Research Article**Curcumin on to hyaluronic acid conjugate enhance cytotoxicity****Manjunatha P. Mudagal*, Suresh Janadri***Department of Pharmacology,**Acharya and B.M. Reddy College of Pharmacy, Soldevanhalli, Acharya Dr. SarvepalliRadhakrishna Road, Bengaluru-560090, Karnataka, India*

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Abstract

Objective: Polymer-drug conjugates have gained much attention largely to circumvent lower drug solubility and to enhance drug stability. Curcumin is widely known for its medicinal properties including its anticancer efficacy. One of the serious drawbacks of curcumin is its poor water solubility which leads to reduced bioavailability. **Materials and methods:** Synthesized hyaluronic acid–curcumin (HA–Cur) conjugate. The drug conjugate was characterized using FT-IR technique. The conjugates, interestingly found to assemble as micelles in aqueous phase. The formation of micelles seems to improve the stability of the drug in physiological pH. We also assessed cytotoxicity of the conjugate using MCF-7, HBL 100 and Hela cell line and quantified by MTT assay. **Results:** The results showed Conjugate (HA–Cur) produced extremely significant reduction in percentage cell viability in dose dependent inhibitory effect on growth of cell lines. **Conclusion:** Conjugated curcumin also showed cytotoxicity reflecting its potential in targeted drug delivery applications.

Keywords: Curcumin, conjugate, cytotoxicity, anticancer

Introduction

Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, Cm) is a hydrophobic polyphenol secluded from the root of the *Curcuma longa*, a spice that has been extensively premeditated and its immense therapeutic potential is entrenched (Simion et al., 2016). Curcumin being an ingredient of turmeric possess a range of pharmacological activities such as anti-oxidant, anti-inflammatory, anti-proliferative, anti-metastatic, anti-cancer and anti-atherosclerotic. It is eminently pleiotropic molecule that inhibits cell proliferation and induces apoptosis in cancer cells. Despite its imperative biological activities, chemical instability, photoinstability and poor bioavailability limits its utilization as an effective therapeutic agent. Therefore, enhancing the bioavailability of curcumin may improve its therapeutic index for clinical setting (Waghela et al., 2015).

An extensive variety of pharmacological activities has been endorsed to curcumin; however due to the low bioavailability of this pigment when administered orally, the translation of its experimental biological benefits into clinical trials is observed difficult in both rodents and humans (Vareed et al., 2008), which is explicated by its poor absorption due to the low solubility in water, limited tissue distribution, and rapid rate of metabolism in liver and intestine followed by the rapid excretion from the body (Anand et al., 2008). So the low bioavailability of curcumin emerges as a foremost barrier to reach its adequate circulating levels related to desirable pharmacodynamic actions, hampering its clinical approval as a therapeutic agent for numerous diseases (Gutierrez et al., 2015).

The progress of a synthetic methodology to produce curcumin conjugates with water-soluble polymers and targeting proteins can potentially augment the therapeutic efficacy of curcumin (Seymour and Miyamoto, 1992). For example encapsulation in polymer nanoparticles, which are aggregates of cross-linked and random copolymers, has shown promise as a potentially effective method of delivering curcumin. In addition, aggregation of surfactants, including sodium dodecyl sulfate (SDS),

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cetyltrimethylammonium bromide (CTAB), and Triton X-100, into micelles also provides desirable effects in solubilizing and stabilizing curcumin (Colangelo and Kee, 2008). Besides that lipid bilayers and vesicles has been suggested as a delivery system for administration of curcumin as a drug (Thangapazham et al., 2008; Sun et al., 2008).

Even though various water-soluble synthetic polymers have been exploited for conjugation of hydrophobic drugs, naturally occurring polymers with intrinsic cell specific binding capacity have remarkable potential as a target-specific drug carrier. For example, hyaluronic acid (HA), a naturally occurring polysaccharide composed of N-acetyl-D-glucosamine and D-glucuronic acid has a strong affinity with cell-specific surface markers such as CD44 and RHAMM (Seymour and Miyamoto, 1992; Laurent, 1987). It is unique among glycosaminoglycans and one of the key components of the extra cellular matrix, hyaluronan contributes drastically to cell proliferation and migration, and may also be involved in the succession of some malignant tumor (Fraser, Laurent and Laurent, 1997). HA is closely related to angiogenesis in several types of tumors, in which HA receptors (CD44 and RHAMM) are copiously over expressed on the surface. Thus, malignant cells with high metastatic activities often exhibit enhanced binding and uptake of HA (Manju and Sreenivasan, 2011). More recently, HA and its derivatives have been popularly used as target-specific drug delivery vehicles for various therapeutic agents due to its affinity towards specific protein found on the surface of diseased cells in larger number compared to normal cells (Lee et al., 2007). Chemical modification and conjugation of hyaluronic acid have been achieved by utilizing reactive functional groups in HA such as carboxylic groups and hydroxyl groups (Manju and Sreenivasan, 2011). In the current study, we report the direct conjugation of hyaluronic acid with curcumin in a homogeneous mixture of water and dimethyl sulfoxide (DMSO) by simple esterification reaction. We studied the cytotoxicity of the conjugate using MCF-7, HBL 100 and Hela cell lines.

Materials and methods

Materials

Hyaluronic acid sodium salt from streptococcus equi sp. (HA) was purchased from Fluka, Bangalore, India. Curcumin 95% total curcuminoid content, from turmeric Sami Labs Pvt. Ltd, 1, 3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) will be obtained from Sigma-Aldrich, Bangalore, India. All other chemicals used for this study will be of analytical grade.

Synthesis of hyaluronic acid-curcumin conjugates (HA-Cur)

1 wt. % solution of Hyaluronic acid, HA (800 mg) in 1:1 V/V

(H₂O/DMSO) mixture was added with DCC (100 mg) and DMAP (40 mg). The solution was stirred for 1 h to activate carboxylic group of HA. 0.203 mM of Curcumin was dissolved in 50 mL of DMSO and slowly added to the above solution under a blanket of nitrogen. The mixture was stirred well at 60–65°C for about 6 h. The resultant solution was dialyzed against DMSO for 1 day and then against deionized water for 3 days using a dialysis membrane (MWCO: 3500 Da) to remove unbound entities. HA-Cur conjugates was lyophilized and kept under refrigeration.

Characterization of HA-curcumin conjugate

HA-CU conjugate will be verified by FT-IR spectrometer.

In-vitro studies

Cytotoxicity studies - MTT assay

10 ml of the suspension cell culture was taken in 15 ml tubes and were centrifuged at 2500 rpm for 10 min. The supernatant was discarded and the cell pellet was resuspended in 1 ml growth medium. The cell viability was checked by counting the numbers of viable cells in the above 1 ml suspension through haemocytometer and diluted the resuspended cells with growth medium to get required cell concentration. 1×10^4 exponentially growing cells were seeded per well in 96 well plates. Cells were exposed to various concentrations of drugs. The plates were incubated at 37°C in 5% CO₂/95% humidified air. After 24 h of incubation, the plates were centrifuged at 2500 rpm for 10 min and the supernatant was discarded. 100 µl of growth medium and 10 µl of MTT was added (5 mg/ml) into each well of 96 well plates and plates were incubated at 37 °C in 5% CO₂/95% humidified air for 4 h. The plates were centrifuged at 2500 rpm for 10 min and the supernatant was discarded. The precipitated formazan salt was dissolved to form a colored solution by adding 100 µl of acid-isopropanol (0.04 N HCl in isopropanol) into each well. The absorbance of this colored solution was measured at a wavelength of 492 nm using a multiwell scanning spectrophotometer (ELISA reader).

Percentage cell viability was calculated using the equation:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of cells (treated)}}{\text{Absorbance of cells (untreated)}} \times 100$$

Statistical analysis

The IC₅₀ for each experiment was repeated at least three times, and each dose group within an experiment was assayed in triplicate. All statistical analysis was performed using chi-square, Fisher's exact test or t-test on raw data, by means of Excel spreadsheet (Microsoft Office 2007, Microsoft Corporation).

Results

Characterization of HA-CU conjugate by FTIR

HA-Cur conjugate was confirmed by infrared spectrum (Figure 1). A broad band around 3258 cm^{-1} was assigned to the O-H groups in HA. A relatively sharp peak at 3321 cm^{-1} is associated with phenolic OH of curcumin in the conjugate. The peaks around 1648 cm^{-1} and 1625 cm^{-1} were assigned to C=O stretching frequency of diketonic functionality of curcumin. The shift in the frequency towards low energy region may be due to the existence of curcumin in the enolic form in the conjugate. Hyaluronic acid shows a peak at 1602 cm^{-1} associated with C=O stretching of carboxylate anion. This peak was vanished once the conjugate was formed reflecting COO^{-1} was the reaction site. A band around 1340 cm^{-1} can be seen which was due to C-O stretching frequency of ester linkage in the HA-Cur conjugate.

Table 1. Effect of Curcumin-HA on percentage cell viability of MCF-7 cell line

Concentration (μM)	% cell viability of Curcumin-HA
Blank	100 ± 0.00
3.906	67.21 ± 0.58
7.812	54.44 ± 1.70
15.625	44.76 ± 0.67
31.25	44.07 ± 1.28
62.5	37.74 ± 2.91
125	34.10 ± 1.64
250	31.95 ± 2.21
500	24.43 ± 1.11
1000	21.61 ± 0.98

IC₅₀ = 30.74 at 250 μM

n=3 and values were expressed as Mean $\pm$ SD.

In-vitro MTT assay

Effect of HA-Curcumin on percentage cell viability of MCF-7

The effect of Curcumin-HA on percentage cell viability on MCF-7 cell lines is given in table 1 and figure 2. The result shown Conjugate (Curcumin-HA) produced extremely significant reduction in percentage cell viability on MCF-7 cell lines (IC₅₀ 30.74 at concentration of 250 μM) in dose dependent inhibitory effect on growth of MCF-7 cells.

Effect of HA-Curcumin on percentage cell viability of HBL-100 cell line

The effect of Curcumin-HA on percentage cell viability on HBL-100 cell line is given in table 2 and figure 3. The result shown Conjugate (Curcumin-HA) produced extremely

Table 2. Effect of Curcumin-HA on percentage cell viability of HBL-100 cell line

Concentration (μM)	% cell viability of Curcumin-HA
Blank	100 ± 0.00
3.906	98.01 ± 1.36
7.812	93.32 ± 1.11
15.625	88.61 ± 0.83
31.25	83.32 ± 1.11
62.5	81.21 ± 1.00
125	79.66 ± 0.69
250	66.10 ± 1.06
500	56.92 ± 1.31
1000	44.69 ± 0.55

IC₅₀ = 93.32 at 7.812 μM

n=3 and values were expressed as Mean $\pm$ SD.

Figure 1. Characterization of HA-CU conjugate by FTIR

Figure 2. Effect of Curcumin-HA on percentage cell viability of MCF-7 cell line

Table 3. Effect of Curcumin-HA on percentage cell viability of Hela cell line (MTT assay)

Concentration (µM)	% cell viability of Curcumin-HA
100	84.86921
50	87.93356
25	86.91211
12.5	92.88093
6.25	91.18444

n=3 and values were expressed as Mean ± SD.

Figure 3. Effect of Curcumin-HA on percentage cell viability of HBL-100 cell line

significant reduction in percentage cell viability on HBL-100 cell line (IC₅₀ 94.01) at concentration of 7.812 µM) in dose dependent inhibitory effect on growth of HBL-100 cells.

Effect of HA-Curcumin on percentage cell viability of Hela cell line (MTT assay)

The effect of Curcumin-HA on percentage cell viability on HBL-100 cell line is given in table 3 and figure 4. The result shown Conjugate (Curcumin-HA) produced extremely significant reduction in percentage cell viability on Hela cell line (IC₅₀ 92.88 at concentration of 12.5 µM) in dose dependent inhibitory effect on growth of Hela cells.

Figure 4. Effect of Curcumin-HA on percentage cell viability of Hela cell line

Discussion

Although curcumin has shown remarkable potential as an anticancer drug, its poor solubility in water leads to poor bioavailability. Previous studies to address this issue of reduced bioavailability include formulating curcumin in various carriers such as polymers, liposomes, polymeric micelles, emulsion and nanospheres. Polymer drug conjugates have distinctive advantages over conventional polymeric nanosized carriers due to its high drug content, good water solubility and increased drug half life in the body. We reasoned that HA-Cur conjugate is a potential curcumin delivery vehicle considering the several advantages of HA. The extreme hydrophilicity of HA and its poor solubility in most organic solvents, however, restricted the direct conjugation of HA with curcumin which is highly hydrophobic. We found that Water/DMSO mixture (1:1 V/V) dissolves both HA and curcumin enabling the conjugation to form the drug conjugate.

The results are a reflection of the stability of the conjugate at physiological pH. However, the degradation profile of pure curcumin shows about 60% degradation within 25 min. After that there is no considerable absorption in the 420 region, showing almost complete degradation of curcumin. One of the studies (Wang et al., 1997) also demonstrated 90% decomposition of curcumin within 30 min. The high stability of the conjugate comparing to curcumin can be traced to the formation of micelles. In the micelle, we presumed that conjugated curcumin exist in the inner core due to its hydrophobicity. The hydrophilic HA molecules, on the other hand, protrude outwardly. The micelle formation thus protects curcumin from the deprotonation and subsequent fragmentation in the alkaline media. The conjugation of curcumin to HA advantageously stabilizes curcumin against hydrolysis and hence enhance its aqueous stability.

Curcumin has two -OH (phenolic) groups and one active methylene group. These points are potential sites for

conjugation. Blocking of –OH groups may reduce medicinal features of curcumin. To get an insight whether the conjugation affected inadvertently the ability of curcumin to kill cells, cytotoxicity of the conjugates was evaluated with MCF-7, HBL 100 and Hela cell line. In the conjugate we synthesized, curcumin is in monofunctional form and the cytotoxic activity shows that the presence of one free phenolic group is enough for its biological activity.

The data indicate that only low quantity of conjugate is required to impart cytotoxicity which we reasoned is due to the high solubility of the conjugate. It is expected that the improvement in the conjugates Cytotoxicity was due to the water solubility and cell internalization ability of HA–curcumin conjugate.

Conclusion

The potentialities of curcumin as a drug have been investigated widely. One of the serious drawbacks of this molecule is its reduced water solubility and instability beyond neutral pH. Conjugated curcumin showed cytotoxicity reflecting its potential in targeted drug delivery applications.

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Conflicts of interest

Nil

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