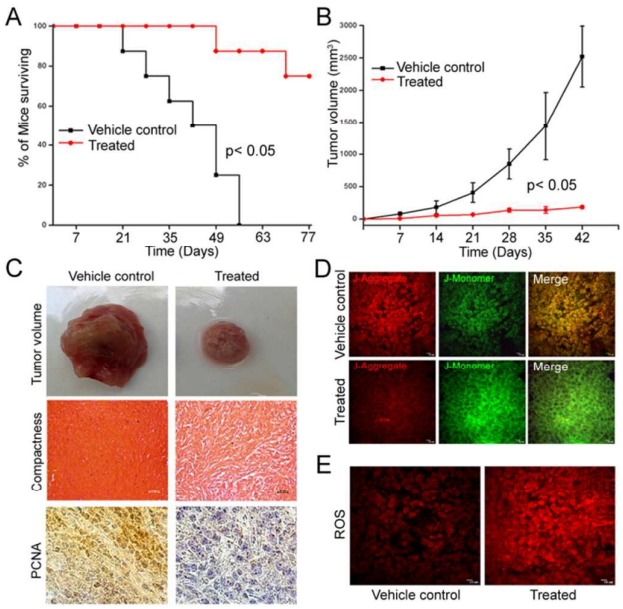

Fig. 5 LDCA inhibits tumor formation *in vivo*. Graph illustrates that treatment (A) increases survivability in mice, *p < 0.05 and (B) limits tumor progression compared to vehicle control. *p < 0.05. (C) 4T1 tumor bearing mice at day 28 were sacrificed; tumors excised, photographed and the representative images have been presented (Upper Panel). H & E staining depicts reduced cellular density in treated tumors (Middle Panel). Representative images demonstrate lowered PCNA expression in LDCA treated tumors (Lower Panel). (D) Freshly resected tumors were sectioned, stained with JC-1 and MMP analyzed. Representative micrographs indicate evident decrease in red fluorescence intensity in 2.5 mg/kg LDCA treated tumors. (E) ROS generation in freshly resected tumors.

to the dosing schedule (Fig. S10, ESI†). Comparative quantification suggestively reveals that treated cohort had increased survivability due to significant decrease in tumor growth kinetics (Fig. 5A and B). Additionally, macroscopic examination depicts that LDCA arrested tumor associated vasculature generation as tumors were too small to require angiogenesis (Fig. 5C, Upper Panel), compromised tumor compactness (Fig. 5C, Middle Panel) and levied a limitation to proliferating cell nuclear antigen (PCNA) expression (Fig. 5C, Lower Panel). Furthermore, expression pattern of cleaved levels of caspase-9/3 and PARP confirm that slow tumor growth was consistently supported by the phenomenology of apoptosis (Fig. S11, ESI†). Additionally histological sections of lung, liver, kidney and spleen showed that LDCA does not garner any toxic manifestations (Fig. S12, ESI†) factually comprehending that LDCA confers an irreversible growth disadvantage without any aversive side effects.

Metabolic reprogramming becomes more complex in the *in vivo* scenario and therefore we ventured to elucidate the potential role of LDCA in disrupting mitochondrial homeostasis. Consistent with the *in vitro* observations, quantitative extrapolation depicts degraded mitochondrial

potential and upregulated radical generation within treated cohort confirming the anti-metabolic effects of LDCA (Fig. 5D, 5E and S13A, S13B, ESI†). Context dependently, we delineated the respiratory status and predictably, LDCA caused a pertinent increase in oxygen consumption both *in vivo* and *in vitro* (Fig. S13C and S13D, ESI†). Results apparently portrays that LDCA subverts apoptosis by blocking LDH-A and hampers the highly resilient mitochondria of the cancer cells comprehending an important milestone in validating LDCA as a potent LDH-A inhibitor that not only deliberates tumor regression but also ensembles its use in clinical setting.

Here, we deliver a substantially important schema that LDCA – is a "dual hit" modulator that is endowed with the ability to specifically and selectively kill cancer cells. The development of a small molecule metabolic modulator that inhibits LDH-A and compensates the hallmark properties of cancer is encouraging as well as a promising strategy to manipulate the metabolism of cancer cell without a manifest in toxicity. A single step reaction benefitted us with a high yield of LDCA and we extend justification in context to our postulation that it might be considered as a "dual hit" agent as it exemplifies competitive inhibition of LDH-A, to simultaneously revert metabolic reprogramming and enforce repression of cancer cell proliferation *in vitro* and oncogenic progression *in vivo*. Our data with clarity exhibit that LDCA might be considered to occupy a centre stage in cancer specific metabolic modulators and targeted therapies to condemn specific hallmarks of cancer, interventions that normalizes metabolic functions in the neoplastic population. Further studies are in prospectus which might open new avenues in the chapter of cancer therapeutics.

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Notes and references

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