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Synthesis, antibacterial and antiviral properties of curcumin bioconjugates bearing dipeptide, fatty acids and folic acid

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1. Introduction

Curcumin, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, commonly known as diferuloyl methane, is a natural yellow pigment derived from rhizomes of the plant Curcuma longa (Zingiberaceae) – a plant grown in tropical southeast Asian and Indian subcontinent, and has been proved as potent antioxidant, anti-inflammatory, antiviral and anticancer agent through modulation of multiple cellular machinery [1–6]. Turmeric, a spice used to provide specific flavor and yellow color to curry, has been used for many centuries as an Indian folklore medicine in Ayurveda – an ancient traditional system of medicine for treatment of wide range of illnesses. Current traditional Indian medicine uses it for biliary disorders, anorexia, cough, diabetic wounds, hepatic disorder, rheumatism, blood purification and rheumatoid arthritis [7–9]. Recent studies have shown curcumin as a potential molecule in the treatment of different forms of cancer, e.g., cervical cancer caused by HPV [10–12].

It has been observed that both curcumin and the oil fraction, suppress the growth of several bacteria like Streptococcus, Staphylococcus, Lactobacillus, etc [12,13] and human pathogenic fungi. Turmeric oil is also active against Aspergillus flavus, Aspergillus parasiticus, Fusarium moniliforme and Penicillium digitatum [14,15].

Results have shown that curcumin treatment effectively reduced Coxsackie Virus B3 replication through desregulation of Ubiquitin–Proteosome System (UPS) and COP9 Signalosome (CSN) performing a critical role in their life cycle, i.e., it strongly reduced viral RNA expression and further protein synthesis [16,17]. Curcumin has been identified as inhibitor of HIV-1 LTR directed gene expression and viral replication. A previous study has shown that curcumin, a pharmacologically safe compound, is able to block HIV replication by inhibiting HIV-integrase and protease [18,19].

The double bonds in curcumin provide definite conformational flexibility to the molecule, which accounts for its various properties. Further, blocking of phenolic groups decreases its antioxidant activity since these groups play critical role in enzymatic activity at receptor sites [20,21]. Studies have revealed that curcumin has very low bioavailability due to its poor absorption and rapid metabolism in the liver and intestinal wall [22,23]. Curcumin is highly hydrophobic and cannot be administered systemically. On intravenous administration, it disappears rapidly from the blood and quickly appears as metabolites in the bile [10,24,25]. Therefore, one of the most appreciable approaches is to make biodegradable conjugates of curcumin molecule with suitable ligands to enhance its cellular uptake. For preparing bioconjugate of curcumin, amino acids and fatty acids – natural...
components of bacterial cell wall, and folic acid – a cofactor in the synthesis of thymidine and other nucleotides, were selected [26–29]. These bioconjugates are supposed to enhance cellular uptake, lipophilicity of the molecule and sustained release of drug molecule to improve the half-life and reduce the rate of metabolism of curcumin molecule inside the cell.

We have previously reported a series of nucleosidic molecules [30–33] of significant therapeutic applications. In the present pursuance, we are focusing on naturally occurring molecules – curcumin and its bioconjugates, e.g., di-O-tryptophanylphenylalanine curcumin (2), di-O-decanoyl curcumin (3), di-O-pamitoyl curcumin (4), di-O-bis-(γ,γ)folyl curcumin (6), C4-ethyl-O-γ-folyl curcumin (8) and 4-O-ethyl-O-γ-folyl curcumin (10). These bioconjugates have been screened for their antibacterial and antiviral activities.

2. Chemistry

Curcumin, 1, has two phenolic groups and one active methylene group which can be utilized as potential sites for chemical variations and covalent linkage with biomolecules. We have synthesized curcumin bioconjugates 2, 3, 4, 6, 8 and 10, wherein the phenolic hydroxyls and active methylene group on curcumin have been utilized.

For the synthesis of compound 2 (Scheme 1), curcumin 1 was reacted with t-boc-N-trp–phe–COOH (indigenously synthesized) in 1:2 molar proportion using dicyclohexylcarbodiimide (DCC) coupling procedure in the presence of DMAP in anhydrous dichloromethane (DCM) to yield di-O-tryptophanylphenylalanine curcumin 2 in 53% yield [34].

Further, curcumin was reacted with decanoyl chloride and palmitoyl chloride in 1:2 molar proportion in the presence of DMAP in anhydrous pyridine to get the molecules 3 and 4 (Scheme 1) in 56% and 44% yield, respectively [35].

For the syntheses of conjugates 6, 8 and 10, folic acid was activated to p-nitrophenyl folate 5 (FA-PNP) (Scheme 2) using p-nitrophenol in the presence of DCC in pyridine and triethylamine (TEA). The completion of the reaction was assessed by precipitation of dicyclohexylurea (DCU) [36].

The activated folate 5 was reacted with curcumin 1 in 2:1 molar proportion in the presence of DCC and DMAP to get the compound 6 (Scheme 3) in 48% yield.

Scheme 1. Synthesis of curcumin bioconjugates with dipeptide, decanoyl chloride and pamitoyl chloride.
For synthesizing the molecule 8, both phenolic groups on curcumin were protected using benzoyl chloride. This di-O-benzoyl curcumin was conferred on a carbanionic character at the active methylene site by using a strong base, like NaOEt and reacted with 2-chloroethanol – a linker unit, followed by reaction with activated folate 5 in 1:1 molar proportion, using DCC and DMAP to yield the compound 8 in 45% yield (Scheme 3).

The compound 10 was synthesized using only one phenolic group on curcumin molecule. Curcumin was taken up in 5% aqueous NaOH and reacted with 2-chloroethanol as linker unit. The resulting product, dissolved in anhydrous pyridine, was added dropwise to the activated folate 5 in 1:1 molar proportion in the presence of DCC and DMAP to yield the compound 10 in 26% (Scheme 4).

All the newly synthesized bioconjugates were characterized by UV, 1H NMR, 13C NMR, mass spectra and elemental analyses. The molecules 2, 3, 4, 6 and 8 have been screened for their antibacterial activities against gram-positive cocci and gram-negative bacilli and the molecules 2, 3, 6, 8 and 10 for their antiviral activities against a range of viruses using standard protocols.

3. Results and discussion

3.1. Antibacterial activity

The molecules 2, 3, 4, 6 and 8 have shown remarkable antibacterial activities with MIC ranging between 0.09 and 0.67 μM (in vitro) against gram-positive cocci (Streptococcus viridans) as well as gram-negative bacilli (Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis) bacterial strains. Results are given in Table 1.

The most encouraging results were found against Streptococcus viridans, E. coli, K. pneumoniae and P. mirabilis with molecules 6 and 8 having MIC 0.27 μM (0.54 μM against P. mirabilis) and 0.09 μM, respectively, for each bacterial strain. The molecules 2, 3 and 4 have shown highly satisfactory results as antibacterials with MIC 0.43, 0.369 and 0.591 μM, respectively, against E. coli and K. pneumoniae (curcumin shows MIC value 1.23–2.47 μM). These results were compared with known antibiotics as standards as shown in Table 1. The antibacterial activity of the bioconjugates was 3.7–27 times higher than that of curcumin itself. The better results obtained with these bioconjugates may be because of their structural similarity with the bacterial cell wall that possesses amino acids and lipids as its integral part and folic acid is a requirement as a cofactor in the synthesis of thymidine and other nucleotides. This ensured high cellular uptake and thus enhanced bioavailability [26–29].

The ester bonds in these molecules are biodegradable, i.e., they get hydrolysed by carboxesterases present in the cells and this results in the enhanced effective concentration of the drug at the target site and sustained release of curcumin further ensures low toxicity, if any.
3.2. Antiviral activity

The molecules 2, 3, 6, 8 and 10 were examined for their cytotoxicity and antiviral activity against a variety of DNA and RNA viruses using different cell cultures. The results have been shown in Table 2. The compound 2 has shown good result against vesicular stomatitis virus and compound 3 against feline corona and feline herpes viruses. The EC\textsubscript{50} value (0.011 \textmu M) of compound 2 was almost nine times lower than its CC\textsubscript{50} value (0.096 \textmu M). Similarly, the EC\textsubscript{50} (0.029 \textmu M) values of compound 3 against feline corona and feline herpes viruses were almost five times lower than its CC\textsubscript{50} (0.146 \textmu M) value. The EC\textsubscript{50} (0.029 \textmu M) of compound 3 has been observed as almost ten times better than that of curcumin (0.271 \textmu M) against FIPV and FHV. Further, the CC\textsubscript{50} values of these bioconjugates were much higher than that of curcumin. Thus, these results confirmed the better acceptability of curcumin bioconjugates by the cells under study than curcumin itself. All these compounds were also tested against Influenza A H1N1/H3N2 subtype and Influenza B in MDCK cell lines. However, none of the test compounds was able to inhibit cytopathic effects of Influenza A or B virus at subtoxic concentrations or the highest concentration tested (100 \mu g/ml).

The positive results with compounds 2 and 3 might be due to their higher lipophilicity, which enhances their cellular uptake. The lipophilicities of compounds 2 and 3 are expressed by log \textit{P} values determined experimentally (Table 3) using the standard stir-shake flask method in an octanol–water system [37]. These values are also compared with values obtained by a computer programme – Molinspiration method. The log \textit{P} values of the compounds 2 and 3 were higher than that of curcumin. As expected, the lipophilicity of the compound 3 was much higher than that of the compound 2. This reaffirmed that the bioconjugates have better biocompatibility when we are concerned about bioavailability of curcumin molecule. Since ester bonds are biodegradable by carboesterases, these molecules ensure higher and sustained bioavailability.

The compound 6 which did not show positive result against the viruses under study has been found to be very active against HPV causing cervical cancer in Indian women [Singh et al. unpublished results]. The bioconjugates, however, did not show any activity against HIV-1 (IIIb and ROD strains) as shown in Table 4.

4. Conclusion

We have prepared a number of curcumin bioconjugates bearing covalent linkage with suitable ligands, viz. dipeptide, fatty acids and folic acid to enhance cellular uptake of curcumin [26–29]. These bioconjugates were screened for their antibacterial activity against gram-positive and gram-negative bacteria using micro-dilution broth susceptibility test method [38].

The molecules 2, 3, 4, 6 and 8 have shown much better antibacterial activities with MIC ranging between 0.09 and 0.67 \textmu M than curcumin (MIC value 1.23–2.47 \textmu M).

Further, the bioconjugate 2 has shown good antiviral property with EC\textsubscript{50} 0.011 \textmu M against vesicular stomatitis virus and similarly, the bioconjugate 3 with EC\textsubscript{50} 0.029 \textmu M against feline corona and feline herpes viruses. The EC\textsubscript{50} values are five to ten times lower than their CC\textsubscript{50} Values.

The bioconjugates 2, 3, 6, 8 and 10 were also screened against HSV, coxsackie virus B\textsubscript{4}, respiratory syncytial virus, parainfluenza virus, reovirus-1, punta toro virus, vaccinia virus and HIV-1 (IIIb and

| Compound | MIC (\textmu M)\textsuperscript{a} | Gram-positive | Gram-negative | \textit{P}. miralells |
|----------|-------------------------------|---------------|---------------|---------------------|
| 2        | 0.43                          | 0.43          | 0.43          | 0.43                |
| 3        | 0.67                          | 0.33          | 0.67          | 0.67                |
| 4        | 0.53                          | 0.53          | 0.53          | 0.53                |
| 6        | 0.27                          | 0.27          | 0.27          | 0.54                |
| 8        | 0.09                          | 0.09          | 0.09          | 0.09                |
| Curcumin | 2.47                          | 1.23          | 1.23          | 1.23                |
| Ampicillin trihydrate | >2.47 | 1.23 | 0.60 | 1.23 |
| Gentamycin sulfate | >1.73 | >1.73 | >1.73 | >1.73 |

\textsuperscript{a} MIC (\textmu M) – Minimum inhibitory concentration, i.e., the concentration in the tube with highest dilution showing no turbidity.
Table 2
Cytotoxicities and antiviral activities of curcumin conjugates 2, 3, 6, 8 and 10.

| Compound | Virus A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R |
|----------|---------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cell     | HeLa EC\textsubscript{50} | >0.019 | >0.019 | >0.019 | >0.038 | >0.038 | >0.038 | >0.038 | >0.019 | >0.019 | >0.019 | >0.019 | >0.019 | >0.019 | >0.019 | >0.096 | >0.096 | >0.096 | >0.096 | >0.096 | >0.096 |
|          | HeLa CC\textsubscript{50} | <0.006 | 0.006 | 0.006 | 0.006 | 0.019 | 0.019 | 0.019 | 0.019 | 0.096 | 0.096 | 0.096 | 0.096 | 0.096 | 0.096 | 0.096 | 0.096 | 0.096 | 0.096 | 0.096 | 0.096 | 0.096 |
|          | D.S-50000 EC\textsubscript{50} | 2 | 4 | 0.8 | - | - | - | - | >100 | >100 | 20 | 60 | 60 | - | - | - | - | - | - | - | - |
|          | D.S-50000 CC\textsubscript{50} | >100 | >100 | >100 | - | - | - | - | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
|          | (S)-DHPA EC\textsubscript{50} | 146 | 250 | 146 | - | - | - | - | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 |
|          | (S)-DHPA CC\textsubscript{50} | >250 | >250 | >250 | - | - | - | - | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 |
|          | Ribavirin EC\textsubscript{50} | 10 | 30 | 30 | 10 | 50 | 85 | >250 | >125 | >95 | >250 | >50 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 |
|          | Ribavirin CC\textsubscript{50} | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 |
|          | Brivudine EC\textsubscript{50} | - | - | - | - | >0.08 | 50 | 2 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 |
|          | Brivudine CC\textsubscript{50} | - | - | - | - | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 | >250 |
|          | HPA EC\textsubscript{50} | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 12.7 | 0.8 | - | - | - | - |
|          | HPA CC\textsubscript{50} | - | - | - | - | - | - | - | - | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
|          | UDA EC\textsubscript{50} | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1.3 | 2.9 | - | - | - |
|          | UDA CC\textsubscript{50} | - | - | - | - | - | - | - | - | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
|          | Ganciclovir EC\textsubscript{50} | - | - | - | - | >0.03 | 0.03 | >0.03 | >0.03 | >0.03 | >0.03 | >0.03 | >0.03 | >0.03 | >0.03 | >0.03 | >0.03 | >0.03 | >0.03 | >0.03 | >0.03 | >0.03 | >0.03 |
|          | Ganciclovir CC\textsubscript{50} | - | - | - | - | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |

A, Vesicular Stomatitis virus; B, Coxsackie virus B4; C, Respiratory syncytial virus; D, Herpes simplex virus-1(KOS); E, Herpes simplex virus-2 (G); F, Vaccinia virus; G, Vesicular stomatitis virus; H, Herpes simplex virus-1TK- KOS ACV; I, Parainfluenza-3 virus; J, Reovirus-1; K, Sindbis virus; L, Coxsackie virus B4; M, Punta toro virus; N, Feline corona virus (FIPV); O, Feline herpes virus; P, Influenza A H1N1 subtype; Q, Influenza A H3N2 subtype; R, Influenza B.

\* 50% Effective concentration [\mu M], or concentration producing 50% inhibition of virus-induced cytopathic effect, as determined by measuring the cell viability with the colorimetric formazan-based MTS assay.

\textsuperscript{b} 50% Cytoxic concentration [\mu M], as determined by measuring the cell viability with the colorimetric formazan-based MTS assay.
with DMSO as solvent using TMS as an internal standard. \( ^{13} \text{C} \) NMR spectra were recorded on Varian XL-300 and Bruker AM-200 spectrometers operating at 75 and 50 MHz, respectively. All solvents were dried and distilled prior to use.

5.1.1. 1,7-Bis-[(4,4-di-O-tryptophanoylphenylalanine)]-3-methoxy phenyl]-1,6-heptadiene-3,5-dione (2)

Curcumin (100 mg, 0.27 mmol) and dipeptide (266 mg, 0.59 mmol) were dissolved in dry DCM (10 ml), and stirred for a few minutes to get a clear solution. Further, DCC (302 mg, 1.47 mmol) and DMAP (66.35 mg) dissolved in DCM (15 ml) were added to the reaction vessel and stirred the reaction mixture overnight at room temperature. The completion of the reaction was assessed by precipitation of dicyclohexylurea (DCU). DCU was filtered off and the filtrate evaporated to a small volume and partitioned between water and ethyl acetate. The organic layer was evaporated and treated with TFA in DCM (1:1, v/v) to remove t-boc group from –NH₂ function. A positive ninhydrin test indicated the presence of free amino group. The residue was dissolved in DCM (30 ml) and this organic fraction was washed consecutively with 5% NaHCO₃ solution (20 ml), NaCl (20 ml), H₂O (20 ml), dried over anhydrous Na₂SO₄, filtered and reduced under vacuum. The title compound was purified by silica gel column chromatography using 1–2% CH₂OH in CH₂Cl₂ as a light yellow solid which was recrystallized from ethanol/water. Yield: 53%; m.p. = 204 °C, \( R_t = 0.27 \) (DCM/MeOH, 9.5:0.5), UV (EtOH) \( \lambda_{max} = 258, 220 \) nm. \( ^{1} \text{H} \) NMR (DMSO-\( d_6 \) and \( D_2 \)O) \( \delta = 2.93 \) (d, 4H, –CH₂-Trp); 3.04 (d, 2H, –CH₂-Ar); 3.74 (s, 6H OCH₃, Ar, Cur); 3.95 (m, 2H, CO–CH–NH₂); 4.48 (m, 2H, –CH–NH₂); 4.59 (s, 2H, C4 of Cur); 6.86 (d, 2H, C2 and C6 of Cur); 6.80 (d, 2H, Trp); 6.87–6.91 (m, 6H, Ar, Cur); 7.08–7.21 (m, 10H, Ar of Phe); 7.28 (m, 8H, Ar-Trp); 7.53 (d, 2H, C1 and C7, Cur); \( ^{1} \text{C} \) NMR (CDCl₃): 34.0, 37.0, 51.9, 56.0, 57.0, 62.1, 111.0, 112.1, 72.75; H, 8.28 found: C, 72.78; H, 8.34.

| Compound       | Measured and predicted log P values of compounds 2, 3 and curcumin. |
|----------------|--------------------------------------------------------------------|
| P             | Log P                 | Log P       |
| 2             | 5.50 \( \times 10^4 \) | 2.74       | 2.82       |
| 3             | 1.69 \( \times 10^4 \) | 8.23       | 9.23       |
| Curcumin      | 1.70 \( \times 10^4 \) | 2.23       | 2.30       |

5. Experimental section

5.1. Chemistry

Silica gel G for TLC and silica gel (60–120) for column chromatography were obtained from E. Merck India Ltd. Curcumin, folic acid, dicyclohexylcarbodiimide (DCC), p-nitrophenol (PNP), trifluoro acetic acid (TFA) and 2-chloroethanol were purchased from Aldrich Chemical Co. USA. The dipeptide, t-boc–N–Trp–Phe–COOH, was indigenously synthesized. Peptone (std.) was purchased from Hi Media laboratory Ltd., Mumbai, India. Melting points were determined by electrothermal apparatus and were uncorrected. UV measurements were carried out on Hitachi 220S spectrophotometer. \( ^{1} \text{H} \) NMR spectra were recorded using DRX 300 Instrument.

Table 3

| Compound | Stir-flask method | Molinspiration method |
|----------|------------------|-----------------------|
| P        | Log P            | Log P                 |
| 2        | 5.50 \( \times 10^4 \) | 2.74       | 2.82       |
| 3        | 1.69 \( \times 10^4 \) | 8.23       | 9.23       |
| Curcumin | 1.70 \( \times 10^4 \) | 2.23       | 2.30       |

ROD strains) in MT4 cells using MTT method but no encouraging results were obtained [39]. However, the present work has shown promise in developing a natural edible substance-curcumin, into a potential antibacterial and antiviral agent and preparing the bioconjugates of curcumin has proved beneficial in order to enhance its cellular delivery.

Further mechanistic studies on compounds 2 and 3 are in progress against vesicular stomatitis, feline corona and feline herpesviruses.

5.1.2. 1,7-Bis-[(4,4-di-O-decanoyl)-3-methoxy phenyl]-1,6-heptadiene-3,5-dione (3)

To curcumin (100 mg, 0.27 mmol) dissolved in dry pyridine (10 ml), added decanoyl chloride (0.12 ml, 0.61 mmol) and DMAP (66.35 mg) under cooled condition and stirred the reaction mixture overnight at room temperature. After completion of the reaction, as indicated by TLC, added chilled water (5 ml) and stirred for 10 min. Further, the volume of reaction mixture was reduced, the residue dissolved in EtOAc (20 ml), washed with water and dried up. The title compound was purified by column chromatography using ethyl acetate (1.5–2%) in hexane as a dark red solid which was recrystallized from EtOAc and Hexane. Yield: 56%; m.p. = 225 °C, \( R_t = 0.35 \) (EtOAc:Hexane, 3:5.5:6.5), UV (EtOH) \( \lambda_{max} = 250, 228, 25.6 \) nm. \( ^{1} \text{H} \) NMR (DMSO-\( d_6 \) and \( D_2 \)O); 0.96 (t, 6H, CH₃ of decanoic acid); 1.28 (m, 20H, –CH₂ of decanoic acid); 1.32 (m, 4H, –CH₂ of decanoic acid); 1.54 (m, 4H, –CH₂ of decanoic acid); 2.72 (s, 6H OCH₃, Ar of Cur); 4.58 (s, 2H, C4 of Cur); 6.67 (d, 2H, C2 and C6 of Cur); 6.78–6.91 (m, 6H, Ar-Cur); 7.54 (d, 2H, C1 and C7 of Cur); \( ^{1} \text{C} \) NMR (CDCl₃); \( \delta = 14.0, 25.4, 29.7, 30.0, 30.3, 33.2, 32.5, 51.9, 56.0, 112.2, 118.9, 123.1, 122.3, 137.2, 137.9, 140.5, 154.8, 169.0, 169.6, MS m/z 1034 (M⁺). Anal. Calcd for C₅₆H₅₆N₀₂: C, 70.78; H, 5.60; N. 8.12. Found: C, 70.78; H, 5.65; N, 8.11.

Table 4

| Compound | HIV-1 Strains | Exp_nr | IC₅₀ᵃ | CC₅₀ᵇ | SIᶜ |
|----------|--------------|--------|-------|-------|-----|
| 2        | IIIb         | P1.4401 | >0.094 | <0.094 | <1  |
|          | ROD          | P1.4402 | >0.093 | <0.093 | <1  |
| 3        | IIIb         | P1.4401 | >0.107 | <0.107 | <1  |
|          | ROD          | P1.4402 | >0.103 | <0.103 | <1  |
| 6        | IIIb         | P1.4401 | >0.036 | >0.036 | <1  |
|          | ROD          | P1.4402 | >0.046 | >0.046 | <1  |
| 8        | IIIb         | P1.4401 | >0.063 | >0.063 | <1  |
|          | ROD          | P1.4402 | >0.065 | >0.065 | <1  |
| Curcumin | IIIb         | P1.4401 | >0.037 | >0.037 | <1  |
|          | ROD          | P1.4402 | >0.034 | >0.034 | <1  |

[a] Compound concentration (\( \mu \)M) required to reduce the viability of mock-infected cells by 50% as determined by MTT method.
[b] Compound concentration (\( \mu \)M) required to achieve 50% protection of MT-4 cells from HIV-1-induced cytopathogenicity as determined by MTT method.
[c] Selectivity index: CC₅₀/IC₅₀ ratio.
overnight at room temperature. The reaction mixture was poured on to chilled water (5 ml) and worked up as in the case of compound 3. The title compound was obtained as a yellow powder, which was recrystallized from a mixture of ethanol and water. Yield: 44%; m.p. = 100 °C, Rf = 0.69 (EtOAc: Hexane, 3:0.7:0). UV (EtOH) λmax 260, 245, nm. 1H NMR (DMSO-d6 and D2O) δ 0.97 (t, 6H, CH3 of palmitic acid); 1.29 (m, 4H, –CH2 of palmitic acid); 1.36 (m, 4H, –CH2 of palmitic acid); 1.58 (m, 4H, –CH2 of palmitic acid); 2.25 (t, 4H, –CH2 of palmitic acid); 3.73 (s, 6H OCH3, Ar of Cur); 4.59 (s, 4H, C9 H, FA); 4.46 (m, 2H, C19, FA); 4.58 (s, 2H, C13 and C15, Ar, FA); 6.61 (d, 4H, C12 and C16, Ar, FA); 6.79 (d, 2H, C2 and C6, Cur); 8.8 (s, 2H, C7, pyrazine, FA); MS 169.0, 154.8, 140.5, 137.9, 132.7, 126.6, 122.3, 118.9, 112.2, 56.0, 36.0, 33.2, 32.5, 32.0, 30.3, 30.0, 29.7, 25.4, 23, 14.0, MS m/z 844 (M⁺). Anal. Calc'd for C53H80O8: C, 75.20; H, 9.46 found: C, 75.32; H, 9.54.

5.1.4. p-Nitro phenyl ester of folic acid (5)
To a stirred solution of folic acid (2 g, 5 mmol) in anhydrous ethyl acetate (10 ml), added dropwise p-nitrophenol (0.834 g, 6 mmol) dissolved in ethyl acetate (10 ml). After 15 min, pyridine and triethylamine (1 ml each) were added to make it more basic, stirred for 15 min and further added DCC (2.579 g, 12.5 mmol). The reaction mixture was stirred for 2.5 h and monitored on TLC. The completion of the reaction was assessed by total consumption of the starting material.

5.1.5. 1,7-Bis-[(4,4'- di-O-bis-(γ, γ)-folyl)-3-methoxyphenyl]-1,6-heptadiene-3,5-dione (6)
Curcumin (94 mg, 0.25 mmol) dissolved in dry pyridine (5 ml) was added dropwise to activated folate ester 5 (323 mg, 0.57 mmol), stirred for 15 min and TEA (0.5 ml), DCC (292 mg, 1.42 mmol) and DMAP (61 mg) were further added. The reaction mixture was stirred for 5 h and monitored on TLC. The precipitate of DCU started to appear after 30 min. At the end of the reaction, DCC was filtered off, the filtrate evaporated and the residue dissolved in EtOAc (20 ml). This organic fraction was washed consecutively with 5% NaHCO₃ solution (20 ml) (to neutralize the residual acid and to separate excess amount of PNP), NaCl (20 ml), H₂O (20 ml), dried over anhydrous Na₂SO₄, evaporated under vacuum, washed with 5% NaHCO₃, dried over anhydrous Na₂SO₄ and concentrated to get the intermediate compound 6. The volume of the reaction mixture was reduced to half and poured on to crushed ice and extracted thoroughly with EtOAc, dissolved in pyridine (5 ml) and further mixed with 2-chloroethanol (0.067 ml, 1 mmol). The reaction mixture was stirred at room temperature for 6 h. After completion of the reaction, the mixture was poured on to crushed ice and extracted thoroughly with EtOAc, dried over anhydrous Na₂SO₄ and concentrated to get the intermediate compound 7 (183 mg, 0.29 mmol). The compound 7, dissolved in dry pyridine (5 ml), was added dropwise to the activated folate ester 5 (286 mg, 0.51 mmol) and stirred the reaction mixture for 15 min. TEA (0.5 ml), DCC (257 mg, 1.25 mmol) and DMAP (61 mg) were further added. The reaction mixture was stirred for 5 h and the reaction was monitored on TLC.

The precipitate of DCU started to appear after 30 min. After the completion of the reaction, DCC was filtered off and the filtrate was evaporated to dryness. After deprotection of both phenolic hydroxyl functions on curcumin with NH₄-phosphate (9:1v/v) at 55 °C, ammonia was removed using water pump, the solvent evaporated and the residue dissolved in EtOAc (20 ml). The product was purified by silica gel column chromatography using ethyl acetate (1:2) in hexane as eluant. The title compound was recrystallized from ethanol/water as light brown solid. Yield: 45%; m.p. = 210 °C, Rf = 0.57 (EtOAc:Hexane, 3:5:6.5). UV(EtOAc) λmax 245, 275, 300 nm. 1H NMR (DMSO-d6 and D2O): δ 1.75 (m, 2H, C2, ethyl), 2.21 (m, 2H, C21, FA); 2.25 (m, 2H, C22, FA); 3.21 (t, 1H, C4, Cur); 4.32 (s, 2H, C9 H, FA); δ 4.46 (m, 1H, C19, FA); 6.54 (d, 2H, C12 and C16 Ar, FA); 6.56 (d, 2H, C2 and C6, Cur); 6.57–6.69 (m, 6H, m/z 835 (M⁺). Anal. Calc'd for C95H80N14O16: C, 60.35; H, 4.91; N, 11.73 found: C, 60.38; H, 4.94; N, 11.77.

5.1.7. 1-(4-Hydroxy-3-methoxyphenyl)-7-(4-O-ethyl-O-folyl-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (10)
To curcumin (184 mg, 0.5 mmol) dissolved inaq NaOH (5%, 10 ml), added 2-chloroethanol (0.05 ml, 0.75 mmol) and stirred the reaction mixture for 7 h. The reaction mixture was extracted with EtOAc. The organic extract was concentrated under vacuum, washed with 5% NaHCO₃, dissolved in pyridine and crystallized with ethanol and water to get the intermediate compound 9. Yield (100 mg, 0.24 mmol, 48%).

Compound 9 (100 mg, 0.24 mmol), dissolved in dry pyridine (6 ml), was added dropwise to the activated ester 5 (157 mg, 0.28 mmol), stirred the reaction mixture for 20 min and TEA (0.5 ml), DCC (123 mg, 0.6 mmol) and DMAP (32 mg) were further added.

The reaction mixture was stirred for 5 h and the reaction was monitored on TLC. DCC was filtered off and the product was obtained following the usual work up procedure and purification by silica gel column chromatography using ethyl acetate (2%) in hexane as eluant and detected by spraying the plates with 1% EtOAc/Hexane. This di-O-benzoyl curcumin (200 mg, 0.34 mmol) was dissolved in ethanol (7 ml) and NaOEt was added to the reaction mixture at room temperature over 20 min and the reaction mixture was stirred for another 30 min. The sodium salt was concentrated under vacuum, washed thoroughly with ethanol, dissolved in pyridine (5 ml) and further mixed with 2-chloroethanol (0.067 ml, 1 mmol). The reaction mixture was stirred at room temperature for 6 h. After completion of the reaction, the mixture was poured on to crushed ice and extracted thoroughly with EtOAc, dried over anhydrous Na₂SO₄ and concentrated to get the intermediate compound 7 (183 mg, 0.29 mmol). The compound 7, dissolved in dry pyridine (5 ml), was added dropwise to the activated folate ester 5 (286 mg, 0.51 mmol) and stirred the reaction mixture for 15 min. TEA (0.5 ml), DCC (257 mg, 1.25 mmol) and DMAP (61 mg) were further added. The reaction mixture was stirred for 5 h and the reaction was monitored on TLC.
5.2. Pharmacology

5.2.1. Antibacterial assay

The in vitro antibacterial activity of each curcumin bioconjugate against Gram-positive cocci (S. viridans) and Gram-negative bacilli (E. coli, K. pneumoniae, P. mirabilis) was evaluated by microdilution broth susceptibility test method. The bacterial strains were isolated from clinical patients following the standard protocols [38].

The antibacterial activities of curcumin bioconjugates, 2, 3, 4, 6 and 8 were compared with that of curcumin. The stock solutions of the conjugates along with curcumin prepared in water/DMSO to ensure complete solubilization were doubly diluted to the concentrations 100 μg; 500 μg, 250 μg, 125 μg and 65.5 μg to which peptone-water (1 ml) was added. The fresh culture of aforementioned strains was prepared and a standardized bacterial suspension (100 μl) of 0.5 McFarland turbidity was added to each dilution. Suitable solvent control (DMSO), positive growth control and standard control were also run simultaneously [39].

The tubes bearing different concentrations of the compounds and control tubes were incubated at 37 °C for 24 h. After incubation, antibacterial activity of molecules in the tube was detected by lack of turbidity which indicated the inhibition of bacterial growth. The concentration in the tube with highest dilution showing no turbidity has been reported as MIC. Each test was performed in triplicate and the MICs reported represent the result of at least two repetitions.

5.2.2. Antiviral assay

The antiviral assay was based on inhibition of virus-induced cytopathogenicity in various cell cultures as described previously [39–43]. The assays were performed against various viruses, viz. influenza virus type 3 (PIV-3) and reovirus type-3, vesicular stomatitis virus (strain G), cytomegalovirus (CMV) from sindbis virus (SV), parainfluenza virus type 1 (strain KOS), herpes simplex virus type 1 (strain KOS), herpes simplex virus type 2 (strain G), cytomegalovirus (CMV) from sindbis virus (SV), parainfluenza virus type 3 (PIV-3) and reovirus type-3, vesicular stomatitis virus (SV), coxsackie virus (Coxs V), respiratory syncytial virus (RSV) using CRFK, HEL, HeLa and Vero cell lines and HIV-1 (IIIB and ROD strains) in MT-4 cells using DMSO method.

5.2.2.1. Virus cytopathogenicity.

The confluent cell cultures were prepared in microdilution trays and inoculated with 100 CCID50 (1 CCID50 corresponding to the virus stock dilution that proved infective for 50% of cell cultures). After 1 h of virus adsorption to the cells at 37 °C, the residual virus was replaced by cell culture maintenance medium without compound was added. All cultures were incubated at 37 °C, and after 2 and 7 days of incubation, compounds were withdrawn and the viability of the cells was determined by the trypan blue exclusion test.

5.2.2.2. Cytotoxicity.

The cytopathicity of the test compounds 2, 3, 6, 8 and 10 was assessed on the basis of two parameters: (i) alteration of normal cell morphology, and (ii) inhibition of macromolecule (DNA, RNA and protein) synthesis. Cytoxicity (CC50) of the compounds was examined by trypan blue exclusion test.

To evaluate cytotoxicity, uninfected confluent cell cultures treated with various concentrations of the test compounds, were incubated in parallel with virus-infected cell cultures prepared in plastic trays containing 24 wells (16 mm diameter; Falcon plastics). After 2 days of incubation at 37 °C in a CO2 incubator, when the cell cultures were confluent, culture medium was removed from each well and 1 ml of maintenance medium containing serial concentrations of the test compounds was added. For the cell control, 1 ml of maintenance medium without compound was added. All cultures were incubated at 37 °C, and after 2 and 7 days of incubation, compounds were withdrawn and the viability of the cells was determined by the trypan blue exclusion method.

5.2.3. Ant-HIV assay

Antiviral screening against HIV – 1 (IIIB and ROD strains) was monitored by the efficiency of drug compounds to inhibit syncytia formation after HIV infection of MT – 4 cells following the MTT method [45–47]. The activity of the compounds against HIV-1 was monitored by inhibition of HIV-1-induced cytopathogenicity in MT-4 cells. Briefly, MT-4 cells (3 × 10^5 cells per well in 96 well plate) were cultured in microdilution trays in the presence of various concentrations of the test compounds added immediately after infection with 50% cell culture infective doses of HIV-1. After 5 days of incubation at 37 °C, the number of viable cells was determined by the MTT (3’-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) method.

5.2.4. Determination of log P value

The partition coefficients of bioconjugates 2 and 3 along with curcumin were determined by the stir-flask method [37]. Octanol and 0.1 NaOH solution were mutually saturated and both phases were separated by centrifugation (5000 rpm, 10 min). A stock solution of each molecule (4 × 10^{-3} M) was prepared using octanol-saturated NaOH solution. A part of these solutions were kept aside for UV measurement. Further, NaOH-saturated octanol was added at various volume ratios to other part of the stock solution. Then the two-phase mixtures were intensively stirred for 4 h at constant temperature (25 °C). The absorbance of the aqueous solutions was measured by UV–vis spectrophotometer at λ_max 258 nm for compound 2 and λ_max 250 nm for compound 3 and 223 nm for curcumin. The P value was calculated as follows:

\[
P = \frac{A_0 - A_1}{A_1} \times \frac{V_w}{V_0}
\]

where \(A_0\) and \(A_1\) represent the absorbance of the molecule in the aqueous phase before and after partitioning, \(V_w\) and \(V_0\) are the water and octanol volumes, respectively. Log P values are an average of three measurements.

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