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Original Article

Synthesis, Characterisation and Cytotoxic Studies of Novel Curcumin-Metformin Conjugate

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ABSTRACT

Background: Curcumin, chemically difeluroyl methane is a yellow polyphenol isolated from the rhizomes of perennial herb *Curcuma longa*. It has been shown highly cytotoxic towards various cancer cell lines but its water insolubility and instability make its bioavailability exceedingly low. Conjugation of curcumin with another drug increases the steric hindrance around the molecule and stabilizes it against chemical and enzymatic degradation. Metformin, chemically N, N'-dimethyl biguanide has been reported to exhibit anticancer potential by activating adenosine monophosphate activated protein kinase (AMPK) pathway. **Methods:** In the present work, novel curcumin-metformin conjugate was synthesised and evaluated for *in vitro* cytotoxic activity. It was prepared through a covalent bond between the phenolic hydroxyl group of curcumin and amino group of metformin using the linker succinic anhydride and by employing carbodiimide coupling agent dicylohexyl carbodiimide (DCC) in the presence of N-hydroxy succinimide (NHS). It was then characterized using IR, ^{1}HNMR and mass spectroscopy analysis. The stability of the synthesized conjugate was studied in acidic and basic pH conditions by UV spectroscopic method. *In vitro* anticancer potential of the synthesised conjugate was studied by MTT assay. **Results:** Curcumin-metformin conjugate exhibited enhanced stability as compared to curcumin. It was found to significantly increase the cytotoxicity against MCF-7 and PA-1 cells as compared to the free drugs. **Conclusion:** It can be concluded that the synthesized conjugate has better stability in the gastrointestinal tract than curcumin and has the potential for optimizing anticancer therapy.

Key words: Conjugation, Curcumin, Drug-drug conjugate, Metformin, MTT assay.

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INTRODUCTION

Cancer is a multifactorial disease requiring treatment targeting multiple intracellular components and signalling pathways. Conventional chemotherapeutic drugs, being low-molecular-weight substances, distribute randomly in the body and have poor tumour selectivity. This leads to a low therapeutic efficacy and toxic side effects, restricting the successful application of anticancer agents. The development of clinical drug resistance has highlighted the need for new chemotherapeutic drugs and new combinations for these agents.[1] Curcumin is a promising anticancer agent exhibiting multi-potent properties and activities against number of molecular targets *in vitro*.[2,3] Translation of curcumin into a drug for treatment is restricted by its poor oral bioavailability due to poor solubility and extensive pre-systemic and systemic metabolism.[4,5] Metformin has been reported to selectively kill cancer stem cells and block tumour growth.[6] In addition synergistic action of metformin was observed with chemotherapeutic drugs in order to reduce tumour mass and prolongation of remission in nude mice. It exhibits anticancer potential by modulating insulin secretion and by insulin mimetic effect.[7] Drug-drug conjugation is a novel strategy that is being evaluated for enhancing the biopharmaceutical properties of selected drugs resulting in compounds with synergistic activity, increased specificity and reduced toxicity.[8] Two or more drug molecules are chemically conjugated to each other via intracellular hydrolysable linkers that allow the therapeutic activity of each constituent drug to be resumed following their delivery into their target cells.[9] The combinatorial conjugation of two drugs leads to the formation of a hybrid conjugate and it has huge potential to improve the solubility and pharmacokinetic-related problems associated with the individual drug molecules while enhancing their therapeutic efficacy. Conjugation of curcumin with water soluble drugs has been reported to increase the solubility of curcumin and prevent its pre-systemic intestinal metabolism.[10] In view of the above, conjugation of curcumin with water soluble drug, metformin may enhance the oral bioavailability of both the drugs and result in synergistic cytotoxic activity.

The present study seeks to exploit the novel approach of conjugation of curcumin with metformin to form a hybrid conjugate. Conjugation of these two drugs with inherent biological properties and activities may result in synergistic activity. The conjugate could increase the stability of curcumin in gastro intestinal tract, thereby improve its pharmacokinetic properties and render it more effective than single drug.

MATERIALS AND METHODS

Curcumin was purchased from Biogenuix (LKT laboratories, Inc., New Delhi) and Metformin was obtained as gift sample from Dr. Reddy's laboratories, Hyderabad. The chemicals and reagents used for the synthesis were of AR and LR grade and procured from Merck, Hi-Media, Rankem and SD Fine Chem. Ltd. Melting points were determined in open capillary tubes using melting point apparatus (RAAGA) and were uncorrected. The purity of the compounds was ascertained by thin layer chromatography using silica gel-G as stationary phase and appropriate mixtures of the following solvents as mobile phase: n-hexane and ethyl acetate. The synthesised compounds were characterised by FTIR spectrometer (FTIR-8400S, Shimadzu) in the range of 400-4000 using diffuse reflectance system and values of V_{max} are reported in cm^{-1}.

^{1}H NMR spectra was recorded on Bruker 400 MHz NMR spectrometer and chemical shifts (δ) were measured as parts per million downfield from the internal reference Tetramethylsilane (TMS). Mass analysis was recorded on Shimadzu MS model 2010A in the scan range 100-1000m/z, using the ESI (electrospray ionisation) mode.

Synthesis of Curcumin-Metformin conjugate

Step 1: Synthesis of curcumin monosuccinate.

1 mmol of curcumin (368 mg) was dissolved in anhydrous benzene and refluxed with 1.5 mmol of succinic anhydride (150 mg) in presence of few drops of pyridine for 24 hrs. Reaction mixture was then cooled and treated with dilute hydrochloric acid in order to remove the excess of pyridine as pyridine hydrochloride. The organic layer was then separated and concentrated. The product was then recrystallized using ethanol.

Step 2: Synthesis of curcumin-metformin conjugate.

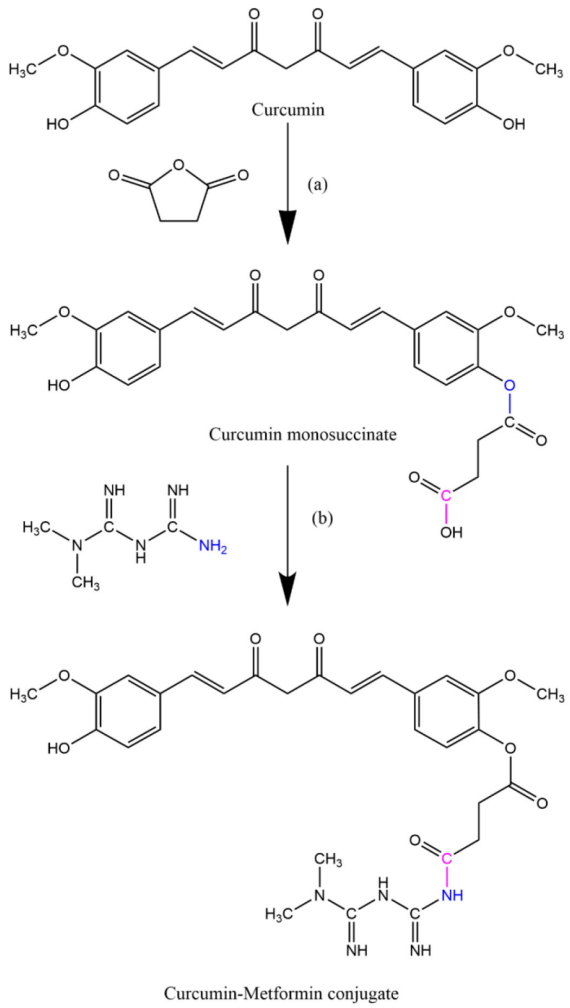
1 mmol of curcumin monosuccinate and *N*-hydroxy succinimide (NHS) were dissolved in 10 ml of dichloromethane in a round bottom flask and then stirred for 15 min. 1.5 mmol of ice cold solution of dicyclohexyl carbodiimide in 5 ml of dichloromethane was then added drop wise. The mixture was stirred at room temperature for 24 hrs after which dicyclohexyl urea formed was filtered off. The reaction mixture was then poured drop wise into ice cold ether to precipitate the NHS ester of curcumin monosuccinate. It was added to three fold molar excess of metformin dissolved in pyridine. The reaction mixture was stirred at room temperature for 24 hrs after which it was added drop wise into ice cold ether to precipitate crude curcumin metformin conjugate. The crude product was dissolved in dichloromethane to precipitate out any unreacted metformin. The reaction mixture was filtered and the filtrate was reprecipitated with ether to isolate unreacted curcumin monosuccinate. The filtrate was then evaporated and the product was collected. The scheme for synthesis of curcumin-metformin conjugate is outlined in Scheme 1.

Stability studies

The stability of conjugate (2 µg/mL solution) in pH 1.2 and pH 7.4 buffers was investigated at different time intervals using UV spectroscopic method. 48 mL of acidic and basic buffer solutions were taken in 250 mL beakers and to each beaker 1 mL ethanol was added. Then, 1 mL of 100 µg/mL solution (in ethanol) of conjugate and curcumin were added to each beaker. Continuous stirring was maintained under temperature conditions of 37±2°C and sample was taken at different time intervals (0, 15, 30, 45, 60, 90, 120 and 180 min). The concentration of conjugate and curcumin, in each sample, were determined using UV spectroscopic method.

In vitro cytotoxic studies by MTT assay

The synthesised curcumin-metformin conjugate, curcumin and metformin were evaluated for their cytotoxicity by MTT assay at the concentration ranging from 200 to 6.25 µg/mL against human breast cancer cell line MCF-7, human ovarian cancer cell line PA-1 and human normal kidney cell line HEK-293. MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] stock solution was prepared by dissolving 500 mg of MTT in 10 mL of phosphate buffer solution. It was stirred for about 1 hr using a magnetic stirrer. The sterilized solution was filtered using 0.2 mm filter and stored at -20°C. The working solution of 5 mg/mL in phosphate buffer solution was freshly prepared when required. Cells were first seeded in a 96-well flat bottomed microtiter plate at a concentration of 5×10^3 cells/well. It was maintained at 37°C in 95% humidity for 24 hr with an atmosphere of 5% CO_2. Different concentrations (200, 100, 50, 25, 12.5, 6.25 µg/mL) of samples were then



Reaction Conditions:
a: benzene, pyridine; 24h reflux
b: N-hydroxy succinimide, dichloromethane, dicyclohexyl carbodiimide, pyridine; 24h reflux

Scheme 1: Synthesis of Curcumin-Metformin conjugate.

added to the wells. After 48 hr of incubation, the cells were washed two or three times with phosphate buffer. 20 µL of MTT (5 mg/mL) was then added to each well which were then incubated for 4 hr at 37°C in a CO_2 incubator. The formazan crystals formed were then dissolved using 100 µL of DMSO as solubilizing agent, per well for 30 min at 37°C in 5% CO_2 incubator. The optical density (OD) of the suspension was recorded using micro plate reader at 570 nm. The % cell viability was then calculated.[11,12]

$$\text{Cell viability in \%} = \frac{\text{Mean OD of test compound}}{\text{Mean OD of negative control}} \times 100$$

The concentration of compound required for 50% inhibition of cell viability was indicated as the half maximal inhibitory concentration (IC_{50}). IC_{50} values of the synthesised compound and free drugs-curcumin, metformin were calculated using GraphPad Prism version 8.0. Morphological observation of cell lines treated with synthesised conjugate was done to determine the changes induced by the conjugate.

RESULTS

Chemistry

Curcumin-metformin conjugate: reddish orange solid, yield-62%, mp 180-182°C, R*f* :0.7; IR (KBr) cm^{-1} amide N-H(s) 3614, O-H(s) 3550, amine N-H(s) 3475, amide C=O(s) 1600, C=O(s) 1750, aromatic C-H (s) 2923, aromatic C=C(s) 1600, C-O-C (s) 1060; ^{1}H NMR: 9.040 (1H, s,-OH), 7.301 -7.474 (6H, m, aromatic C-H), 6.254,6.265 (2H, d, H-7,7'), 6.272,6.284 (2H, d, H-8,8'), 4.183 (2H, s, -CH_2, H10), 3.865,3.766 (6H, s, -OCH_3, H-11,11'), 2.223 (2H, t, -CH_2, H-13), 2.218 (2H, t, -CH_2, H-14), 1.208 (3H, s, H-18), 1.038 (3H, s, H-19); ^{13}C NMR: 55.95 (C-11/C-11'); 183.13 (C-9/C-9'); 48.71 (C-10); 127.10 (C-8/C-8'); 140.44 (C-7/C-7'); 115.20 (C-6/C-6'); 121.39 (C-5/C-5'); 122.79 (C-4/C-4'); 115.05 (C-3/C-3'); 148.37 (C-2/C-2'); 147.23 (C-1/C-1'); 172.08 (C-12); 29.00 (C-13); 33.75 (C-14); 174.61 (C-15); 157.30 (C-16/C-17); 39.71 (C-18/C-19); MS: m/z 578 (M-1).

Stability studies

The results obtained from the stability studies are shown in Figure 1. Curcumin-metformin conjugate showed greater stability at pH 1.2 than at pH 7.4 as compared to curcumin.

Cytotoxic studies

The synthesised curcumin-metformin conjugate, curcumin and metformin were evaluated for their cytotoxicity by MTT assay at the concentration ranging from 200 to 6.25 µg/mL against human breast cancer cell line MCF-7, human ovarian cancer cell line PA-1 and human normal kidney cell line HEK-293. IC_{50} values obtained are summarised in Table 1 and graphical representation for comparative cytotoxic effect of test compounds on three cell lines is shown in Figure 2. The morphological changes of cells treated with conjugate was observed to determine the changes induced by the conjugate. The morphological observations of untreated and treated cell lines are depicted in Figure 3.

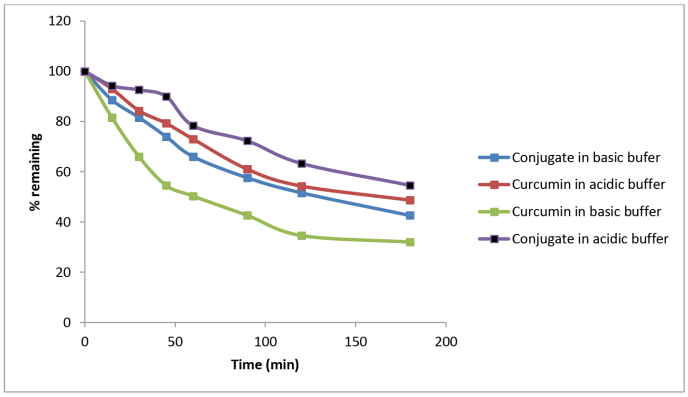


Figure 1: Comparative stability studies.

Table 1: IC_{50} values of synthesised compound and free drugs in µg/mL.

Sample Code	MCF-7		PA-1		HEK293	
	Mean	SD	Mean	SD	Mean	SD
C	14.17	0.61	4.56	1.30	155.60	18.95
M	10.16	0.35	2.00	0.32	104.60	12.80
CM	5.701	0.82	1.52	0.15	51.23	9.65

(Note: C-curcumin, M-metformin, CM-curcumin-metformin conjugate)
All values are represented as mean ± SD (n=3)

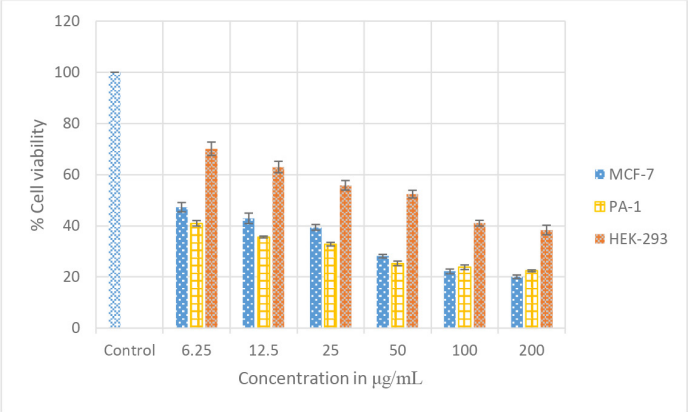


Figure 2: Comparative graphical representation of cytotoxic effect of conjugate on different cell lines.

DISCUSSION

The site for conjugation in curcumin is phenolic hydroxyl group and metformin has an amino group. Curcumin-metformin conjugate was prepared through a covalent bond between these two active groups. As curcumin cannot form direct amide linkage with metformin, a linker succinic anhydride was used. The reaction of curcumin with the linker succinic anhydride in presence of pyridine resulted in the formation of acid derivative of curcumin, curcumin monosuccinate by ring opening elongation of the phenolic hydroxyl group with succinic anhydride. The reaction with succinic anhydride was intended to take place with one of the phenolic hydroxyl groups in curcumin which was achieved using 1.5 mmol equivalents of succinic anhydride. The intermediate was then transformed to its activated ester using standard protocol of carbodiimide activation.[13] It was then treated with metformin to give curcumin metformin conjugate. The synthesised conjugate was confirmed by NMR, Mass and IR spectral data.

The synthesised conjugate was found to be more stable in acidic environment than in basic environment as compared to curcumin. It shows that basic hydrolysis is predominant in case of curcumin-metformin conjugate. The increased stability in acidic medium may be attributed to the steric hindrance around curcumin by metformin in curcumin-metformin conjugate. This would stabilize the conjugate against enzymatic degradation.

Curcumin has been reported to be cytotoxic against various cancer cell lines including breast cancer and ovarian cancer. Metformin has been reported to inhibit the proliferation of cancer cell lines in dose and time dependent manner in ovarian cancer.[14] Preclinical studies corroborates the effectiveness of metformin in breast cancer.[15] *In vitro* and *in vivo* studies revealed that combination of curcumin and metformin resulted in synergistic effect in cancer therapy.[16] So in the present study curcumin was conjugated with metformin as a single molecule and evaluated for *in vitro* cytotoxicity against breast cancer and ovarian cancer cell lines. The cytotoxicity of the synthesised conjugate was compared with that of free drugs against MCF-7, PA-1 and normal cell line HEK-293 by MTT assay. The conjugate exhibited higher cytotoxicity against MCF-7 cell lines (IC_{50} value of 5.70 µg/mL) than that of curcumin and metformin (IC_{50} values of 14.17 and 10.16 µg/mL respectively). The conjugate significantly increased the cytotoxicity effect on PA-1 cells (IC_{50} value of 1.52 µg/mL) when compared with either drugs alone (IC_{50} values of 4.56 and 2.00 µg/mL for curcumin and metformin respectively). The conjugate significantly reduced the viability of MCF-7 and PA-1 cells in

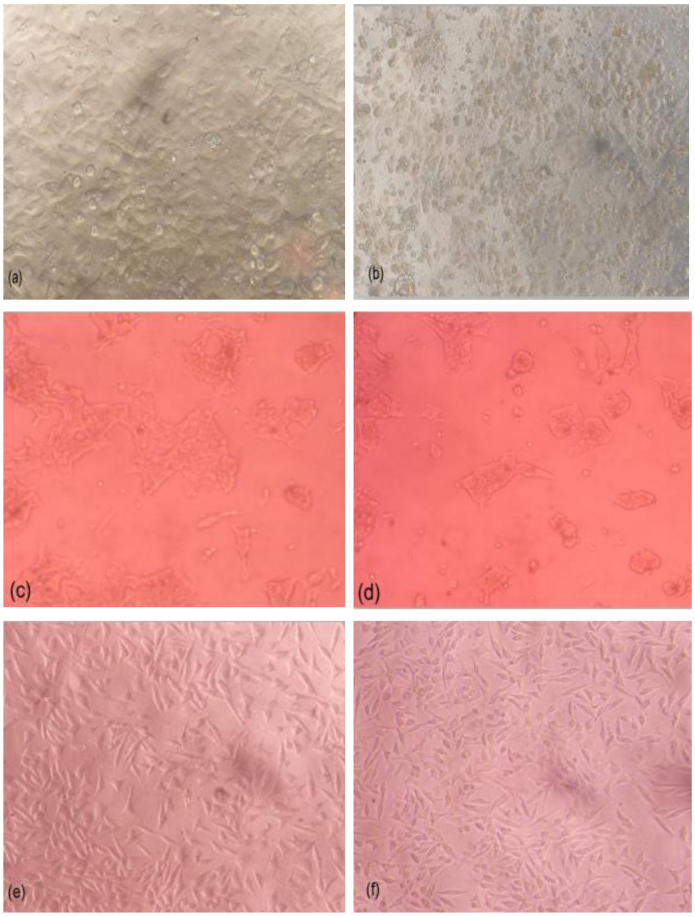


Figure 3: Morphological observation of untreated cells (left) and cells treated with conjugate (right) using MTT assay. (a, b) MCF-7; (c, d) PA-1; (e, f) HEK-293.

a concentration dependent manner, while found to be less toxic to the normal cell HEK-293 (IC_{50} value of 51.23 µg/mL). The results revealed that morphological changes were observed in MCF-7 and PA cells after being treated with IC_{50} of synthesised conjugate.

CONCLUSION

Curcumin-Metformin conjugate was synthesised by employing succinic anhydride as linker and dicyclohexyl carbodiimide as coupling agent. It was then evaluated for *in vitro* anticancer activity on two cancer cell lines. The conjugate has enhanced stability in acidic and basic environment than curcumin. The hybrid conjugate was found to significantly increase the cytotoxicity against MCF-7 and PA-1 cells than the individual drugs. Hence, based on stability and cytotoxic studies, it can be concluded that the conjugate has the potential for optimizing anticancer therapy. Further studies to evaluate the pharmacokinetic profile of the conjugate are required to correlate the findings with observed anticancer efficacy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

FT-IR: Fourier transform infrared; **NMR:** Nuclear magnetic resonance; **UV:** Ultraviolet; **DMSO:** Dimethyl sulphoxide.

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