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Predicted role of Acetyl-CoA synthetase and HAT p300 in extracellular lactate mediated lactylation in the tumor: *In vitro* and *in silico* models

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Major highlights of paper:

- This study presents new data that addresses significant gaps in our understanding of key substrates and enzymes involved in a novel epigenetic process called lactylation, which modulates the cellular landscape in both cancer and non-cancer cells.
- Specifically, the study found that breast cancer cells secrete lactate into the extracellular environment during drug-induced cell death.
- Molecular dynamics simulations were then used to predict the potential role of acetyl-CoA synthetase in generating lactyl-CoA by macrophages and microbiota.
- Additionally, the study suggests that HAT p300 may use lactyl-CoA as a substrate, similar to the known substrate acetyl-CoA.
- Finally, this paper proposes novel missing links in the shuttling of lactate and its derived products within the tumor microenvironment, taking into account the roles of tumor-associated macrophages and microbiotas.

ABSTRACT

Background

As per the Warburg effect, cancer cells are known to convert pyruvate into lactate. The accumulation of lactate is associated with metabolic and epigenetic reprogramming, which has newly been suggested to involve lactylation. However, the role of secreted lactate in modulating the tumor microenvironment through lactylation remains unclear. Specifically, there are gaps in our understanding of the enzyme responsible for converting lactate to lactyl-CoA and the nature of the enzyme that performs lactylation by utilizing lactyl-CoA as a substrate. It is worth noting that there are limited papers focused on metabolite profiling that detect lactate and lactyl-CoA levels intracellularly and extracellularly in the context of cancer cells.

Methods

Here, we have employed an in-house developed vertical tube gel electrophoresis (VTGE) and LC-HRMS assisted profiling of extracellular metabolites of breast cancer cells treated by anticancer compositions of cow urine DMSO fraction (CUDF) that was reported previously. Furthermore, we used molecular docking and molecular dynamics (MD) simulations to determine the potential enzyme that can convert lactate to lactyl-CoA. Next, the histone acetyltransferase p300 (HAT p300) enzyme (PDB ID: 6GYR) was evaluated as a potential enzyme that can bind to lactyl-CoA during the lactylation process.

Results

We collected evidence on the secretion of lactate in the extracellular conditioned medium of breast cancer cells treated by anticancer compositions. MD simulations data projected that acetyl-CoA synthetase could be a potential enzyme that may convert lactate into lactyl-CoA similar to a known substrate acetate. Furthermore, a specific and efficient binding (docking energy -9.6 kcal/mol) of lactyl-CoA with p300 HAT suggested that lactyl-CoA may serve as a substrate for lactylation similar to acetylation that uses acetyl-CoA as a substrate.

Conclusion

In conclusion, our data provide a hint on the missing link for the lactylation process due to lactate in terms of a potential enzyme that can convert lactate into lactyl-CoA. This study helped us to project the HAT p300 enzyme that may use lactyl-CoA as a substrate in the lactylation process of the tumor microenvironment.

Keywords: Metabolic reprogramming, Glycolysis, Lactic acid, Epigenetic modification, Histone modification, Warburg effect.

Introduction

A tumor contains various kinds of cancer cells and non-cancer cells including immune cells, stromal cells, and in close vicinity, microbiotas (1-3). There are strong suggestions that intracellular and intercellular shuttling of lactate may be required to meet the diverse metabolic, signaling, and epigenetic regulations of cancer and cancer associated cells (4-7).

Lactate generated by cancer cells may be shuttled to the extracellular milieu so that lactate could influence metabolic adaptations by macrophages and microbiotas (8-11). Besides the clear role of lactate in the metabolic rewiring of cancer cells and cancer-associated cells such as pro-inflammatory macrophages, there is a clear gap in our understanding of lactate-mediated non-metabolic implications including lactylation form of epigenetic modifications of histones.

The coherence between metabolic and epigenomic process that supports the various tumor hallmarks including proliferation, invasiveness, metastasis and drug resistance is being unraveled (8-14). Emerging observations support the role of lactate as a non-metabolic signaling molecule that promotes lactylation and post-translational modification of a lysine residue on histone and other target proteins such as PKM2 and beta-catenin (11-14). At the same time, lactylation is reported in certain types of cells including macrophages, and is linked to the transcriptional changes that help the M1 macrophages to be changed into M2 macrophages (15-19).

In essence, M2 macrophages are shown to share similar attributes to that of pro-inflammatory and tumor-associated macrophages (20-22). A view on the role of extracellular lactate as a metabolic fuel by microbiotas is proposed. In this way, an indirect role of lactylation in cancer growth and proliferation is proposed by activating M1 macrophages in the tumor microenvironment which is in turn transformed into pro-tumor M2 macrophages (15-22). Among many distinctive features of M1 macrophages, acetyl-CoA synthetase is highly active and overexpressed which is known to catalyze the formation of acetyl-CoA from acetate (23-30). However, the role of acetyl-CoA synthetase in lactylation is not explored. The constraints in the detection of lactate and lactate-derived metabolic products could be due to the highly shuttled and dynamic nature of lactate in the tumor microenvironment. Therefore, a link that can connect the generation of lactyl-CoA from lactate by specific enzymes of cancer cells, tumor-associated macrophages, and microbiotas in the tumor niche is not evident.

In spite of increasing understanding on the novel evidence on the lactylation in various cellular components of tumor microenvironment, there is a gap regarding the nature of the enzymes may use lactyl-CoA for lactylation and report of lactyl-CoA in the physiological setting. At the same time, the possibility of the involvement of histone acetyltransferase (HAT) p300 which is well-known as a regulator of chromatin remodeling is indirectly proposed, but not explored (31-37).

In this study, we aimed to investigate lactate excretion in the extracellular conditioned medium of cancer cells treated with anticancer compositions. To explore the missing link in lactylation, we utilized molecular docking and molecular dynamics (MD) simulations to identify potential enzymes capable of generating lactyl-CoA from lactate. Additionally, we conducted molecular interaction studies to determine the binding affinities of lactate and its derived products with HAT p300. Our findings shed light on the significant gaps in our understanding of lactylation, particularly regarding the enzyme that can catalyze lactylation using lactyl-CoA.

Materials and Methods

Materials

Cell culture reagents were purchased from Invitrogen India Pvt. Ltd. and Himedia Laboratories Pvt. Ltd. MCF-7 breast cancer cells were procured from the National Centre of Cell Science (NCCS) Pune, India. DMSO, agarose, acrylamide, and other chemicals were of molecular biology grade and obtained from Himedia Laboratories Pvt. Ltd. India and Merck India Pvt. Ltd.

Extracellular metabolite profiling

The extracellular conditioned medium of MCF-7 breast cancer cells treated by DMSO and cow urine DMSO fraction (CUDF) (50 µg/ml) final concentration was collected as per the previously published procedure (41-43). Then, 750 µl of extracellular conditioned medium was mixed with 4X loading buffer (0.5 M Tris, pH 6.8, and Glycerol). Next, the conditioned medium along with the loading buffer was loaded on the vertical tube gel electrophoresis (VTGE) purification system with a matrix of 15% polyacrylamide gel (acrylamide: bisacrylamide, 30:1) (42-43). The fractionated extracellular metabolites were collected in the running buffer (96 mM glycine, pH 8.3). The detailed procedure was adopted from previously published in-house VTGE-assisted purification of metabolites (42-43). Furthermore, LC-HRMS analyses of VTGE-

purified extracellular metabolite elutes were performed by Agilent TOF/Q-TOF Mass Spectrometer station Dual AJS ESI ion source. During LC separation, RPC18 Hypersil GOLD C18 100 x 2.1 mm-3 µm and mobile phase of 100% Water (0.1% FA in water) and 100% Acetonitrile (90% ACN +10% H2O+ 0.1% FA) were used in the proportion of 95% and 5% (40). Mass spectrometry was performed in a positive mode and analyzed as per the procedure adopted from previously reported methodology (43).

Molecular Docking

Potential oncometabolites including lactate (PubChem CID: 91435), acetate (PubChem CID: 175), lactyl-CoA (PubChem CID: 3081970), and acetyl-CoA (PubChem CID-444493) were retrieved from the database as ligands for molecular docking. The PubChem database was used to download the structure of ligands in SDF format. Then conversion of ligands into PDB format took place using the software OpenBable. Before performing molecular docking, both ligands were energy minimized to obtain stable conformation using Avogadro software (44) with the steepest descent method and MMFF94s force field. Protein Data Bank (PDB) (https://www.rcsb.org) was used to download the receptor protein. Here, HAT p300 (PDB ID-6GYR) and acetyl-CoA synthetase (PDB ID: 2P2F) were taken as target proteins. Hetatoms are removed from the protein before performing docking. This protein was subjected to the AutoDock Tool 4.2. to perform the steps of protein preparation, which include the removal of water molecules, bond correction, assigning AD4 type atoms, adding polar hydrogens, and adding Kollman charges (45). AutoDock Vina Software was used to perform molecular docking (46). After the successful docking, confirmation of the binding position of oncometabolite into the receptor protein and calculation of bond distance has been done by Discovery Studio Visualizer v3.0 (DSV3) and Accelrys software (47).

Molecular Dynamics (MD) Simulations

The 20ns Molecular Dynamics (MD) simulation of metabolite lactate (PubChem CID: 91435), and acetate (PubChem CID: 175) with acetyl-CoA synthetase (PDB ID: 2P2F) was performed with the help of Desmond software to confirm the binding stability and strength of the complex (48). Desmond has inbuilt functions to add pressure, volume system, temperature, and many functionalities to accomplish protein-ligand binding. Ligand-protein complex was plunged into a water-filled orthorhombic box of 10 Å spacing. The conformational changes upon binding

of ligands with acetyl-CoA synthetase were recorded by using the 1000 trajectories frames generated during the 20ns MD simulation and the Root Mean Square Deviation (RMSD) was calculated to reveal the binding stability of lactate and acetate.

RESULTS

The relevance of intracellular, extracellular, and inter-tissue lactate as metabolic fuel and signaling molecules that link the epigenetic-metabolic axis in cancer is considered challenging (4-10). The reason behind the gaps in understanding the role of lactate and its derived metabolic products such as lactyl-CoA is the lack of detection of lactate and lactyl-CoA at the physiological concentration at intracellular, extracellular, and inter-tissue levels. The lack of clear evidence on the levels of lactate and lactyl-CoA could be linked with constraints such as specific and efficient metabolite profiling methodologies and the highly shuttling and diffusive nature of lactate and lactyl-CoA in the tumor microenvironment.

Detection of lactate in the extracellular conditioned medium

In this regard, we have attempted to detect the levels of lactate and lactyl-CoA at the intracellular and extracellular levels of breast cancer cells treated by DMSO and anticancer drug compositions enriched with free fatty acids and tripeptides (41-43). The use of anticancer drug composition CUDF is taken as one of the candidate drug models to see the difference in the detection of lactate and lactyl-CoA compared to the DMSO control. Extracellular metabolite profiling of MCF-7 breast cancer cells suggested surprising observations that lactate was not detected in DMSO control (Figure 1A). At the same time, CUDF-treated MCF-7 breast cancer cells previously reported for inducing cell death indicated the presence of lactate (m/z 89.0239, RT-1.483, mass-90.0131) in the LC-HRMS derived total ion chromatogram of extracellular conditioned medium (Figure 1B). The detailed MS and MS/MS spectra displayed clear evidence of lactate-specific negative ESI fragment ion spectra such as 89.0235 and 96.9594. Interestingly, LC-HRMS profiling of VTGE-purified intracellular metabolites did not show a detectable level of lactate and lactyl-CoA. At the same time, extracellular profiling showed the presence of lactate in the case of breast cancer cells under drug-induced stress and cell death (Figure 2). But, we did not detect traces of lactyl-CoA in the case of both DMSO and CUDF-treated breast cancer cells.

Molecular docking on lactate and acetyl-CoA Synthetase

In a quest to find the relevance of lactate as an extracellular signaling molecule, we found that acetyl-CoA synthetase is known to promote pro-tumor phenotype in macrophages. Also, acetyl-CoA synthetase is known for the metabolic activities in bacterial cells that could be linked with the microbiotas in the niche of the tumor microenvironment. By employing molecular interaction studies, data indicated that lactate (Figure 3A) occupies the same substrate site as in the case of acetate (Figure 3B). The binding affinity and interactions at the active sites of acetyl-CoA synthetase are projected to be almost similar from lactate (Figure 3C) over acetate (figure 3D) in terms of residues such as TRP413, TRP414, GLN415, ARG515, ASN512, and ARG526. The number and nature of interacting forces including hydrogens bonds projected identical. Besides a missing link on the suitable enzyme that performs the lactylation process, a debate is pertinent on the existence of an enzyme that converts lactate to lactyl-CoA. By using molecular docking studies, we have screened various potential enzymes as potential players that may catalyze the formation of lactyl-CoA from the lactate (data not shown). During the screening, a key enzyme is predicated as acetyl-CoA synthetase that may potentially prefer the lactate as a substrate during the formation of lactyl-CoA that is similar to the acetyl-CoA formation from acetate (Figure 2). Data obtained from DSV3 predicted the almost similar binding pockets with hydrogen bonds between acetate (GLN415, ARG515, ARG526) and lactate TRP413, TRP414, GLN415, ARG515, ASN521) for acetyl-CoA synthetase (Figure 3 and Table 1).

Molecular Dynamics (MD) Simulation

To analyze the stability of the lactate-acetyl CoA complex, the MD simulations were carried out for a length of 20 ns. For comparison, a known substrate acetate is selected as a positive control for MD simulations of acetyl-CoA synthetase. We have analyzed the conformation of the protein-ligand complex obtained during the simulation period of 20ns. Root mean square deviation (RMSD) was calculated during the simulation trajectory of 20ns for the ligands such as lactate and acetate against acetyl-CoA synthetase. The RMSD evolution plot of acetyl-CoA synthetase on the Y-axis suggests that values (1.2 to 2.4 Å) for lactate (Figure 4A) is within the well-accepted range, 1-3 Å and almost similar to acetate (Figure 4B) RMSD value (0.9 to 2.7 Å) Furthermore, it is important to note that simulations are converged and appear to be stabilized at the end of simulations of length between 12 to 20 ns for lactate and a known substrate of acetyl-CoA synthetase.

Next, root mean square fluctuation (RMSF) values of acetyl-CoA synthetase in complex with lactate suggested a well-acceptable range of fluctuations for lactate (0.4 to 1.8 Å) and acetate (0.5 to 1.6 Å) specifically in the region spanning from 280 to 550 amino acid residues (Figure 5A and 5B). These residues from 280 to 550 position of acetyl-CoA synthetase is known for substrate binding and catalytic activity. The MD simulations explain the interaction fraction of the acetyl-CoA enzyme residues with the lactate and acetate, which means how much % of the simulation time the specific interactions of these residues maintained ligand-protein complexes. Here, the ligand-protein contact map of lactate (Figure 6A) and acetate (Figure 6B) showed similar interaction profiles in terms of ranges on Y-axis from 0 to 2. Most notable amino acid residues that contributed to the stable ligand-protein complexes are found common such as THR416, ASN521, GLY524, and ARG526.

Molecular docking on lactyl-CoA and HAT p300

Concerning the recent discovery of an epigenetic lactylation process, there is a substantial gap between the substrate and the enzyme which is known to play a vital role in lactylation for histone modification. However, suggestions are put forth on the involvement of lactate-derived metabolites such as lactyl-CoA during the lactylation process (12-18).

In this direction, we have employed *in silico* approach to understanding the epigenetic modification by the process of lactylation. Autodock Vina was used to perform molecular docking experiments due to its better accuracy in predicting binding patterns, less run time, higher reproducibility, and its ability to powerfully search for potential energy surfaces (30). The molecular binding patterns of lactyl-CoA and acetyl-CoA against HAT p300 showed similar attributes in terms of binding energy and interacting amino acid residues (Table 1). DSV3 was used after molecular docking to find the binding residues and details of polar bonds including hydrogen bonds. Lactyl-CoA binds through six strong conventional hydrogen bonds to the binding residues ARG1312, GLU1423, LYS1426, LYS1427, GLU1477, ARG1478) of HAT p300 protein (Figure 7A and Figure 7C, Table 1). The binding affinity (-9.6 kcal/mol) and specificity of lactyl-CoA within the active site of HAT p300 shared almost identical binding attributes such as docking energy (-10.3 kcal/mol) and key residues (ARG1305, ASP1306, ARG1312, GLU1416, GLU1423, GLU1477, ARG1478) responsible for hydrogen bonds for a stable acetyl-CoA and HAT p300 enzyme complex (Figure 7B and Figure 7D). Molecular

interactions suggested that lactyl-CoA may display equivalent binding affinity that occupies the similar binding pocket of acetyl-CoA in the substrate binding site of the HAT p300 enzyme.

DISCUSSION

Cancer cells within the tumor microenvironment achieve metabolic reprogramming by concerted contributions from cellular and non-cellular factors (1-4). Indeed, the requirements of cancer cells are met through various metabolic networking including glucose metabolism and distinctive metabolic products including lactate (5-11). Given Warburg's effects on cancer cells, the production of lactate is suggested as a waste product. Currently, there is an emergence of understanding that cancer cells may export metabolic waste lactate to fuel the growth and metastasis by supporting various intracellular metabolic and non-metabolic epigenetic regulations of cancer-supporting cells such as macrophages within the tumor microenvironment (12-19).

Epigenetic alterations such as methylation, acetylation, succinylation, and newly included lactylation of histones and other protein targets are known to alter the pro-tumor attributes of cancer and cancer-supporting cells including macrophages (8-10). Furthermore, accumulating shreds of evidence have shown the existence of an axis between epigenetic changes and metabolic adaptations (11-19). At the same time, however, the key insights on the nature of substrate, the biological abundance of a substrate, and associated enzymes are missing.

Here, our data suggested a possible role of acetyl-CoA synthetase as a key enzyme that allows the binding of lactate to the same binding site as acetate. Interestingly, active site binding amino acid residues such as TRP413, TRP414, GLN415, ARG515, ASN521 are earlier reported in case of acetyl-CoA synthetase as in case of lactate vs. acetate (23-30). These findings hinted at the potential uses of acetyl-CoA synthetase by cancer cells to generate lactyl-CoA for the proposed lactylation process because of metabolic reprogramming. Then non-cancer cells including microbiotas with suitable enzymes may convert lactate to lactyl-CoA and then lactyl-CoA is released into the tumor microenvironment (23-27). The role of acetyl-CoA synthetase is depicted in the activation of macrophages mediated by pro-inflammatory bacteria (28-30).

In recent, modulation of epigenetic writer enzymes such as HATs are implicated in the inflammatory landscape of tumor microenvironment specifically in the context of polarization of M1 macrophage to M2 macrophage (31-34). Among various HATs, the enzymatic role of HAT p300 to alter the acetylation mark on histone and other tumor suppressors is highlighted (35-37.

But the relevance of HAT p300 in contributing lactylation marks on histone and other proteins using lactyl-CoA as a substrate remains obscure. The possibility of metabolic strategies by cancer cells to use lactylation as a part of mitigating acetylation marks on histone proteins could be explored. This would be almost similar to shunting off HATs by cancer and cancer-associated cells as a part of the activation of oncoproteins during chromatin remodeling to achieve pro-tumor attributes. In recent, non-histone proteins such as PKM2 and beta-catenin are suggested as protein targets that modulate the macrophage phenotype transition in cancer and other human disease conditions (11-19). The relevance of HAT p300 in modulating the transcriptional landscape of cancer cells and tumor-associated immune cells such as macrophages is considered as a link between the metabolic-epigenomic axis that may use metabolites such as acetyl-CoA and lactyl-CoA

In a direction to the unresolved question of the nature of substrate and enzymes in lactylation, there is a hint of the potential enzymatic role by HAT p300 (15-19). Among various HATs, HAT p300 is suggested to modulate the transcription of genes that are linked to the development of various human diseases including cancer and immune-related diseases that involve macrophages in their tissue environment (31-37). HAT p300 is determined to comprise several domains including HAT domain (1285-1664) amino acid residues (24-27). HAT p300 catalytic domain is antagonized by various small molecules including A-485, I-CBP112, natural products, and bi-substrate analogs (Lys-CoA) (31-37). However, binding affinity and position by lactyl-CoA to p300 HAT is not known and may be potentially linked with the lactylation process. Although the active site on HAT p300 is reported in earlier works by showing key amino acid residues such as PHE1374, LEU1398, SER1400, ARG1410, THR1411, TYR1414, HIS1415, ARG1312, GLU1423, LYS1426, LYS1427, GLU1477, and ARG1478 (31-37). Importantly, molecular docking data on specific and similar binding by both lactyl-CoA and acetyl-CoA indicated a strong binding to the reported amino acid residues such as ARG1312, GLU1423, LYS1426, LYS1427, GLU1477, and ARG1478. These amino acid residues interact with several known natural and synthetic substrates including acetyl-CoA, lysyl-CoA, A-485, and I-CBP112. It is interesting to note that lactate did not have any binding affinity within the active site of HAT p300

The above observations are in coherence with a recent finding that the lactylation process modulates the transcriptional gene regulation in M1 macrophage and allows it to change into M2

macrophage (18-19). Zhang et al.[12] discovered the process of lactylation with experimental evidence at molecular and cellular levels. However, pertinent questions were not answered on the nature of the enzyme and biological source and relevance of lactyl-CoA that may potentially act as a substrate for a potential enzyme such as HAT p300. Our data is the first and novel proposition on the biological possibilities of lactyl-CoA within the tumor microenvironment and the mode of lactylation mediated by the HAT p300 enzyme.

One of the crucial messages that could be derived from this work is that extracellular lactate due to drug-induced stress and death may be an additional adaptive mechanism by cancer cells to suppress and fine-tune immune cells including the transition of macrophage phenotype (11-22).

Future propositions

- It would be interesting to explore the relevance of extracellular lactate in the tumor niche and conversion into lactyl-CoA by pro-tumor microbiotas (35-37) by utilizing acetyl-CoA synthetase enzyme. This will uncover additional mechanisms that can explain the availability of lactyl-CoA.
- Since both lactyl-CoA and acetyl-CoA bind to the same active HAT domain of HAT p300, it would be interesting to evaluate whether lactylation epigenetic marks may inhibit acetylation marks on chromatin that could drive certain cells such as macrophages toward polarization from anti-tumor M1-macrophage to pro-tumor M2 macrophage in the tumor microenvironment.
- In addition, the authors make a proposition that the availability of lactyl-CoA within the tumor microenvironment may be linked to the nature of microbiotas that are equipped with an enzyme that may convert lactate into lactyl-CoA. Hence shuttling of lactate and lactyl-CoA is proposed between cancer cells, microbiotas, and immune cells such as macrophages within the tumor microenvironment.
- The impact of these findings will have a significant contribution to solving the unanswered questions on the molecular mechanisms of lactylation in the context of the tumor microenvironment. Herein, the authors propose a future model in that lactate is shuttled into the tumor niche harboring microbiotas during external agents-mediated cell death to cancer cells.

- In this way, microbiotas are equipped with the metabolic machinery including lactyl-coenzyme A and acetyl-CoA enzyme to generate lactyl-CoA from lactate (35-36).
- In the future, research attempts would be challenging to understand and evaluate the shuttling of lactate and lactyl-CoA among cancer cells, cancer-supporting macrophages, and microbiotas that could contribute to the pro-tumor microenvironment.
- Hence, this study proposes future investigations to understand the lactylation process in the tumor microenvironment by exploring cellular links among cancer cells, macrophages, and pro-tumor microbiotas.
- A future link between the overexpression of P-gp in cancer cells and the distribution of CoA, lactate, lactyl-CoA, and acetyl-CoA among cellular components of the tumor microenvironment may be explored and linked with pro-tumor metabolic and epigenetic reprogramming.
- In the future, small pharmacological inhibitors of HAT p300 could be employed to quench the polarization of macrophage and in turn, this would be an additional avenue for combinatorial therapies along with chemotherapy to lead to drug resistance by upregulating lactylation mediated process.
- A concept of mimetic of lactate and lactyl-CoA could be considered to hinder the polarization of macrophages and metabolic reprogramming of microbiotas in the tumor niche to achieve new avenues of anticancer therapies.
- A very recent paper revealed the mechanism of lysine lactylation in *E. coli* (34) and it would be fascinating to see the effects of microbiotas with the machinery of lactylation enzymes such as YiaC, CobB, and YdiF that can use extracellular lactate in the tumor microenvironment and providing signaling molecules to cancer cells and cancer-associated immune such as macrophages as pro-tumor phenotype.
- A proposed model on the relevance of extracellular lactate in the lactylation process in cancer and cancer-supporting cells such as macrophages that shape up the pro-tumor microenvironment is presented (Figure 8).

CONCLUSION

In conclusion, our data suggested the presence of extracellular lactate in response to anticancer drug compositions enriched with free fatty acids and tripeptides. Importantly, lactate was not noticeable in the extracellular compartment of DMSO-treated breast cancer cells.

Additionally, lactyl-CoA was not detected in the extracellular compartment of breast cancer cells. Molecular docking and MD simulations predicted that lactate could serve as a substrate for acetyl-CoA synthetase besides its known substrate acetate. These projections helped to hypothesize that acetyl-CoA could have the potential to convert lactate into lactyl-CoA in the tumor microenvironment and the ensuing lactylation-mediated epigenetic process. Furthermore, molecular interaction studies helped to propose that HAT p300 may serve as a potential enzyme that can use lactyl-CoA to transfer the lactate group for the lactylation of histones and other target proteins. The nature of the data is based on *in vitro* detection of lactate and molecular docking and MD simulations, and these findings are novel and could be a meaningful incremental step to resolve gaps in the lactylation and tumor microenvironment.

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Authors Declarations:

"Data availability" statements

- The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.
- All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Authors' contributions:

Rushikesh Patel: Data collection, Methodology, Interpretation

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Kiran B Lokhande: Data collection, Methodology, Manuscript draft preparation

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Jayanta K. Pal: Interpretation, Manuscript draft preparation

Nilesh Kumar Sharma: Conceptualization/Idea, Interpretation, Manuscript draft preparation

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Details of Figures and their legends:

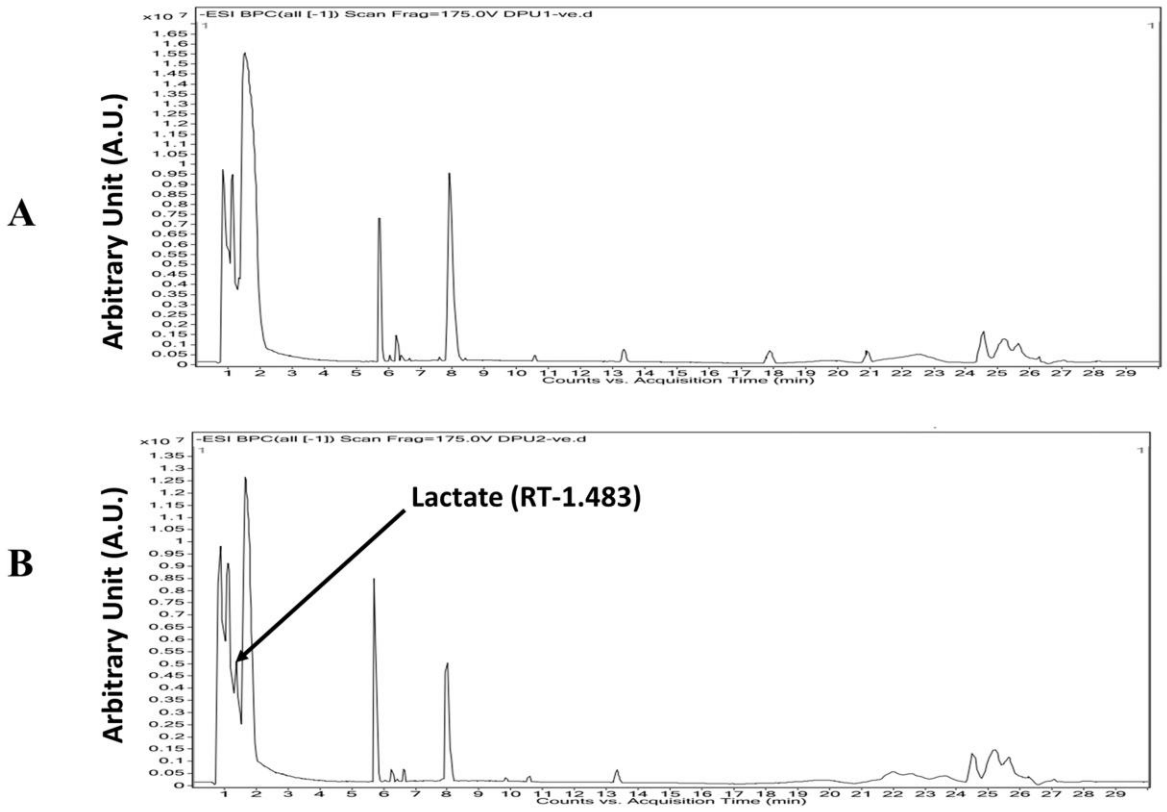
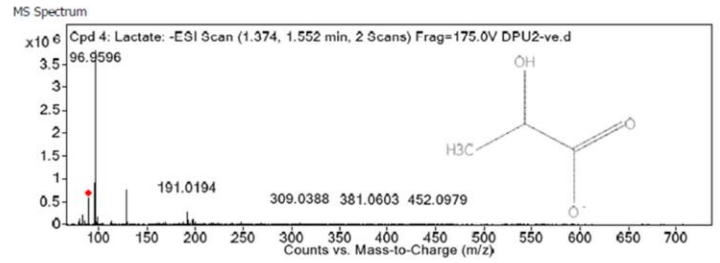
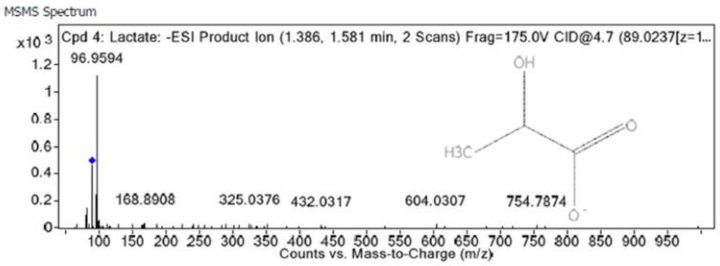


Figure 1. Distinctive extracellular metabolite profiling of MCF-7 breast cancer cells treated by DMSO and CUDF, anticancer drug compositions.

Negative ESI total ion chromatogram of extracellular conditioned medium of MCF-7 breast cancer cells. A. DMSO treated MCF-7 breast cancer cells. B. CUDF treated MCF-7 breast cancer cells.

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 4: Lactate	**Lactate**	89.0239	1.483	Auto MS/MS	90.0311





MS/MS Spectrum Peak List

m/z	z	Abund
78.9584		37.96
79.9554		97.13
82.9524		147.94
89.0235	1	454.38
95.9507		244.39
96.9594		1124.1
97.9474	1	46.2
98.9542		54.64
168.8908		30.34
325.0376		26

Figure 2. Lactate is detected in the extracellular conditioned medium of MCF-7 breast cancer cells.

Negative ESI MS and MS/MS fragment ion spectra of lactate by LC-HRMS.

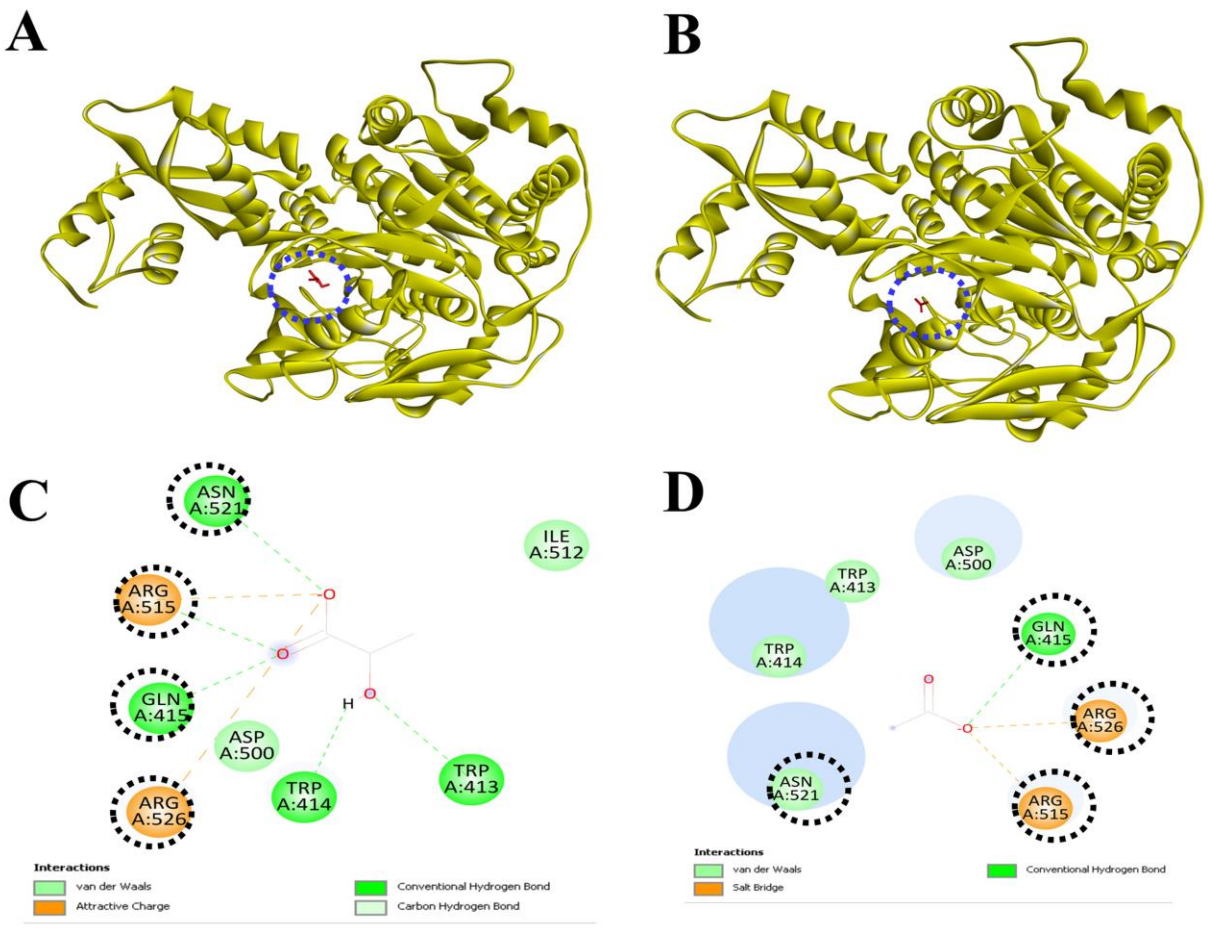


Figure 3: A metabolite lactate (PubChem ID-91435) shows similar molecular interactions as acetate (PubChem ID-30089) with Acetyl-CoA Synthetase (PDB ID: 2P2F)

(A). An emphasized 3-D ribbon structure on a docked site of lactate with Acetyl-CoA Synthetase bound (B). DSV3 assisted 3-D image of Docked pocket surface structure between lactate and Acetyl-CoA Synthetase showing H-bond interaction (Green colour) and steric interaction (Violet colour) between ligand and target protein. (C). An emphasized 3-D ribbon structure on a docked site of acetate with Acetyl-CoA Synthetase. (D). DSV3 assisted 3-D image of docked pocket surface structure between acetate and Acetyl-CoA Synthetase showing H-bond interaction (Green colour) and steric interaction (Violet colour) between ligand and target protein.

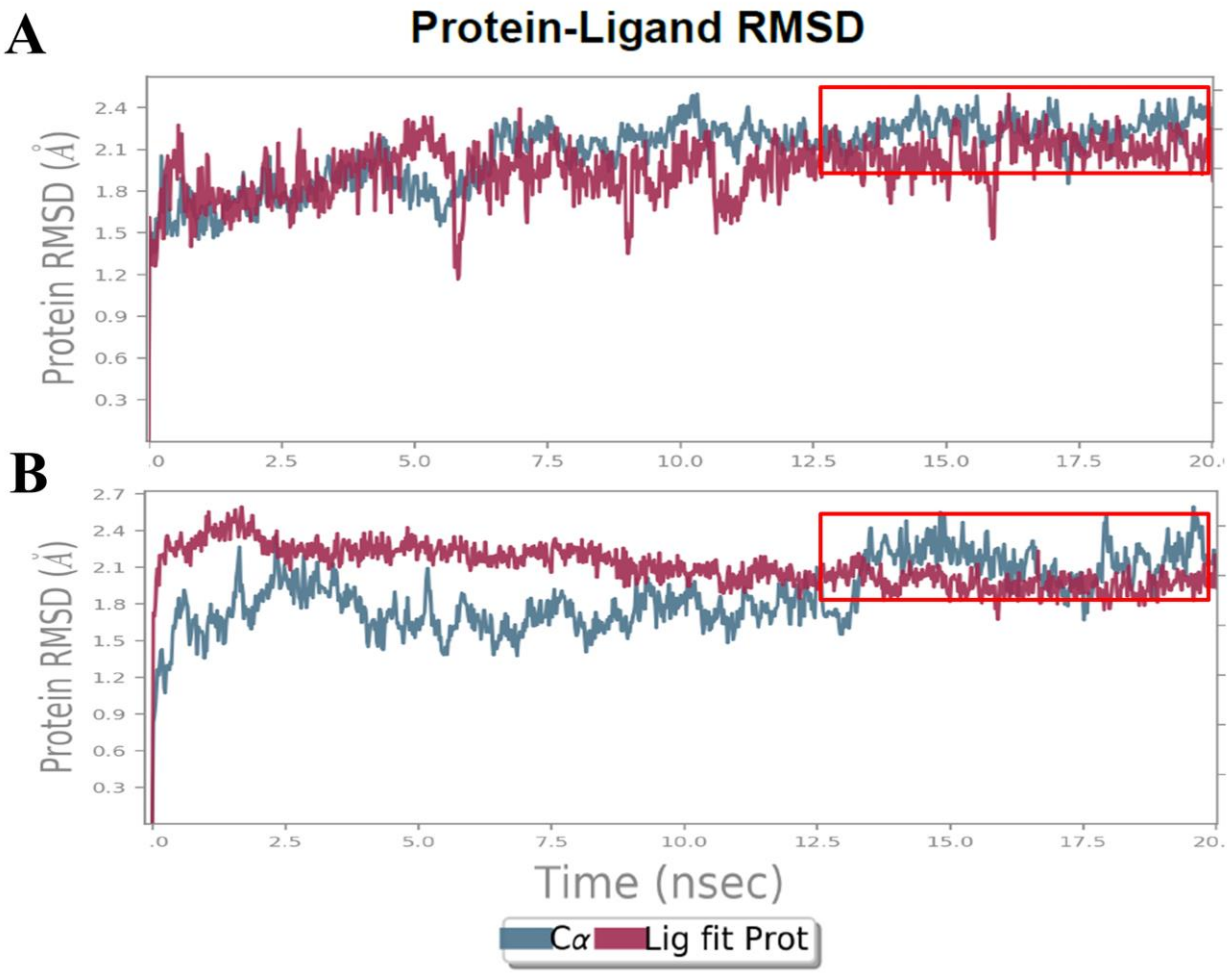


Figure 4. Protein-ligand Room Mean Square Deviation (RMSD) shows the stable complex between lactate and Acetyl-CoA Synthetase and attributes match with a known substrate acetate. In this figure, the left-Y axis denotes the RMSD evolution of Acetyl-CoA Synthetase because of structural conformation during simulation for a duration 20ns. The order of changes of RMSD values of protein is within the acceptable range of 1-3Å. (A). RMSD plot of lactate-acetyl-CoA synthetase complex. (B). RMSD plot of acetate-acetyl-CoA synthetase complex

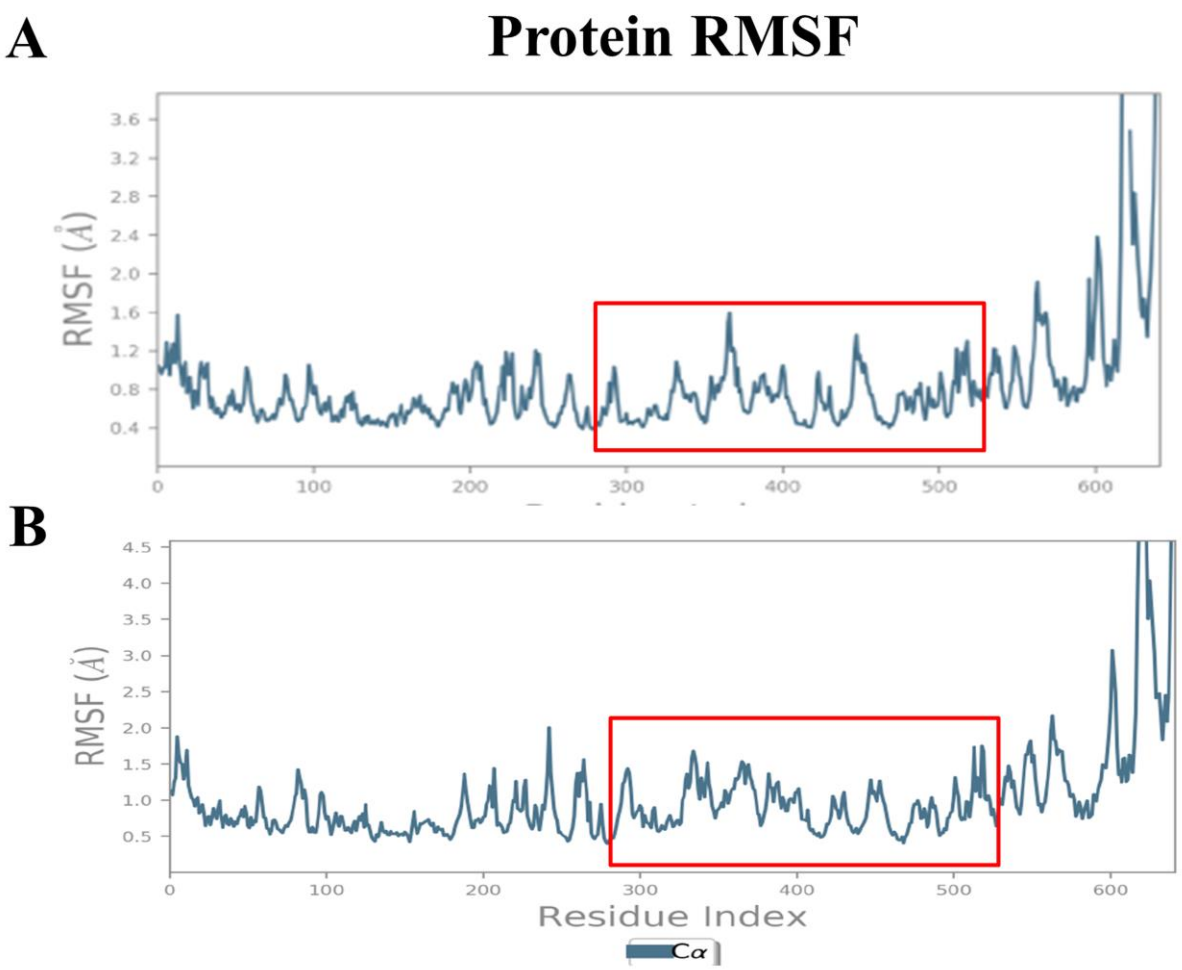


Figure 5. Protein-ligand Room Mean Square fluctuations (RMSF) plot depicts the good stability between lactate and Acetyl-CoA Synthetase and the least fluctuation attributes match with a known substrate acetate.

In this figure, the left-Y axis denotes the RMSF evolution of Acetyl-CoA Synthetase because of structural conformation during simulation for a duration 20ns. The order of changes of RMSF values of protein is within the acceptable range of 1-2Å. (A). RMSF plot of lactate-acetyl-CoA synthetase complex. (B). RMSF plot of acetate-acetyl-CoA synthetase complex

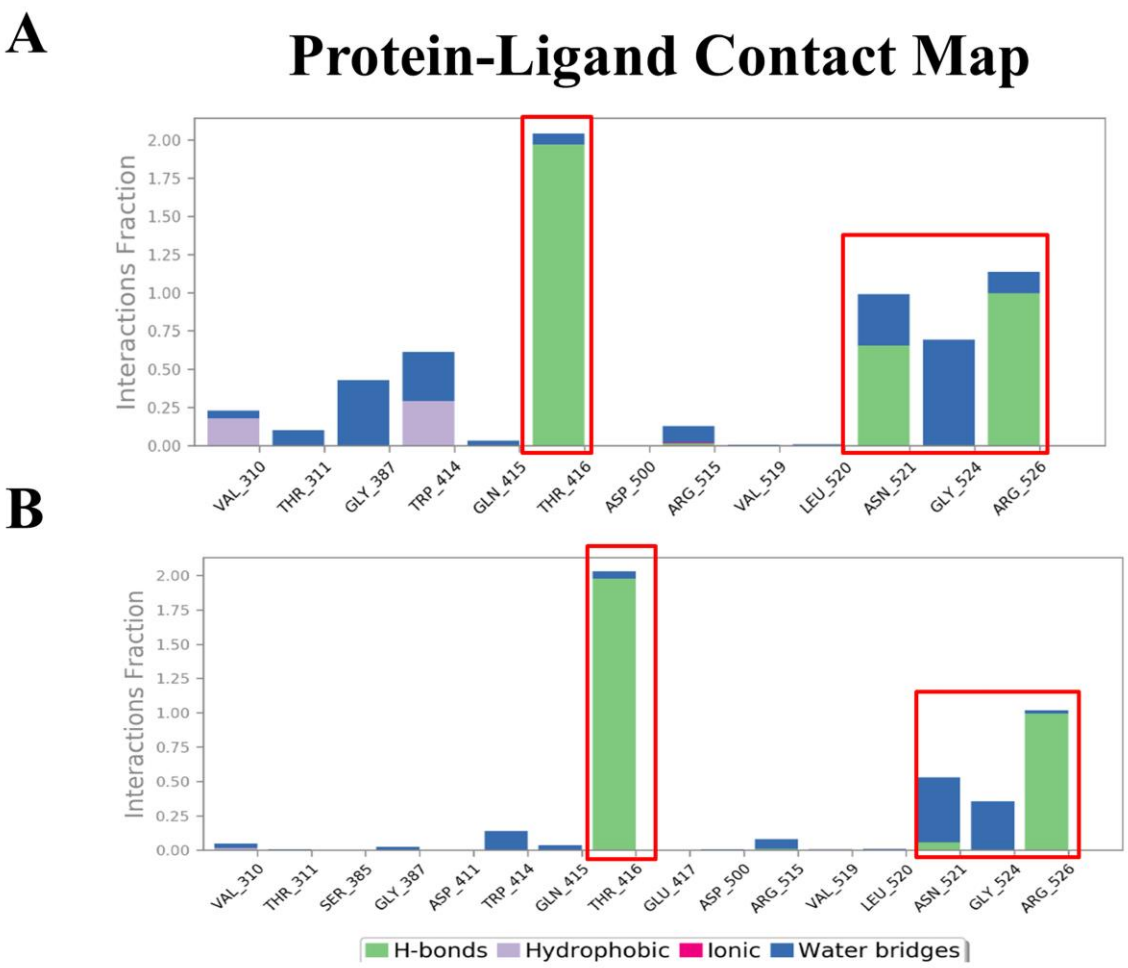


Figure 6. Protein Acetyl-CoA synthetase shows an appreciable and similar contact map with lactate and acetate, a known substrate.

(A). Protein-ligand contact map lactate-acetyl-CoA synthetase complex. (B). Protein-ligand contact map of acetate-acetyl-CoA synthetase complex

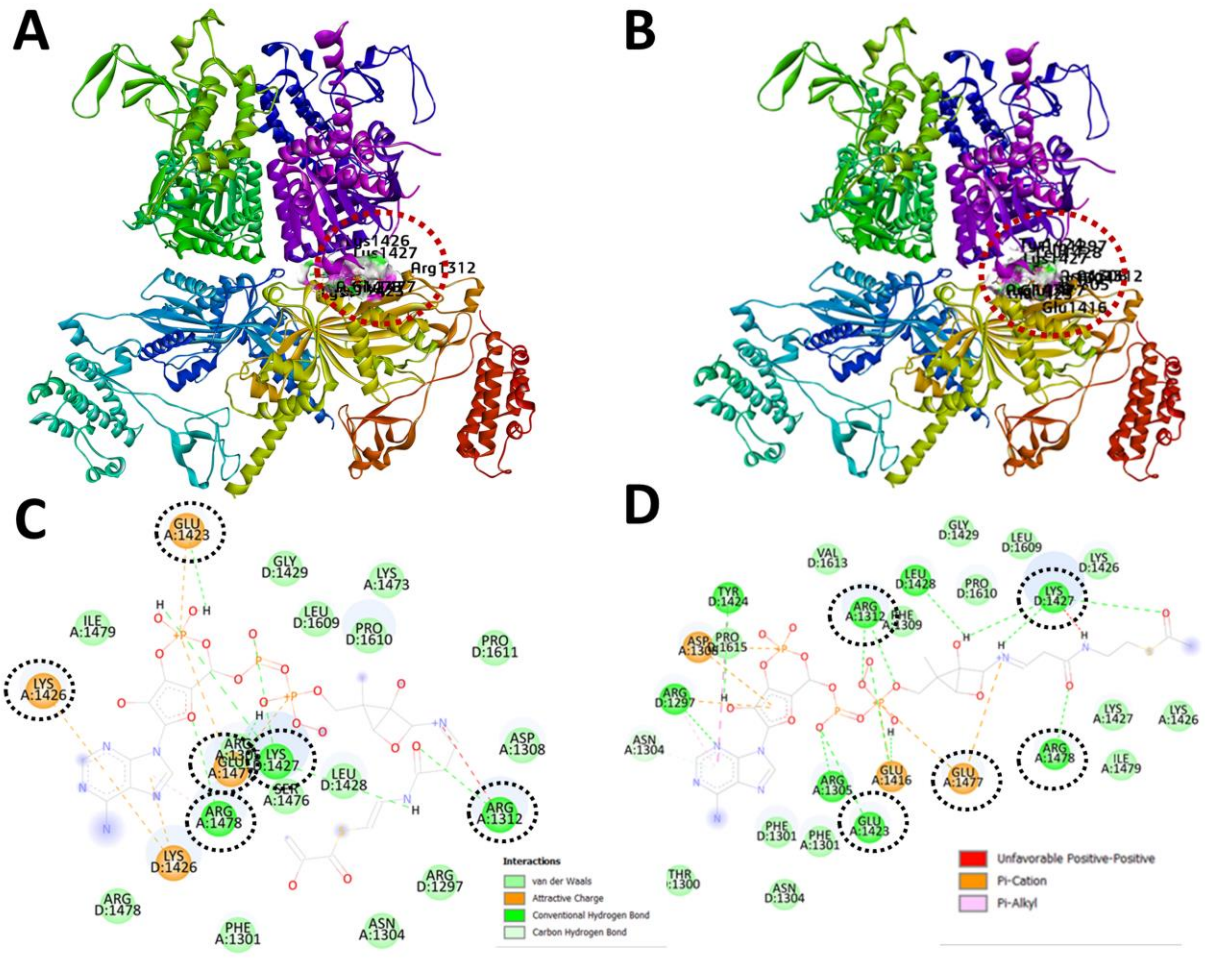


Figure 7. A lactate-derived metabolic product lactyl-CoA (PubChem CID: 3081970) showed strong and specific binding to acetyl-CoA synthetase and lactyl-CoA is predicted as a substrate for lactylation.

(A). A ribbon structure with a full 3D view between lactyl-CoA and acetyl-CoA synthetase (B) A ribbon structure with a full 3D view between acetyl-CoA and acetyl-CoA synthetase (C). DSV3 assisted 3-D image of Docked pocket surface structure between lactyl-CoA and acetyl-CoA synthetase (D) DSV3 assisted 3-D image of Docked pocket surface structure between acetyl-CoA and acetyl-CoA synthetase.

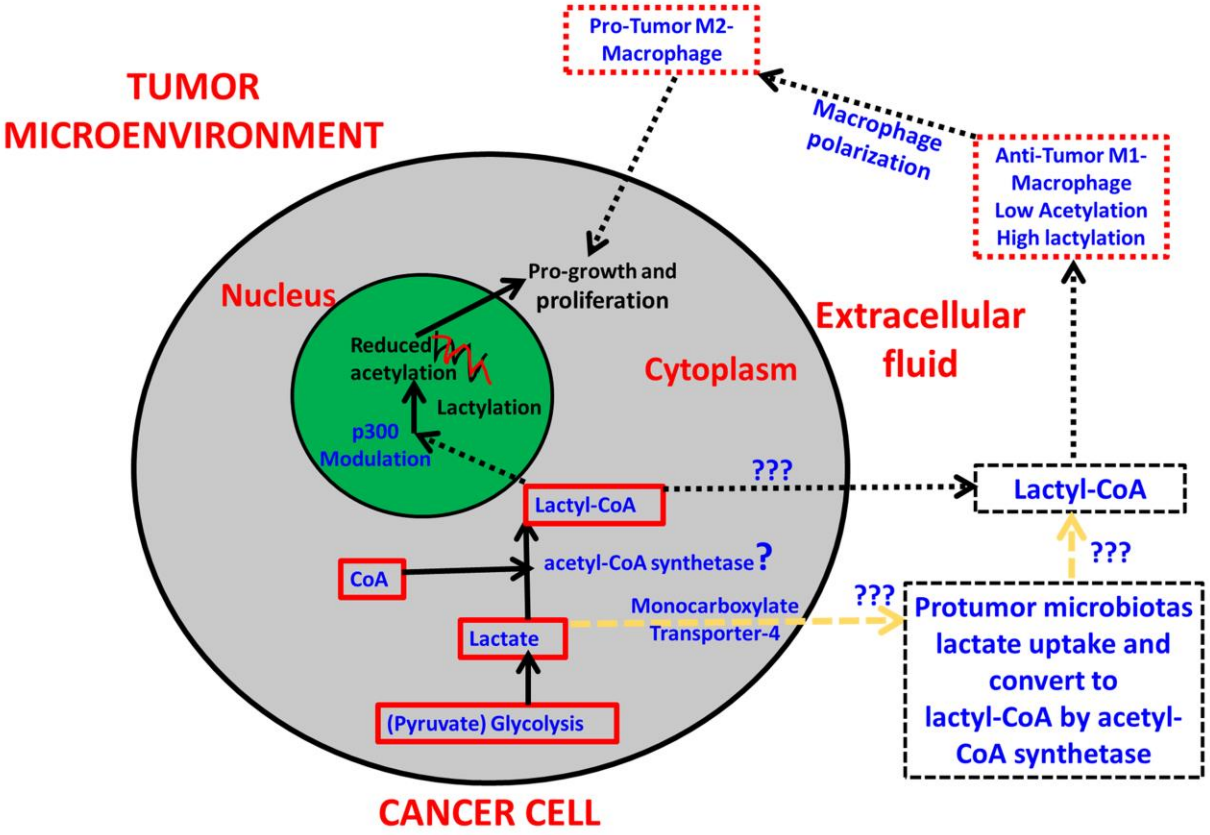


Figure 8. A proposed model on the role of lactate and lactyl-CoA in the lactylation process during epigenetic changes in the tumor. A possible pathway is speculated within the tumor microenvironment that hosts cancer cells and non-cancer cells including immune cells, stromal cells, and microbiotas.

Table 1. Molecular interactions of lactate, acetate, lactyl-CoA and acetyl-CoA to HAT p300 and acetyl-CoA synthetase using AutoDock Vina and DSV3.

Name of LIGAND	Protein PDB ID and name of chain	Binding energy (-kcal/mol)	Interacting residues	No. of Hydrogen bonds	RMSD value l.b.	RMSD value u.b
lactate (PubChem CID: 91435)	acetyl-CoA synthetase (PDB ID: 2P2F)	-3.9	TRP413 TRP414 GLN415 ARG515 ASN521	5	0.0	0.0
acetate (PubChem CID: 175)	acetyl-CoA synthetase (PDB ID: 2P2F)	-3.1	GLN415 ARG515 ARG526	3	0.0	0.0
lactate (PubChem CID: 91435)	HAT p300 (PDB ID: 6GYR)	-4.0	GLN1455	1	0.0	0.0
lactyl-CoA (PubChem CID: 3081970)	HAT p300 (PDB ID: 6GYR)	-9.6	ARG1312 GLU1423 LYS1426 LYS1427 GLU1477 ARG1478	6	0.0	0.0
acetyl-CoA (PubChem CID-444493)	HAT p300 (PDB ID: 6GYR)	-10.3	ARG1305 ASP1306 ARG1312 GLU1416 GLU1423 GLU1477 ARG1478	7	0.0	0.0