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Dual Tumor Targeting Ability of Fatty Acyl (Amide) Amino Acid Alkyl Ester Conjugates: *In vitro* Cytotoxicity and DNA Cleavage based Bioactivity

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In this work, the bioactivity of fatty acyl (amide) amino acid alkyl esters through cytotoxicity and DNA cleavage properties were assessed. The synthetic method utilized HBTU-catalyzed amide formation between the carboxyl group of stearic acid and linoleic acid and the amine group in L-glutamic acid dimethyl ester and L-methionine methyl ester. The conjugates were elucidated using FTIR, NMR and mass and evaluated for cytotoxicity (COLO-205, A549 cell lines). Conjugates **10a-c** exhibited selective growth inhibitory effects on cancer cell lines, while no impact was detected on normal cells. Overall, there was a significant reduction in the proliferation of human colorectal (COLO-205) and lung cancer (A549) cancer cells. Conjugates **10a-c** also exhibited favourable cytocompatibility on normal murine fibroblast (L929) cells. Conjugate **10c** showed promiscuous *in vitro* cytotoxicity against colorectal (COLO-205) and lung (A549) cancer cells compared to conjugates **10a** and **10b**.

Keywords: Cytotoxicity, DNA cleavage, Fatty acid, Amino acid methyl ester.

INTRODUCTION

Colorectal cancer (CRC) is the third most prevalent malignant tumor and the second most lethal kind of cancer. It is responsible for approximately 1.9 million new cases and 0.9 million deaths globally in 2020 [1,2]. Colorectal cancer (CRC) is more common in highly developed countries and is increasing in middle- and low-income countries due to the adoption of western lifestyles [3]. The significant prevalence of CRC cases is an escalating worldwide public health dilemma. Increasing awareness of CRC is crucial for adopting healthy lifestyle choices, innovative approaches to managing CRC and establishing worldwide screening initiatives. These efforts are essential for decreasing the incidence and death rates associated with CRC in the future. Thus, colorectal cancer is a diverse illness with several subtypes that have an impact on prognosis and response to treatment [4]. Current therapeutic limitations further need to be rectified by designing suitable therapeutic alternatives containing scaffolds of amino acids, fatty acids and bioactive molecules.

Amino acids are essential organic compounds for living things and are exciting building blocks for designing biodegradable and biocompatible polymeric biomaterials with different physico-chemical and biological properties [5]. The polymerization of drugs with amino acids or their derivatives proved to be good choice in targeted drug delivery. These systems possess responsive features, including temperature, pH and enzyme reactions, which may be tailored to specific sites [6].

Fatty acids can undergo simple transformations that produce pharmacologically significant molecules due to the presence of a hydrocarbon chain and a reactive carboxylic group [7]. A frequently used approach involves conjugation of the carboxyl end of lipid with a hydroxyl or amine group of the drug to produce a desired ester or amide linkage [8]. Lipid-drug conjugates containing hydrazone bonds enable effective decomposition at a lower pH [9]. These modifications augmented the lipophilicity of hydrophilic drugs and improved their compatibility with lipophilic cell membranes and components of drug delivery carriers [10]. Fatty acid-drug conjugates in the form of emulsions or micelles have several benefits for the improve-

ment in oral bioavailability of drugs [11]. Additionally, these conjugates reduce the toxicity of anticancer drugs as reported by Bradley *et al.* [12].

Tumor cells have an increased uptake of natural fatty acids for energy and biochemical processes, making these conjugates an effective delivery system as explained by Dauchy *et al*. [13]. Lipophilic prodrugs are utilized to induce prolonged drug release by involving in the lipid metabolism pathways that evade hydrolysis and displays enhanced interactions with cell membranes [14]. In addition, specific membrane protein modifications with lipid membrane have been identified as possible targets for cancer treatment. It is based on the idea that specific lipids can be used to change the content and structure of cancer cell membranes. This process may break down the structure of lipid rafts and modify the positioning and function of proteins that are connected to the cell membrane. As a result, it disrupts the pathways that are essential for the proliferation of tumor cells [15]. The combination of fatty acids with biologically active substances leads to the formation of hybrid molecules that exhibit a wide range of improved biological activities, such as antibacterial and antifungal effects [16]. Fatty acid derivatives are known to possess several biological features, including anti-inflammatory, antiproliferative [17] and antioxidant activities [18]. According to literature reports, several modified fatty acids show promising features for treating malignancies [19].

These factors have spurred interest in the tactical development of novel drug conjugates to facilitate more cost-effective scaling. However, despite significant advancements, ongoing research aims to discover a more effective anticancer agent with diminished adverse effects, enhanced bioavailability and heightened clinical efficacy by generating new drug conjugates. Based on the information mentioned above and as part of our ongoing study on designing anticancer agents, we developed conjugates consisting of fatty acid (amide) amino acid alkyl esters. Furthermore, conjugates were evaluated for their anticancer properties against human colorectal (COLO-205), lung cancer (A549) and normal murine fibroblast (L929) cells. Furthermore, DNA cleavage studies was performed on the most cytotoxic conjugate to assess DNA cleavage ability.

EXPERIMENTAL

Sigma-Aldrich supplied *N*,*N*,*N*′,*N*′-tetramethyl-O-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), triethylamine (TEA), L-glutamic acid dimethyl ester hydrochloride and L-methionine methyl ester hydrochloride. Stearic and linoleic acids were procured from TCI Chemicals, Japan. A Perkin-Elmer FT-IR spectrophotometer recorded spectra from KBr discs containing ~5% w/w samples. The NMR study was performed in DMSO-d_6 and tetramethylsilane as internal standard (Bruker AC spectrometer recorded ^{1}H 300 MHz, ^{13}C 75 MHz spectra). HRMS was recorded on a Shimadzu QP2010 and the melting points were recorded using the MEL-TEMP capillary apparatus.

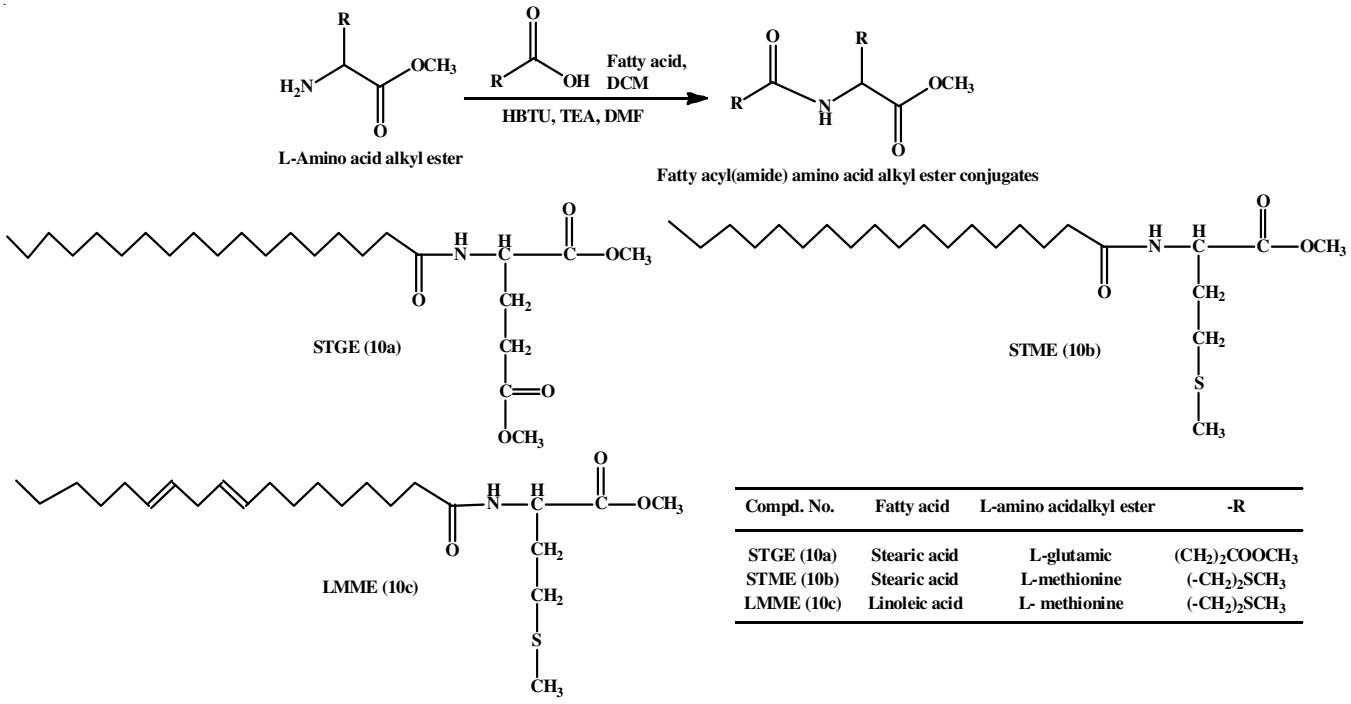
General procedure

Synthesis of fatty acid-based amide containing amino acid alkyl ester (FAAE, 10a-c): Fatty acyl amide conjugates containing amino acid alkyl ester (**10a-c**) were synthesized in the presence of HBTU reagent *via* a coupling reaction between amino acid alkyl ester and the fatty acids. The reaction vessel containing dried DCM (25 mL), HBTU (0.33 mmol) and fatty acid (1 mmol) were mixed slowly with continuous stirring. After that amino acid alkyl ester (1 mmol), TEA (0.77 mmol) and DMF were suspended in the reaction mixture and the reaction was continued under a nitrogen atmosphere for 5 h. The completion of the reaction was confirmed by TLC in a DCM/methanol (7:3 v/v) solvent system. The crude product was poured in 50 mL of water and extracted with DCM (100 mL). Then, the organic phase was passed over sodium sulfate and evaporated to dryness under reduced pressure (**Scheme-I**). Finally, the crude product was purified with column chromatography using silica gel adsorbent and a CH_2Cl_2:CH_3OH (7:3) solvent system [20].

Stearic acid (amide)glutamic acid dimethyl ester (STGE, 10a): Pale green crystal; m.p.: 122-124 ºC; IR (KBr, ν_{max}, cm^{-1}): 3356, 2945, 2867, 2565, 2515, 1717, 1754, 1666, 1645, 1434, 1323; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 0.84 (s, 3H, -CH_3), 1.49 (s, 26H, -CH_2), 2.24 (s, 2H, -CH_2), 3.94 (s, 2H, CH_2), 3.06 (s, 2H, -CH_2), 3.08 (s, 2H, CH_2), 3.11 (s, 2H, -CH_2), 3.58 (s, 6H, 2× -$COOCH_3$), 4.10-4.14 (m, 1H, -COCH), 8.17 (s, 1H, CONH); ^{13}C NMR (75 MHz, DMSO-d_6): 14.2 (-CH_3), 22.7 (-CH_2), 25.9 (-CH_2), 26.7 (-CH_2), 27.6 (-CH_2), 28.5 (-CH_2), 29.1 (-CH_2), 29.4 (-CH_2), 29.6 (-CH_2), 29.7 (-CH_2), 29.9 (-CH_2), 30.0 (-CH_2), 30.1 (-CH_2), 30.4 (-CH_2), 30.5 (-CH_2), 30.7 (-CH_2), 30.9 (-CH_2), 31.7 (-CH_2), 36.7 (-CH_2), 51.5 (-OCH_3), 51.8 (-OCH_3), 52.1 (-CH), 171.5 (-C=O), 172.6 (-C=O), 173.2 (-C=O); HRMS: *m/z* 442.3566 (M+H)$^+$. Elemantal analysis calcd. (found) % for $C_{25}H_{47}NO_5$ (*m.w.* 441.35): C, 67.99 (68.01); H, 10.73 (10.55); N, 3.17 (3.20); O, 11.75 (11.80).

Stearic acid (amide)methionine methyl ester (STME, 10b): Yellowish green crystal; m.p.: 138-140 ºC; IR (KBr, ν_{max}, cm^{-1}): 3326, 2935, 2878, 2845, 2570, 1757, 1735, 1653, 1614, 1474, 1359; ^{1}H NMR (300 MHz; DMSO-d_6) δ ppm: 1.09 (s, 3H, -CH_3), 1.25-1.29 (m, 26H, CH_2), 1.89 (s, 2H, CH_2), 1.91 (s, 2H, CH_2), 1.94 (s, 2H, CH_2), 2.04 (s, 2H, -CH_2), 2.09 (s, 2H, -CH_2), 2.29 (s, 3H, S-CH_3), 4.36 (t, 1H, -COCH), 3.74 (s, 3H, -COO-CH_3), 8.08 (s, 1H, -CONH); ^{13}C NMR (75 MHz; DMSO-d_6) δ ppm: 14.4 (-CH_3), 17.7 (-S-CH_3), 22.6 (-CH_2), 25.5 (-CH_2), 25.8 (-CH_2), 28.9 (-CH_2), 29.1 (-CH_2), 29.4 (-CH_2), 29.6 (-CH_2), 29.7 (-CH_2), 29.9 (-CH_2), 30.0 (-CH_2), 30.2 (-CH_2), 30.5 (-CH_2), 30.7 (-CH_2), 30.9 (-CH_2), 31.0 (-CH_2), 31.4 (-CH_2), 31.8 (-CH_2), 36.7 (-CH_2), 51.4 (-CH), 51.7 (-OCH_3), 171.8 (-C=O), 172.5 (-C=O); HRMS: *m/z* 430.3375 (M+H)$^+$. Elemantal analysis calcd. (found) % for $C_{24}H_{47}NO_3S$ (*m.w.* 429.33): C, 67.08 (67.11); H, 11.02 (11.04); N, 3.26 (3.24); O, 11.72 (11.80); S, 7.20 (7.25).

Linoleic acid (amide) methionine methyl ester (LMME, 10c): Dark green crystal; m.p.: 145-147 ºC; IR (KBr, ν_{max}, cm^{-1}): 3256, 2896, 2843, 2862, 2560, 2535, 1869, 1770, 1728, 1663, 1644, 1450, 1354; ^{1}H NMR (300 MHz; DMSO-d_6) δ ppm: 0.97 (s, 3H, -CH_3), 1.15 (s, 3H, S-CH_3), 2.10-1.17 (m, 26H, 13× -CH_2), 2.55 (s, 2H, -CH_2), 3.76 (s, 3H, $COOCH_3$), 3.00 (t, 1H, -COCH), 5.09 (q, 2H, 2× -CH=CH), 5.34 (m, 2H, 2× -CH=CH), 8.10 (s, 1H, -CONH); ^{13}C NMR (75 MHz; DMSO-d_6) δ ppm: 14.5 (-CH_3), 17.5 (-S-CH_3), 22.7 (-CH_2), 25.6 (-CH_2), 28.5 (-CH_2),



Compd. No.	Fatty acid	L-amino acidalkyl ester	-R
STGE (10a)	Stearic acid	L-glutamic	$(CH_2)_2COOCH_3$
STME (10b)	Stearic acid	L-methionine	$(-CH_2)_2SCH_3$
LMME (10c)	Linoleic acid	L- methionine	$(-CH_2)_2SCH_3$

Scheme-I: Synthesis of fatty acyl(amide) amino acid alkyl ester conjugates (**10**)

29.3 ($-CH_2$), 29.6 ($-CH_2$), 29.7 ($-CH_2$), 29.8 ($-CH_2$), 30.0 ($-CH_2$), 31.2 ($-CH_2$), 32.1 ($-CH_2$), 33.7 ($-CH_2$), 33.9 ($-CH_2$), 36.6 ($-CH_2$), 37.7 ($-CH_2$), 51.4 (-CH), 51.8 ($-OCH_3$), 127.2 (-CH), 127.3 (-CH), 132.0 (-CH), 132.2 (-CH), 171.5 (-C=O), 172.6 (-C=O); HRMS: *m/z* 426.6784 $(M+H)^+$. Analysis calcd. (found) % for $C_{24}H_{43}NO_3S$ (*m.w.* 425.67): C, 67.72 (67.75); H, 10.18 (10.15); N, 3.29 (3.27); ; O, 11.57 (11.52); S, 7.16 (7.10).

Cell cultures: National Centre for Cell Science (NCCS), Pune, India provided the human colorectal cancer cell line (COLO-205), lung carcinoma (A549) and normal mouse fibroblast (L929). The cells were cultivated in 75 cm^2 cell culture flasks using DMEM medium. Fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (10,000 units of penicillin and 10 mg of streptomycin in 0.9% NaCl) were added as supplements. The culture flasks were maintained at 37 ° C in a humidified environment with 5% CO_2, 95% air.

***In vitro* anticancer activity using MTT assay:** The cytotoxicity assessment for doxorubicin and synthesized conjugates (STGE **10a**, STME **10b** and LMME **10c**) was performed on colorectal (COLO-205), lung (A549) cancer cells and normal mouse fibroblast (L929). In brief, the colorectal cancer (COLO-205), lung carcinoma (A549) and normal mouse fibroblast (L929) cells were cultured into a 96-well plate and incubated for 24 h at 37 ºC in an atmosphere containing 5% CO_2 to promote cell adhesion in culture medium (DMEM supplemented with 10% fetal bovine serum, 1% antibiotics containing penicillin and streptomycin). Following the incubation period of 24 h, each well of the cells was supplemented with 100 µL of fresh culture medium containing conjugates **10a-c** and doxorubicin at 31.25-1000 µg/mL concentrations in triplicate. After incubation of 48 h, the medium was withdrawn and cells were washed with phosphate buffered saline (PBS). Then, MTT solution was added to each well and the cells were further incubated for an additional 4 h. After that cell medium was disposed off and 100 µL of DMSO was added to each well to dissolve the formazan dye crystals. The absorbance was measured using the microplate reader at 490 nm. Furthermore, the cytocompatibility of synthesized conjugates **10a-c** and doxorubicin (31.25 µg/mL, 1000 µg/mL concentrations) were determined on normal mouse fibroblast (L929) cells [20].

DNA cleavage study of conjugate 10c (LMME): The agarose gel electrophoresis monitored the cleavage activity of conjugate **10c** at different concentrations *viz*. 100, 200 and 400 µg/mL). Supercoiled pUC19 plasmid DNA (350 ng) in Tris-HCl (100 mM, pH 8.0) buffer was treated with specified concentrations of conjugate **10c** in a typical experiment. The samples were kept at room temperature and loaded onto a 1.5% agarose gel with a 0.5X loading buffer of 40% sucrose and 0.02% bromophenol blue. Electrophoresis was conducted at a voltage of 100 V for 90 min in a TAE (tris acetate EDTA) buffer. The sample was stained with ethidium bromide. Experimental conditions were kept consistent for the control assays. The gels were observed using a UV transilluminator and the images were acquired with an attached camera and processed by using Alpha DigiDoc TM RT. Version V.4.1.0 PC-Image software [21].

RESULTS AND DISCUSSION

The systematic modification of various fatty acid skeletons with different L-amino acid alkyl esters using a chemically fertile carboxylic group led to corresponding conjugates. The synthesis was carried out by following literature methods with slight modifications. Initially, a standard coupling agent HBTU activated the free carboxylic groups in fatty acids. Then the addition of amino acid alkyl ester in the presence of TEA reacted to the activated carboxylic group leads to the formation

of the corresponding fatty acyl (amide) amino acid alkyl ester conjugates (**10a-c**). The structures of all the three conjugates were confirmed using FTIR, NMR and mass spectroscopy.

***In vitro* cytotoxic activity:** The cytotoxic potential and cytocompatibility of conjugates were determined by monitoring the impact of synthesized conjugates **10a-c** on COLO-205, A549 and L929 cells. In this study, we determined the tumor tissue selectivity of conjugates **10a-c** by comparing the cytotoxicity (IC_{50}) on the human cancer cell lines (COLO 205) and lung (A549) (Table-1). The conjugate **10c** (IC_{50} = 9.63 ± 0.90 μM) showed more cytotoxicity than conjugate **10b** (IC_{50} = 24.55 ± 1.67 μM) and conjugate **10a** (IC_{50} = 35.91 ± 3.40 μM) on COLO-205 cells, while being less cytotoxic than standard doxorubicin (IC_{50} = 3.70 ± 0.50 μM). The order of cytotoxic potency on COLO-205 was doxorubicin > **10c** > **10b** > **10a** in the study. Conjugate **10c** also exhibited more selectivity on COLO-205 cells than conjugate **10b**, conjugate **10a** and doxorubicin. Besides this, conjugate **10c** (IC_{50} = 17.82 ± 1.54 μM) showed more cytotoxic efficiency in comparison to conjugate **10b** (IC_{50} = 23.76 ± 2.74 μM) and conjugate **10a** (IC_{50} = 40.68 ± 4.35 μM) in A549 cells. All the conjugates (**10a-c**) revealed less potency than doxorubicin in lung cancer cells (A549) (Table-1). The cytotoxic effect was doxorubicin > **10c** > **10b** > **10a** in A549 cells.

The selectivity index (SI) values indicated the selective targetability of conjugate **10c** over conjugates **10b** and **10a** and doxorubicin on both cancer cell lines (COLO-205 and A549) suggesting the cytotoxic potential of conjugate **10c**. Furthermore, all the synthesized conjugates (**10a-c**) also expressed cytocompatibility in normal mouse fibroblast (L929) compared to standard doxorubicin. The structure-activity relationship (SAR) study of the fatty acyl (amide) amino acid alkyl ester conjugate (**10a-c**) was also derived based on cytotoxicity data and supportive literature. Herein, the antiproliferative effect of fatty acid (amide) amino acid alkyl ester conjugates (**10a-c**) was attributed to the presence of the C_{18} carbon chain of fatty acid in the conjugate [22]. Similarly, the presence of the unsaturated fatty acid carbon chain of linoleic acid (n-6) in conjugate **10c** was responsible for the significant cytotoxic effect on COLO-205 and A549 cells when compared to stearic acid-based conjugates (**10a** and **10b**). The chain length, saturation degree and double bond configuration are all factors that influence toxicity [23]. The most bioactive conjugate conjugate **10c** was further evaluated in the DNA cleavage study.

DNA cleavage ability of conjugate 10c: In this method, changes in the DNA mobility were observed under the applied electric field. The gel electrophoresis results in conjugate **10c** showed the concentration-dependent cleavage of pUC19 plasmid DNA at 100, 200 and 400 μg/mL. Herein, an increase in the concentration of conjugate **10c** demonstrated the conversion of electrophoretic bands (Fig. 1) from supercoiled (form I) into the nicked (form II), while the appearance of a linear form (form III) suggested that the complexes induced lethal double-strand scission. Typically, during electrophoresis plasmid DNA undergoes migration with the fastest movement observed when the supercoiled form (form I) transforms into a relaxed nicked form (form II), indicating the occurrence of single-strand DNA scission. The study observation for the linearized form of DNA (form III) inferred cleavage of both DNA strands.

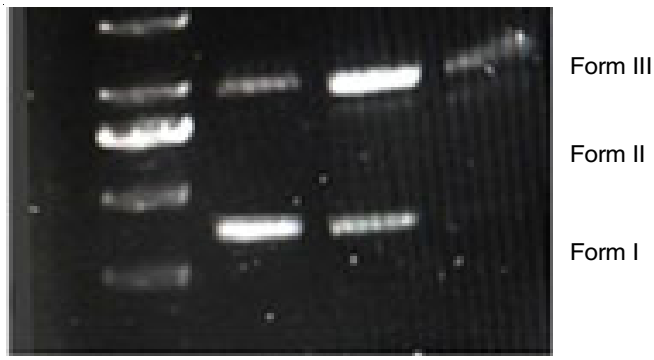


Fig. 1. Agarose gel electrophoresis for the cleavage pattern of pUC19 plasmid DNA (350 ng) by complex LMME (**10c**). Lane 1 (DNA control), Lane 2 (100 μg/mL), Lane 3 (200 μg/mL), Lane 4 (400 μg/mL)

Conclusion

In summary, a coupling agent HBTU facilitates the coupling of fatty acids such as stearic acid and linoleic acid with corresponding amino acid methyl esters like L-glutamic acid dimethyl ester and L-methionine methyl ester. This coupling reaction resulted in the formation of amide conjugates (**10a-c**). The findings show that the conjugates had different cytotoxic effects on colorectal (COLO-205) and lung (A549) cancer cells, depending on the type of fatty acid chain in the STGE (**10a**), STME (**10b**) and LMME (**10c**) conjugates. Moreover, the unsaturated linoleic acid chain (**10c**) acts as a factor for promising cytotoxicity on COLO-205 and A549 rather than conjugates **10a** and **10b**. The most bioactive conjugate LMME (**10c**) exhibited good DNA cleavage ability in a concentration-dependent manner. Thus, the conjugation of stearic acid and linoleic acid with the corresponding L-glutamic acid dimethyl ester and L-methionine methyl ester could successfully target COLO-205 and A549 cells. This finding can potentially develop conjugate LMME (**10c**) as a possible target in anticancer developments. Further research is needed on developed conjugates to achieve new therapeutic outcomes in the tumor-targeted delivery.

TABLE-1
THE DATA IS PRESENTED IN THE FORM OF IC_{50} (μM), WHICH INDICATES THE CONCENTRATION OF THE COMPOUND RESPONSIBLE FOR THE CYTOTOXIC EFFECT ON COLO-205, A549 AND L929 CELL LINES (RELATIVE TO THE CONTROL) AFTER 72 h OF CELL CULTURE WITH THE (STGE (**10a**), STME (**10b**), LMME (**10c**) CONJUGATES

Name of conjugate	COLO-205 (μM)	SI	A549 (μM)	SI	L929 (μM)
10a	35.91 ± 3.40	1.28	40.68 ± 4.35	1.13	46.32 ± 4.20
10b	24.55 ± 1.67	2.25	23.76 ± 2.74	2.32	55.30 ± 3.89
10c	9.63 ± 0.90	6.49	17.82 ± 1.54	3.50	62.53 ± 5.45
Doxorubicin	3.70 ± 0.50	0.39	3.25 ± 1.10	0.44	1.46 ± 0.30

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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