Antibacterial Activities of Curcumin Bioconjugates

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Abstract: Curcumin is a wonder molecule as it is used for prevention and cure of multiple diseases such as cancer, diabetes, inflammation, cataract, rheumatoid arthritis, HIV etc. However, it has not been possible so far to assign a definite drug profile because of low bioavailability, fast metabolism, poor adsorption, low solubility and lack of targeted delivery. Several bioconjugates of curcumin with amino acids and nucleotides have been prepared so far to overcome these limitations. In the present study the two new conjugates of curcumin have been synthesized by esterifying its phenolic group functions with nicotinic acid (vitamin B3) and its isomer picolinic acid viz. 1,7-bis (4-O-nicotinoyl-3-methoxyphenyl)-1, 6-heptadene-3, 5-dione (2) and 1, 7-bis (4-O-picolinoyl-3-methoxyphenyl)-1, 6-heptadene-3, 5-dione (4). These bioconjugates were evaluated for antibacterial activity against medically important gram positive cocci (Enterococcus faecium) and gram negative bacilli (Klebsiella pneumoniae, Escherichia coli and Pseudomonas aeruginosa). Antibacterial activity was performed by microdilution broth susceptibility method in vitro. Results demonstrated that both bioconjugates possess high antibiotic potential as evidenced by enhanced antibacterial activity in comparison to pure curcumin. On the basis of results it may concluded that lipophilic nature of these conjugates enhances the cellular uptake and may be better option for medicinal and pharmaceutical applications.

Key words: Curcumin, bioconjugate, nicotinic acid, picolinic acid, antibacterial property

INTRODUCTION

The plant kingdom represents the rich store house of organic compounds which have nutritional as well as medicinal value. Turmeric (Curcuma longa) used as dietary spice, has medicinal properties (Srivastava et al., 2011) so, it is used as herbal medicine in Ayurveda-the traditional Indian system of medicine. Curcumin, 1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadene-3, 5-dione, the main coloring yellow component is isolated from the rhizomes of turmeric. Curcumin possesses antioxidant (Weber et al., 2005) anticancer (Aggarwal and Shishodia, 2006), anti-inflammatory (Chainani-Wu, 2003), antidiabetogenic (Arun and Nalini, 2002; Sivabal and Anandh, 2010; Sovia et al., 2011), antileishmaniasis (Saleheen et al., 2002), Anti-schistosomal (El-Sherbiny et al., 2006) and antimalarial (Reddy et al., 2005) activity. It also reduces blood cholesterol (Asai and Miyazawa, 2001; Hasimun et al., 2011), suppresses rheumatoid arthritis (Deodhar et al., 1980), Alzheimer disease (Yang et al., 2005), enhances wound healing (Sidhu et al., 1998), protects from liver injuries (Morikawa et al., 2002) and cataract formation (Suryanarayana et al., 2005). Curcumin is effective to prevent nicotine induced blood cell damage (Banerjee et al., 2010).

There are certain limitations which prevent curcumin to use as a drug (Pandey et al., 2010) which are as follows:

- It is highly hydrophobic (lipophilic) because of the presence of two free phenolic hydroxyls which make it too polar, restricting its uptake into the cells
- It has low absorption through intestinal gut wall causing its poor systemic bioavailability
- In presence of reductases (Lin et al., 2000), it is rapidly metabolized to reduced derivatives like dihydro, tetrahydro and hexahydro curcumin.

Recently it has been observed that few endophyte fungi have a potency to generate curcumin analogues by transformation (Prana et al., 2010; Simanjuntak et al., 2010)

The above limitations can be overcome by attaching curcumin to such ligands which can enhance its solubility, bioavailability and slow down the metabolism. The reactive sites of attachment of ligands to curcumin

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molecule can be either at phenolic hydroxyls, keto or active methylene group. The double bonds are essential for proper conformation of the molecule. The masking of phenolic groups with ligands such as amino acids (Dubey et al., 2007) nucleosides (Kumar et al., 2001) and peptides (Singh et al., 2010) are already reported. Conjugates of curcumin with nicotinic acid (vitamin B₃) and its isomer picolinic acid having biodegradable ester linkage has been synthesized (Fig. 1).

These bioconjugates have been screened for their antibacterial activities. The bacterial resistance is increasing day by day against standard antibiotics so researchers are screening plants (Krishnan et al., 2010; Sonibare et al., 2011) natural phytochemicals and their derivatives as an option for antibiotics.

Several bacteria are responsible for causing severe infections. Escherichia coli cause urinary tract infections and diarrhea (Crichton, 2008). Pseudomonas aeruginosa cause primary wound infection, urinary tract infection, and blood stream infection in burn patients (Ekrami and Kalantar, 2007) and respiratory infections (Govan, 2008). Klebsiella pneumonia can cause nosocomial infections, urinary tract infections and respiratory tract infections (Crichton, 2008). Enterococcus faecium can also cause urinary tract infections and meningitis (Fisher and Phillips, 2009; Ryan and Rey, 2004).

The objective of the present study was to synthesize such conjugates of curcumin which may be lipophilic causing better cellular uptake and show promising antibacterial activity.

MATERIALS AND METHODS

The synthesis, purification and characterization (by spectroscopy) were done in three months duration.
i.e., from April-June 2009. Testing of antibacterial activity was performed during April to July 2010.

Curcumin, nicotinic acid, picolinic acid, silica gel G for TLC and silica gel (60-120) for column chromatography were purchased from E. Merck India Ltd. Synthetic grade solvents used were purchased from Qualigens, purified and dried prior to use. UV visible spectra were recorded on Hitachi 220 S double beam spectrophotometer. NMR spectra were recorded using DRX 300 instrument with DMSO as solvent using TMS as internal standard. The Nutrient agar, MacConkey agar, Mueller Hinton agar, Brain Heart Infusion broth and dimethyl sulfoxide (DMSO) were purchased from Himedia Laboratories Pvt. Limited, Mumbai, India.

Nicotinic chloride: Redistilled thionyl chloride (1.09 mL, 0.015 mol) was added to nicotinic acid (1.23 g, 0.01 mol) and refluxed for 30 min. The reaction mixture was evaporated to dryness on rotary evaporator under reduced pressure. Yield = 72% (0.890 g, 6 mmol).

1, 7-bis (4-O-nicotinoyl-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione: Curcumin (100 mg, 0.27 mmol) was taken in dry DMP (dimethyl formamide; 5 mL) and mixed with nicotinic chloride (90 mg = 0.6 mmol). Few drops of triethylamine were added as a catalyst. The reaction mixture was stirred overnight at room temperature. The completion of reaction was checked by TLC. Now the reaction mixture is poured into chilled water (10 mL), shaken well and then extracted with DCM (dichloromethane) thrice (5 mL each time). Added Na$_2$SO$_4$ (1 g) to the extracted DCM and kept overnight. Filtered the dried reaction mixture, evaporated to dryness and then purified by silica gel column chromatography using DCM/MeOH gradient. Yield 42%, Rf = 0.81 (DCM/MeOH; 98: 2). Elemental analysis: Observed-C, 68.49; H, 4.51; N, 4.89% calculated for C$_{32}$H$_{26}$O$_8$N$_2$: C, 68.51; H, 4.49; N, 4.84%. UV λ max (MeOH) = 260 (s), 290, 320 (s) nm. $^1$H-NMR (DMSO-d$_6$ and D$_2$O); δ 3.75 (s, 6H, two-OCH$_3$); 4.52 (s, 2H, C$_1$ of cur); 6.53 (d, 2H, C$_3$ and C$_5$ of cur); 6.80-6.95 (m, 6H, Ar-cur); 6.99 (m, 2H, C$_2$ of pyridine ring); 7.00 (d, 2H, C$_3$ of pyridine ring); 7.52 (d, 2H, C$_1$ and C$_2$ of cur); 8.20 (d, 2H, C$_5$ of pyridine ring); 8.50 (s, 2H, C$_2$ of pyridine ring).

Picolinic chloride: Preparation of picolinic Chloride is similar to that of nicotinic chloride.

1, 7-bis (4-O-picolinoyl-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione: Method of preparation is similar to that of (2).

RF = 0.80 (DCM/MeOH; 98: 2). Elemental analysis: Observed-C, 68.50; H, 4.48; N, 4.86% calculated for C$_{32}$H$_{26}$O$_8$N$_2$: C, 68.51; H, 4.49; N, 4.84%. UV λ max (MeOH) = 260 (s), 292, 326nm. $^1$H-NMR (DMSO-d$_6$ and D$_2$O); δ 3.72 (s, 6H, two OCH$_3$); 4.58 (s, 2H, C$_1$ of cur); 6.67 (d, 2H, C$_3$ and C$_5$ of cur); 6.78-6.91 (m, 6H, Ar-cur); 6.80 (m, 2H, C$_2$ of pyridine ring); 6.99 (m, 2H, C$_5$ of pyridine ring); 7.00 (d, 2H, C$_2$ of pyridine ring); 7.54 (d, 2H, C$_1$ and C$_2$ of cur); 8.20 (d, 2H, C$_5$ of pyridine ring).

Antibacterial assay

Minimum inhibitory concentration (MIC): The antibacterial activity of curcumin and its synthetic bioconjugates (2) and (4) were evaluated by microdilution broth susceptibility method against gram positive and gram negative bacteria. The bacterial strains used for testing antibacterial activity were Klebsiella pneumoniae (clinical isolates), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853) and Enterococcus faecium (ATCC 33186).

The antibacterial activity of curcumin bioconjugates (2) and (4) were compared with that of curcumin. Stock solution of test agents (1 mg mL$^{-1}$ concentration) was made in DMSO (dimethyl sulphoxide) to ensure complete solubilization (Liang et al., 2008). Sterile BHI broth (500 µL) was taken in a set of five sterile test tubes for one bacteria and one compound. To the first tube of each set 500 µL of 1 mg mL$^{-1}$ of stock solution was added and then doubly diluted to the effective final concentrations of 500, 250, 125, 62.5 and 31.2 µg mL$^{-1}$ in the subsequent test tubes. The fresh cultures of aforementioned bacterial strains were prepared and a standardized bacterial suspension of 0.5 Mc Farland turbidity was prepared and 100 µL of this was added to each tube. Now each five tube has a final volume of 600 µL with effective compound concentrations of 300, 150, 75, 37.5 and 18.75 µg, respectively. Suitable solvent control DMSO (to check sterility), DMSO+bioconjugate+BHI, without bacteria (to checks sterility) and positive growth control (DMSO+BHI + bacteria, without bioconjugate + to check bacterial growth) were also run simultaneously (Talaro and Talaro, 2002). The tubes bearing different concentration of curcumin bioconjugates and three above mentioned control tubes were incubated at 37°C for 24 h. After incubation, antibacterial activity of curcumin bioconjugates in the test tube was detected by lack of turbidity which indicates the inhibition of bacterial growth (Talaro and Talaro, 2002; Forbes et al., 2007). The concentration in the test tube with highest dilution showing no turbidity was recorded as MIC. Each test was performed in triplicate and the result of at least two repetitions was reported as MIC of the bioconjugates against that particular bacterial strains.
Minimum bactericidal concentration (MBC): The solution of tube with no turbidity and a solution of tube with one higher concentration were sub-cultured on suitable culture media (Klebsiella pneumoniae and Escherichia coli on MacConky agar, Pseudomonas aeruginosa on nutrient agar and Enterococcus faecium on blood agar) and incubated overnight at 37°C. The highest dilution showing no growth on subculture was recorded as MBC.

RESULTS AND DISCUSSION

In order to prepare curcumin bioconjugates, nicotinic acid is selected as:

- It is a constituent of vitamin B-complex and it can be taken orally like curcumin.
- It contains heterocyclic nucleus which is generally found in antibacterial compounds.

So, the hypothesis was that the curcumin bioconjugate with nicotinic acid may show enhanced antibacterial activity as it is evident from results of this experiment, shown in Table 1. Picolinic acid is the isomer of nicotinic acid so it is also used as ligand. Curcumin is linked with these ligands by ester bond which may get hydrolyzed by esterase enzymes present in the cells and these results in the increased effective concentration causing enhanced antibacterial activity.

The systemic bioavailability of curcumin is due to the fast metabolism as it is biotransformed to dihydro, tetrahydro and hexahydro-curcumin in presence of reductases, but the curcumin bioconjugates can not be recognized by the above enzymes (as enzymes are specific in reactions) so their biotransformation may be prevented. Also the lipophilicity of curcumin bioconjugates is supposed to be more than the curcumin itself so their cellular uptake may be better.

The antibacterial activity of curcumin bioconjugates (2) and (4) in comparison to curcumin was investigated in vitro against Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa and Enterococcus faecium (Table 1). The curcumin and its bioconjugates (2) and (4) exhibited antibacterial activity against all the four tested microorganisms. The antibacterial activity assay was done to determine the MIC. The bioconjugate (2) and (4) have shown remarkable antibacterial activity with MIC 0.26 μmol against gram positive cocci (Enterococcus faecium) as well as against gram negative bacilli (Klebsiella pneumoniae, Escherichia coli and Pseudomonas aeruginosa). The MBC values of all the three samples are higher than that of MIC against all the four mentioned bacteria. It is expected also because the concentration required to kill a microorganism would be definitely higher than the concentration required inhibiting its growth. The MBC value of compound (2) and (4) is found to be 0.52 μmol against Klebsiella pneumoniae and Pseudomonas aeruginosa, but in case of Escherichia coli compound (2) and in case of Enterococcus faecium compound (4) shows >0.52 μmol.

On the other hand the MBC value of pure curcumin is 0.82 μmol against Klebsiella pneumoniae and Escherichia coli and >0.82 μmol for Enterococcus faecium. These MBC value are higher than the synthesized bioconjugates. However, it is found to be 0.41 μmol in case of Pseudomonas aeruginosa.

The MIC of curcumin bioconjugates (2) and (4) for Klebsiella pneumoniae and Escherichia coli was found to be lower (0.26 μmol) than that of curcumin (0.82 μmol). The antibacterial activity of bioconjugates (2) and (4) was found to be 3.15 times higher than that of curcumin itself against Klebsiella pneumoniae and Escherichia coli.

Several other conjugates of curcumin (Kumar et al., 2001, 2000; Mishra et al., 2005; Tajbakhsh et al., 2008; Singh et al., 2010; Kapoor et al., 2006, 2007) also showed more antibacterial activity. In case of Pseudomonas aeruginosa and Enterococcus faecium, the MIC of curcumin was found to be 0.20 μmol which shows that the activity of bioconjugates (2) and (4) will be 0.76 times less than that of curcumin.

The reason for higher MIC of curcumin bioconjugates against Pseudomonas aeruginosa and Enterococcus faecium needs further evaluation. Bacterial factors may be playing a pivotal role here since these two bacteria are different in many ways from Klebsiella pneumoniae and Escherichia coli, both of which belong to same family enterobacteriaceae.

| Table 1: Antibacterial activity, MIC and MBC of curcumin bioconjugate (2) and (4) in comparison with curcumin |
| --- | --- | --- | --- |
| Bacteria | Compound | Activity | MIC (μmol) | MBC(μmol) |
| Klebsiella pneumoniae (clinical isolates) | Curcumin | + | 0.82 | 0.82 |
| | (2) | + | 0.26 | 0.52 |
| | (4) | + | 0.26 | 0.52 |
| | Escherichia coli | Curcumin | + | 0.82 | 0.82 |
| | (ATCC 25922) | (2) | + | 0.26 | >0.52 |
| | (4) | + | 0.26 | 0.52 |
| | Pseudomonas aeruginosa | Curcumin | + | 0.20 | 0.41 |
| | (ATCC 27853) | (2) | + | 0.26 | 0.52 |
| | Enterococcus faecium | Curcumin | + | 0.20 | >0.82 |
| | (ATCC 33186) | (2) | + | 0.26 | 0.52 |
| | (4) | + | 0.26 | >0.52 |

(2): 1, 7-bis (4-O-nitrosoxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione
(4): 1, 7-bis (4-O-picolinoyl-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione

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Enterococcus faecium is a gram positive cocci and has a very different cell wall structure with a very high concentration of peptidoglycan and teichoic acid in their cell wall. Pseudomonas aeruginosa is however, gram negative having the same cell wall composition as Klebsiella pneumoniae or Escherichia coli, but its biochemical nature is very different. It is a non-fermenter and a strict aerobe as compared to other two gram negative bacteria which ferment sugar and are facultative anaerobe. May be these differences are responsible for their different activity against curcumin and its bioconjugates. These bioconjugates can be subjected for clinical trials. If encouraging results are found, these conjugates may be used as antibiotics.

CONCLUSION

In the present investigation the bioconjugates of curcumin (2) and (4) have shown remarkable antibacterial activity. The bioconjugates (2) and (4) showed more antibacterial activity than curcumin against Klebsiella pneumonia and Escherichia coli while these conjugates are less active than curcumin against Pseudomonas aeruginosa and Enterococcus faecium.

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