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Honokiol Loaded MPEG-PCL Micelles for the Treatment of Age-Related Macular Degeneration

by

Amna Shahid

A thesis submitted in partial fulfilment
of the requirements for the degree of
Master of Science in Pharmaceutical Nanotechnology
Department of Pharmaceutical Science
Taneja College of Pharmacy
University of South Florida

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ABSTRACT

Age related Macular Degeneration (AMD), a multi-factorial age-related retinal hypoxic disorder resulting in irreversible loss of vision is the foremost cause of blindness in United States. Current treatment strategies used to treat disease involve multiple intraocular injections of Anti – VEGF agents into the vitreous of eye. In addition to the issues of drug localization and targeted delivery, the need to frequently inject drug into the eye raise patient compliance issues. These challenges call for sustained drug delivery system. In this study, a sustained drug delivery system was prepared by loading an anti- HIF agent, honokiol into MPEG-PCL polymer. These HON-MPEG-PCL micelles were characterized by measuring Size, PDI & zeta potential. In-vitro drug release study was conducted. DSC was performed to confirm entrapment of a drug in micelles. Micelles formed had a particle size of 30.8±0.8 nm with the PDI of 0.19 ± 0.0004 and Zeta potential was calculated to be -5.46 ±0.49 mv. Entrapment efficiency was calculated to be 64±0.135 %. In vitro drug release showed sustained release of formulation that reduces the need of frequent injections. Cell line studies for in-vitro characterization were conducted in human epithelial retinal cells (ARPE). Result from Cytotoxicity showed the formulation to be less toxic as compare to drug solution. Furthermore, ELISA studies performed showed the periodic downregulation of HIF and VEGF which are involved in underlying mechanism of AMD. Therefore, Honokiol-Loaded MPEG-PCL micelles were successfully developed and characterized. Cell line studies further showed the formulation to be effective in the treatment of AMD.

INTRODUCTION

AMD (age – related Macular Degeneration) is a multi-factorial age-related ophthalmic disorder that is characterized by substantial, progressive, and irreversible loss of central vision due to macular degeneration. (Lim, Mitchell, Seddon, Holz, & Wong, 2012). AMD is the leading cause of blindness in United States with an estimated 10 million people being affected by this disease. (Ambati & Fowler, 2012). As the population ages, the prevalence rate of AMD is also seen to escalate. (Friedman et al., 2004). In another report presented on prevalence rate of AMD, AMD is found to be the cause of blindness in more than 8 million Americans which is projected to escalate by more than 50% by the year 2020. (Jager, Mieler, & Miller, 2008). About 60% of people suffering from vision loss have significantly reduced ability to participate in day to day life activities with one third of population reported to be suffering from clinical depression(Friedman et al., 2004). In addition to elder being more affected by this disease, Studies have shown that AMD is more common in females as compare to males and is more prevalent in white population (Friedman et al., 2004).

Pathophysiology of Age- Related Macular Degeneration (AMD):

Based on Pathophysiology, AMD can be broadly classified into Dry AMD & Wet AMD. The first clinically significant manifestation of AMD is drusen, which is an extracellular deposition(Bhatt, Fnu, Bhatia, Shahid, & Sutariya, 2020). Dry AMD is non-vascular form of AMD and is characterized by degeneration of retinal pigment epithelial cells (RPE) & photoreceptors.

Late stage of Dry AMD is called Geographic Atrophy (GA), while Wet AMD is vascular form of AMD and is characterized by choroidal Neo vascularization (CNV). (Ferris III et al., 2013). Dry AMD is a chronic disorder and leads to some degrees of visual disturbances. If not treated this may progress to blindness. In contrast Wet AMD is an acute disorder and rapidly escalates leading to blindness. (Gottlieb, 2002)

Physiologically, Retina is multi- layered metabolically active structure lining the back wall inside the eye. Macula, an important component of retina is approximately 0.6 mm in size, in the middle of macule. Fovea, a photo-receptor dense area in center of macula is responsible for 'high-definition vision'. (Bellezza, 2018) Photoreceptors are acellular in nature and are depended on Retinal Pigment epithelial (RPE) cells for trophic support. These RPE cells are monolayer of cells and are located behind photoreceptors (Karlsson, 2014) Neurons present in Outer retina converts light photon into Action potential. (Bird, 2010) Bruch membrane, a penta-laminar structure is located between retinal pigment epithelial (RPE) & Choroidal membrane of eye. It acts as molecular sieve for transport of important nutrients and oxygen across the membrane. (Booij, Baas, Beisekeeva, Gorgels, & Bergen, 2010). These structures are inter- linked and are depended on each other in terms of metabolic needs. Photoreceptors are composed of discs like structures comprising of pigments that are involved in absorbance and initiation of visual process. RPE (retinal pigment Epithelial) cells phagocytose distal part of outer segment of photoreceptors everyday as part of normal process. This shredded waste material is recycled by phagolysosome and some of it is sent back to photoreceptor. The leftover material is passed on to Bruch membrane to be disposed of by choriocapillaris. The material that escapes degradation forms an "electron dense residual bodies", which over times accumulates in RPE cells resulting in Drusen formation. (Bird, 2010). As person age, there is a thickening of Bruch's membrane which further reduces the

supply of oxygen and nutrients, thus increasing more stress. (Pauleikhoff, Harper, Marshall, & Bird, 1990). Studies have shown that these event result in oxidative stress. Lipofuscin is nondegradable debris accumulates in eye with age. Presence of light results in Reactive oxygen species (ROS) formation from lipofuscin. (Scherz- Shouval et al., 2007) Reactive oxygen species (ROS) production is further amplified in post-mitotic cells because of decline in autophagic clearance and built up of waste material (Cuervo et al., 2005). These Reactive oxygen species (ROS), because of their un stability and potent oxidizing abilities are highly reactive and very prone to interact with other molecules. (Cheeseman & Slater, 1993). This worsens the drusen accumulation between Bruch membrane and RPE cells, this results in reduced oxygen supply to RPE cells from choriocapillaris eventually causing hypoxia. (Arjamaa, Nikinmaa, Salminen, & Kaarniranta, 2009).

Role of HIF and VEGF in AMD:

The cellular reactions to hypoxia results in expression of many genes. Hypoxia induced factor or HIF is responsible for oxygen hemostasis and is involved in maintaining and ensuring cell survival under hypoxic conditions. (Blasiak, Petrovski, Veréb, Facskó, & Kaarniranta, 2014). HIF is heterodimeric transcription factor consisting of HIF alpha and HIF beta. Physiologically, HIF alpha is an oxygen sensitive part and is regulated by proline and asparagine residues hydroxylation. This hydroxylation step is carried out by oxygen dependent prolyl hydroxylase & factor inhibiting HIF (FIH) hydroxylate, respectively. Proline hydroxylation is followed by recruiting of HIF alpha by Von Hippel Lindau for Proteasomal degradation in cytoplasm. In case of HIF alpha escape, asparagine hydroxylase plays role in preventing its binding to co -activator, thus HIF -mediated transcription is stopped. However, under hypoxia hydroxylases are inactivated resulting in HIF alpha hydroxylation. These series of events result in HIF alpha translocation into

nucleus and its dimerization with HIF beta, resulting in active HIF complex. This activated complex then binds to hypoxia response element (HRE) present in hypoxia response genes thus initiating angiogenesis. (Vavilala, Ponnaluri, Kanjilal, & Mukherji, 2014) Role of VEGF in AMD:

Under normal circumstances, there exist a balance between pro- angiogenic factors for instance, Vascular Endothelial Growth Factor (VEGF) and anti – angiogenic factors such as pigment epithelium derived factor (PEDF) factors. However, studies have shown that hypoxia plays a role in upregulation of pro -angiogenic factors e.g., VEGF, integrins and proteinases. (Campochiaro, 2004). This ultimately leads to choroidal neo vascularization (CNV), clinical hallmark of wet AMD & serious complication of AMD that can lead to blindness. (Lim et al., 2012). Choroidal capillaries are fenestrated in nature and are responsible for supplying nutrients and oxygen through blood circulation to retina. (Jo, Kim, & Kim, 2010). These chorio- capillaries lies beneath retina and plasma leaks out of these capillaries and accumulates beneath RPE cells. These cells have tight junctions and form outer blood-retinal barrier. Angiogenesis of these choroidal blood vessels damage the photoreceptors that can results in permanent damage and thus blindness r. (Jo et al., 2010).

Current Treatments:

Based on pathophysiology, anti – VEGF therapies have been approved by FDA to target choroidal Neo-vascularization (CNV), (1) bevacizumab/Avastin (2) aflibercept/VEGF trap eye (3) Pegaptanib/Macugen (4) ranibizumab/Lucentis (Subhani, Vavilala, & Mukherji, 2016).

The first anti – VEGF therapy PEGAPATINIB was approved in 2004 by FDA. The underlying mechanism of action for this drug involves binding to $VEGF_{165,}$ variant of VEGF alpha dominant in CNV. Down the road, Bevacizumab approval in same year and its promising potential

to fight colon cancer led to its off-label use for AMD as anti – VEGF therapy. (Rosenfeld, Moshfeghi, & Puliafito, 2005). Ranibizumab, a smaller fragment of Bevacizumab was developed next owing to initial believe that bevacizumab could not cross retina. Ranibizumab is an FDA approved therapy for AMD now. Aflibercept aka VEGF trap is newly approved anti VEGF therapy for AMD. It is a fusion protein and has shown to trap the VEGF. (Stewart, Grippon, & Kirkpatrick, 2012). Use of these Anti-VEGF medications has helped achieving good result for the treatment of this disease. However, a study reports the need of repetitive ranibizumab and bevacizumab administration either on monthly basis or in some cases may require the setup of personal dose regimen based on severity or progression of disease (Kovach, Schwartz, Flynn, & Scott, 2012).

Route of Drug delivery

The physiology of eye is a complex and achieving therapeutic dose at posterior side of eye can be complicated affair offering fair challenges. While eye's anterior segment is easily assessable for topical delivery, multiple clearance mechanism, physical barriers, transient residence time & corneal epithelium impermeability makes it harder for drugs to cross these barriers (Kaur, Garg, Singla, & Aggarwal, 2004) and reach at the posterior segment of eye which is desirable target location in the case of AMD. Topical delivery though is convenient approach but nasolacrimal drainage and tear dynamics can result in poor bioavailability as reported less than 5% of topically applied drug permeates and reaches intraocular tissues.(Ding, 1998; Geroski & Edelhauser, 2000) Furthermore, systemic delivery of drug requires high doses which puts patient at several toxic side effects.(Geroski & Edelhauser, 2000) Intra-vitreal route bypass all these physiological barriers to achieve desired therapeutic concentration in eye. However, liquefaction of vitreal fluid due to aging may result in non-uniform drug delivery. (Tan et al., 2011) Studies have reported that increased or repetitive intravitreal injections may not be very practical approach

and can compromise patient's compliance in addition to putting patient at risk of infections, endophthalmitis, vitreous floaters, intraocular inflammation, retinal detachment, and cataract. (Ventrice et al., 2013; Wong et al., 2008). Thus, the development of sustained release drug formulation to treat age related macular degeneration is an ideal drug delivery system.

Hypoxia-Induced Factor – AMD Target

Age – related macular degeneration (AMD) is a multi – factorial disease and involves the complex interplay of many factors that is why the use of anti-VEGF therapy alone has only been able to achieve partial success. (Subhani et al., 2016). Same is reported by group of scientists who targeted VEFG and PDGF-B, another pro-angiogenic factor to achieve better results. (Enge et al., 2002).

In a study reported Kelly et.al in 2003, it was proven that HIF alpha is a master regulator of angiogenesis. Study showed that constitutive form of HIF alpha alone was able to regulate expression of pro-angiogenic factors such angiopoietin (ANGPT1), ANGPT2, Placental growth factor & platelet-derived growth factor -B in animal model, thus causing neo-vascularization. However, on the other hand some studies have shown that VEGF expression alone is insufficient to cause ocular neovascularization as in some cases of non-proliferative retinopathy, elevated level VEGF were observed without any retinal neovascularization.(Ohno-Matsui et al., 2002)(Kelly et al., 2003). Implantation of intra-ocular Sustained release pellets of VEGF were failed to cause retinal neovascularization (Ozaki et al., 1997). Thus, an approach based on targeting HIF alpha, a master regulator of many angiogenic factor leading to CNV can prove to be potentially beneficial in treatment of CNV, underlying pathology in AMD.

Honokiol

Ischemic tissue is one of the important pathological factors that results in upregulation of Hypoxia induced Factor (HIF) which initiates the cycle of events leading to upregulation of many pro-angiogenic factors causing neo vascularization in AMD .(Vavilala, Ponnaluri, Kanjilal, & Mukherji, 2014). Honokiol is a lignin isolated from the bark of Magnolia and has been the part of Chinese and Japanese medicine regimen. This biphenolic phytochemical has been shown to have potent Anti – HIF properties. (Vavilala et al., 2012). Studies have shown that Honokiol inhibits hypoxia induced HIF resulting significant decrease in Choroidal neo –vascularization.(Vavilala et al., 2013).

Methoxy poly (ethylene glycol) Polycaprolactone) MPEG-PCL:

MPEG-PCL polymer is an FDA approved bio-compatible, amphiphilic polymer. It combines hydrophilic PEG and hydrophobic PCL which imparts them advantage of encapsulating wide range of drugs. These well researched polymers are widely used in biomedical field for drug delivery purposes and have properties like biocompatibility and controllable biodegradable nature.(Streets, Bhatt, Bhatia, & Sutariya, 2020). These polymer because of their slow degradation rate are favored for sustained release drug delivery system. Research has shown these polymers to be capable to localize in inflamed areas which helps to achieve targeted actions. These polymers have been widely used in field of nanotechnology, pharmaceutics and medicinal chemistry (Danafar, 2016). Outer covering is constituted by the PEG part of polymer which provide "stealth" effect thus making them less immunogenic(Ma, Miao, & Song, 2010)

Nanotechnology:

Nanotechnology is relatively new and advanced science that operates at nanoscale $10^{-9.}$ This field takes advantage of size to volume ratio and can achieve alteration of physical properties to meet therapeutic needs. The vast applications of this technology in medical field has potential to revolutionized health care facilities provided to patients. Encapsulated drug in nanoparticles has shown to decrease toxic effects along with achieving high efficiency and targeted delivery (Hirani, Grover, Lee, Pathak, & Sutariya, 2016) Furthermore, the use of micelles for ocular drug delivery offers advantages such as increased solubility, surface area and drug dissolution.

Therefore, loading micelles with Honokiol has potential to show promising therapeutic effects by achieving sustained release localized effect with reduced toxicity and side effects.

MATERIALS AND METHODS

Materials

Honokiol (CAS No, 35354-74-6) was purchased TCI. Methoxy poly (ethylene glycol)-block-poly (ε- caprolactone) m PEG-b-PCL in different ratio combination (2K-2K, 5K-2K,5K-10K) were purchased from Sigma Aldrich, St. Louis, MO, USA. HPLC grade Acetone was purchased from Sigma- Aldrich. HPLC grade Methanol was ordered from Fisher Scientific. Dimethyl sulfoxide (DMSO) and polyvinyl alcohol (PVA) were also ordered from Fisher chemicals and Tween -80 was purchased from Sigma Aldrich. Slide-A- Lyzer® Dialysis Cassette (MWCO, 10,000 Da) were purchased form Thermo Scientific (IL, UASA). ARPE -19 cell line derived from human retinal pigment epithelial cell line was ordered from ATCC© CRL2302™ (American Type Culture Collection). Trypsin (0.05%) was purchased from Thermo Fischer scientific (Lansing, MI, USA). Fetal Bovine Serum (FBS) along with penicillin-streptomycin (10,000 U/ml) were ordered from Gibco Thermo Fisher Scientific, USA. Incomplete media (i.e.) DMEM F12 medium was also bought from ATCC (VA, USA). Further, phosphate-buffered saline (PBS) was purchased form Corning cellgro (Manassas, VA, USA). For cytotoxicity studies, an MTT reagent (3-(4,5-dimethylthiazole-2-yr)-2,5-diphenyltetrazolium) bromide salt reagent was ordered from Sigma Aldirch (St. Lious, MO, USA). Nucleus stain DAPI (4’,6-diamindion-2-

phenylindole) (CN:5748) was ordered from Tocris Bioscience (MN, USA). Cell mask ™ deep red plasma stain (CN:C10046) was bought from Molecular probes, Invitrogen™ Thermo Fisher Scientific, USA. For ELISA studies, an Invitrogen™ e Bioscience™ human HIF kit was ordered from Fisher Scientific (Lansing, MI, USA).

Cell Culture

Cell culture studies were performed using ARPE-19 (ATCC© CRL2302™) cells. These cells derived from human epithelial cell line were grown in complete media, formulated by adding 10% FBS and 1% 10,000 U/ml pen – strep antibiotics in DMEM F12 medium. The cell cultures were allowed incubation period of 24 h at 37°c temperature supplied with 5% CO2.

Method of preparation

Synthesis of HON-MPEG-PCL Micelles

The Honokiol- MPEG-PCL micelles were synthesized using solvent evaporation technique that involved slowly and steadily injecting organic phase into aqueous phase. Briefly, micelles were prepared by using 1:10 drug to polymer ratio. For preparing organic phase, 2mg of Honokiol drug was weighed on weighing balance and added into 5 ml of centrifuge tube. 200 µL of DMSO was used to dissolve drug by sonicating the mixture for 5 minutes. In the next step, 20 mg of MPEG-PCL polymer along with 1.8 ml of acetone was added to same 5 ml centrifuge tube containing dissolved honokiol drug. To obtain homogeneity, the final mixture was vortexed. 4 ml filtered distilled water was used as an aqueous phase. The 2ml organic phase was added into 4 ml aqueous phase dropwise using a 23G syringe under magnetic stirring. The emulsion formed was left stirring on magnetic stirrer (Thermo Fisher, Lansing, MI) for 24 h at 700 RPM to allow time for formation of micelles and evaporation of organic solvents. Next day, the formulation was

centrifuged at 5000 rpm for 15 min to remove any free drug followed by centrifuging supernatant at 18000 RPM for 20 min at RT. The micelles pellet obtained after centrifugation was resuspended in saline for further studies. Blank micelles were prepared following the same protocol except for the addition of drug (Honokiol).

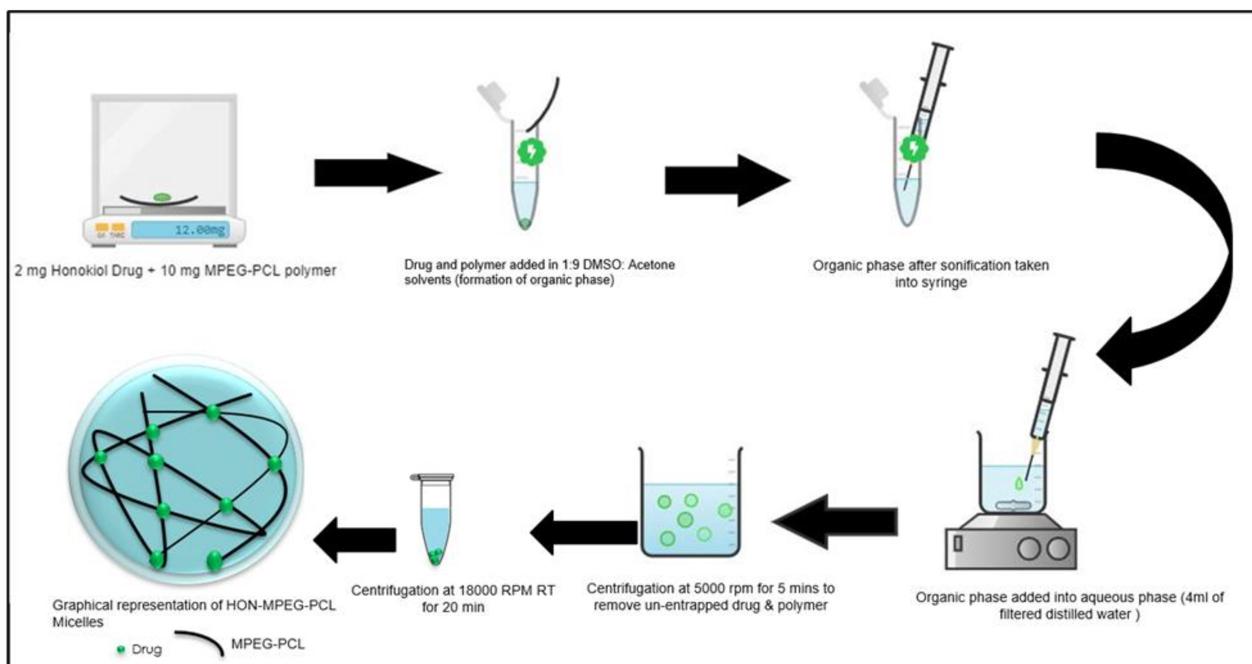

Figure 1: Graphical presentation of preparation of Hon-MPEG-PCL micelles

Development of Analytical method

Analytical method was developed using UV-Vis spectroscopy. Honokiol drug solution was prepared by dissolving 2 mg of drug in 2 ml methanol. 1 ml of this drug solution was taken to make a stock solution by diluting it in 9 ml of methanol. Different solution concentrations from 1 ppm to 50 ppm were prepared. Samples were analyzed using 96-well plate. Each sample was analyzed in triplicate through UV-Visible spectroscopy at 292 nm wavelength. The absorption obtained for each sample was quantified and calibration curve was obtained. The R^2 value was calculated to be 0.9993.

Characterization of MPEG-PCL Micelles

Dynamic Light Scattering (DLS) & Zeta- Potential (ZP)

Dynamic Light scattering (DLS) principle was used for the determination of nanoparticle size. 633 nm He-Ne laser was used as a light source and scattering angle was 90°. (Nano ZS90, Malvern Instruments Ltd., UK, Zeta Sizer Software Ver. 7.10). Each sample was diluted 1:10 using filtered distilled water. Samples were run in triplicate at 25°c temperature and results were reported as an average of size in nm along with poly dispersity index (PDI).

Smoluchowski equation was used to obtain zeta potential of micelles using particle's electrophoretic mobility. Samples were appropriately diluted (1:10) by adding 100 µl of micelles formulation into 900 µl of distilled water. Zeta cuvettes were used to analyze samples in triplicate at 25°C (Malvern Instruments Ltd., UK, Zeta Sizer software Ver. 7.10).

Transmission Electron Microscopy

The morphology of HON-MPEG-PCL was characterized using transmission electron microscopy (TEM). For taking images, samples were prepared using Cu- film square grids. Formulation was diluted using filtered distilled water (1:10) 5ug of sample was taken on grid. Samples were allowed to settle for approximately 20 minutes. Further, samples were negatively stained using 2% w/v phosphotungstic acid (PTA). 400 K× magnification was used to take images at an accelerating voltage of 120 kV.

Entrapment Efficiency (EE)

Entrapment efficiency was analyzed to check the drug loading capacity of HON-MPEG-PCL micelles. The formulation was centrifuged at 5000 rpm for 15 min to separate free drug solution or polymer. Supernatant obtained was collected and centrifuged further at 18000 rpm to obtain pellet which was further washed thrice with filtered distilled water to get rid of any free drug or polymer. Samples were prepared by re-constituting micelles with methanol using 1:10 dilution. 100 µL of formulation was diluted into 900 µL of methanol. Samples were analyzed in triplicate using UV spectrophotometer at 292 nm wavelength. Calibration curve equation was used to quantify the results.

% Entrapment Efficiency (EE) is calculated as

$$\%\text{Entrapment Efficiency} = \frac{\text{Amount of Drug loaded in micelles}}{\text{Actual drug amount used for preparation}} \times 100$$

Differential Scanning Calorimetry

Differential Scanning Calorimetry Q-20 (TA instruments, New Castle, DE USA, Q series Q-20-2288-DSC Software) was used for thermal analysis to achieve physicochemical analysis of HON-MPEG-PCL micelles. Honokiol drug, MPEG-PCL (5K-2K) polymer and formulation were analyzed. Appropriate quantity of all three samples (5 mg) were individually placed in an aluminum pan which was later hermetically sealed. These samples were heated gradually at rate of 10°C min−1 from 30°C to 300°C in a nitrogen atmosphere. The flow rate of nitrogen was 50 ml/min. Empty Aluminum pan was used as control.

In-Vitro Drug release studies:

In vitro drug release studies were conducted to determine the sustained release action of HON-MPEG-PCL micelles formulation. This experiment comprised of comparison between drug release profile of free drug and prepared micelles formulation. Release media was formulated by adding 1 L of phosphate buffer along with 0.1% V/V of Tween -80 (pH 7.4). To dissolve Tween-80 into phosphate buffer, further optimization was done under magnetic stirring at 150 at 37°c. Evaporated volume was adjusted as per needed to 1 L through the experiment to achieve consistency in results. Samples were prepared as follows: Drug solution used as control was prepared following the same protocol as formulation except for the addition of MPEG-PCL (5K-2K) polymer in preparation Drug release pattern was calculated using dialysis cassettes. 0.5 ml of both the drug solution and HON-MPEF-PCL micelles were injected into cassette using syringe having 23 G needle. These cassettes were suspended into the release media. 100 ml of release media was taken in a beaker and sample filled cassette was immersed in this release media under magnetic stirring at 150 rpm and 37◦c temperature. Samples were collected at different time periods. For each 1 ml sample drawn at each time interval, it was replaced with fresh 1 ml phosphate buffer release media. Samples were drawn at 0.25. 0.75, 1, 2 h for the first day. Later samples were drawn every 24 h for 10 days (144 h). Samples obtained were diluted with methanol and analyzed under UV spectrophotometer at 292 nm. Finally, Results were quantified by plotting a graph.

Cell line Studies:

Cytotoxicity study

ARPE-19 cells were used to perform MTT Analysis for calculating cytotoxicity of prepared HON-MPEG-PCL formulation in comparison with pure honokiol drug solution. First day, Cells were seeded in 96 – well plate at a density of 5000 cells/well. These cells were supplemented with 200 µL of Complete media i.e., DMEM F12 and 10% FBS and were later allowed to grow in incubator supplied with 5% CO_2 at 37°c for 24 h. Next day, cells were treated with formulation and drug samples in triplicate. These samples were prepared in ICM (in complete media) at a different concentration ranging from 0.001, 0.01, 0.1, 1.0,10,20 µM of drug solution and formulation, respectively.

After treatment, cells were allowed to incubate for 4 hr. After 4 h treatments were removed and replaced with fresh complete media. Again, cells were incubated for 24 h. After 24 h, complete media was removed and MTT reagent 100 µL (1mg/ml) was added to the 96 well plate which was left to incubate for 4 hrs. After 4hrs, each well was replaced with 100 µl of DMSO after removing MTT agent. DMSO was used to dissolve formazan crystals. The crystal displays cell viability by showing purple color. The cell viability was the quantified by analyzing the 96 well plate through UV spectrophotometer at a wavelength of 595 nm. Cells treated with just DMEM-12 was used as negative control and 0.1% Triton × 100 was considered positive control. Results were quantified by plotting a graph between the concentrations Vs Viability while considering the cell viability of negative control as 100% viable.

Cellular uptake

Cellular uptake capacity of the formulation HON-MPEG-PCL was measured to determine the localized action of formulation in the cell. This was achieved using the confocal microscopy. ARPE-19 cells were seeded at 2×10^5 density per well in a three well plate and incubated for 24h to achieve confluency. Free drug solution, dye loaded coumarin Honokiol micelles and Blank micelles were used as treatments. Further, cells were washed thrice next day with PBS to remove any suspended dead cells. Each well was treated with different treatment for about 15 mins. DAPI was used to stain nucleus while Cell Mask ™ deep red plasma stain was used for cell membrane staining. The results were analyzed under confocal microscope FV1200 (Olympus, Tokyo, Japan) at 400k magnification for period of 80 min.

Anti HIF ELISA

This experiment was performed to study the anti HIF activity of Honokiol. Cells were seeded at a density of 5×10^3 cells/mL in 96 well plate. The plate was incubated for period of 24h in an incubator supplied with 5% CO_2 at 37 °c to achieve confluency. Next day, cells were treated with Honokiol drug solution and MPEG-PCL HON Micelles at a concentration of 10 µm. The treatment was given for a period of 24h,48h,72h & 96 h. Invitrogen ELISA HIF Kit Was used to calculate the expression of HIF protein. Experiment was performed following the given protocol within kit and plate was studied at 450 nm and 550 nm in UV spectrophotometer. Graph was plotted by taking Control as expressing 100% HIF expression and results were analyzed.

Anti- VEGF ELISA

The effect of HON – MPEG-PCL micelles % Honokiol drug solution on expression of VEGF was studied by seeding APRE-19 cells in 96 well plate at a density of 5000 cells/well. The

96 well plate was incubated for 24 h supplied with 5% CO_2 at 37°c. After 24 h, cells were treated with different treatment groups for the period of 24h,48h,72h & 96 h. Invitrogen Human VEGF ELISA ready to use Kit was used to analyze the expression of VEGF in samples collected at different timepoints. Plate was analyzed in UV spectrophotometer at 450 nm wavelength. Complete media and Incomplete media were taken as negative and positive control. Graph was plotted by taking control as 100% expression of VEGF.

Statistical analysis

All sample were run in triplicate and data are represented as mean ± standard deviation. ANOVA, and GraphPad Prism (version 6, USA) were used to analyze data statistically. P value <0.05 and < 0.01 , <0.001 were considered significant

RESULTS

Characterization of MPEG-PCL Micelles

Dynamic Light Scattering and Zeta Potential:

Pre-formulation trials were conducted using different variations of MPEG-PCL polymer composition including MPEG-PCL (5K-2K, 5K-5K &10K-5K ratio). These studies concluded MPEG-PCL (5K-2K) polymer in a 1:10 drug to polymer ratio to be optimum for micelles formulation. Particle size of micelles along with poly dispersity index (PDI) was determined using dynamic Light Scattering. Size was found to be of order 30.8±0.8 nm and PDI was 0.19 ± 0.0004. The low PDI value obtained from results was indicative of homogeneity and monotonous distribution of micelles in a formulation. The smaller particle size of micelles and lower value of PDI made formulation optimum for ocular delivery.

The mean zeta potential was determined using Malvern Zeta sizer Nano ZS90 was calculated to be -5.46 ±0.49 mV for the HON-MPEG-PCL micelles as shown in Figure 2

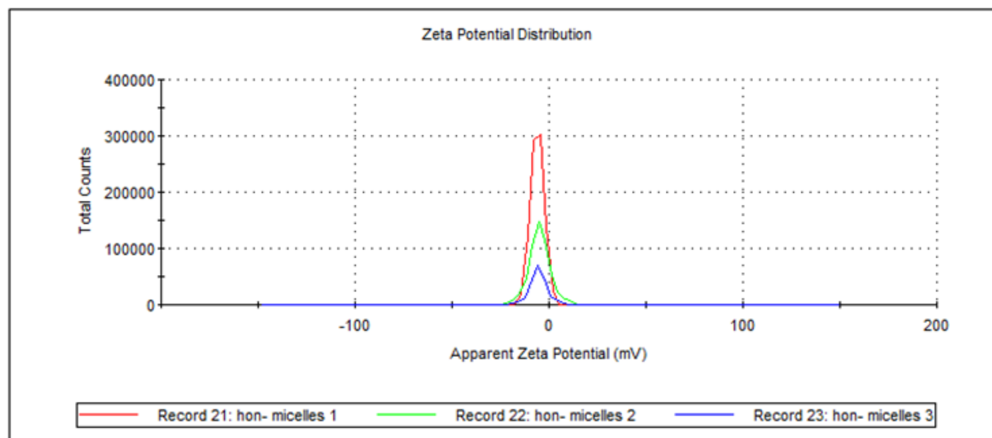

Figure 2: Z-average of HON-MPEG-PCL micelles (SD ± mean)

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed to check the morphology of micelles. The TEM images obtained after analyzing Cu grid containing samples showed micelles size to be around 30 nm. The obtained result falls in line with previous result obtained from the dynamic Light Scattering (DLS) studies which confirms the size of micelles. TEM image (figure 3) further confirmed the uniformity of preparation along with spherical shape.

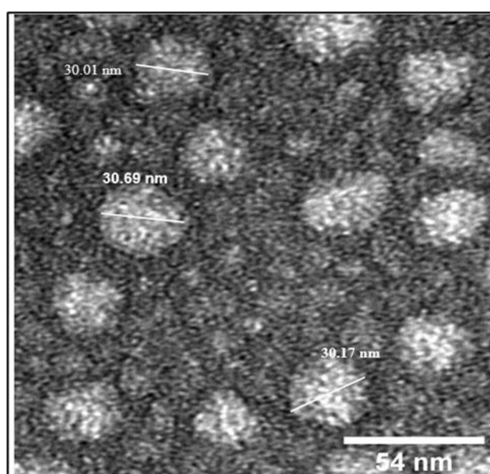

Figure 3: TEM image of HON – MPEG- PCL Micelles at am accelerating voltage of 120KV at 400 KX magnification

Entrapment Efficiency:

Entrapment efficiency of HON-PEG-PCL micelles was obtained by re-suspending the micelles in methanol in 1:9 ratio. Entrapment efficiency was calculated using the equation previously developed by dissolving varying concentrations of honokiol in methanol at 292 nm (λmax) and plotting a calibration curve r value. Reading were obtained by analyzing samples in UV spectrophotometer at 292 nm (λmax). Entrapment efficiency was calculated to be 64% ±0.135.

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry was performed to confirm loading of honokiol in micelles. This thermal analysis was performed on Honokiol drug, polymer MPEG-PCL (5K-2K) & formulation (HON-MPEG-PCL) which helped to identify the physical state of a drug in polymer that can in turn dictate the drug releasing pattern of the formulation. In case of Honokiol drug, Peak was appeared at 84.85°c while for the polymer the characteristic peak appeared at 55.16°c however, the absence of these characteristic peaks in DSC spectra of Hon-MPEG-PCL formulation which showed peak at 112.67°c confirmed the entrapment of Honokiol drug in Honokiol-MPEG-PCL micelles (figure 4).

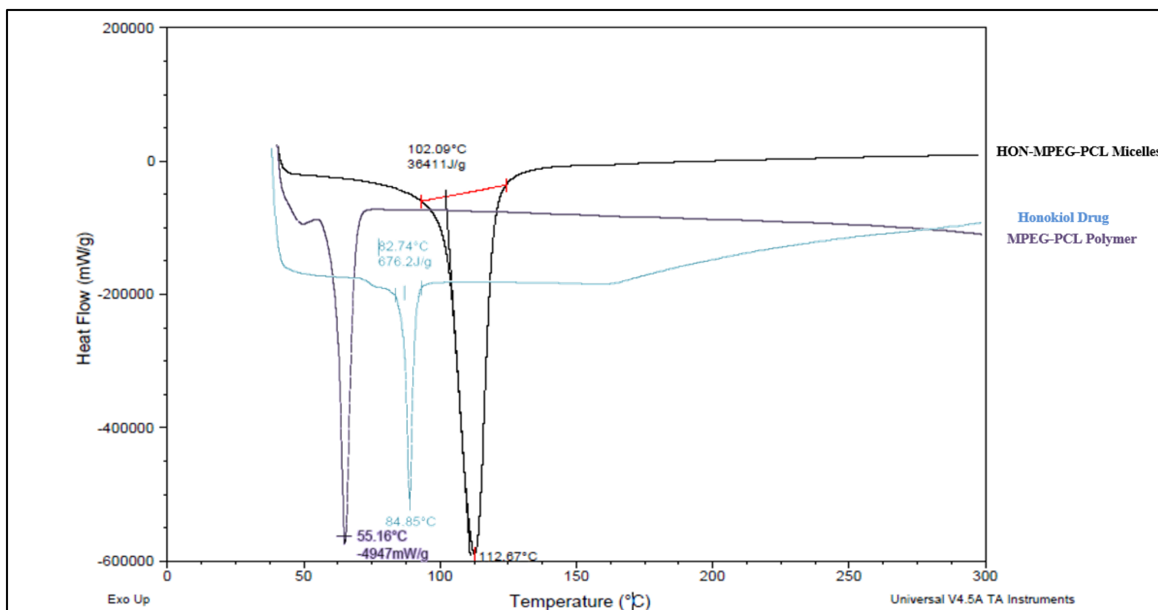

Figure 4: DSC spectra of Honokiol drug, MPEG-PCL polymer and Honokiol -MPEG-PCL micelles

In-vitro drug release:

Sustained release action of Hon-PEG-PCL micelles was analyzed through in vitro release studies. Dialysis cassettes containing with 0.5 ml of both drug solution and Formulation were immersed in 100 ml of release media comprising of PBS along with (0.1% v/v) Tween-80 to create sink condition. For the first day, samples were withdrawn at 0.25 h, 0.5 h, 1h and 2 h time point. From following day, sample were withdrawn after every 24 h for total period of 240 h. Each time removed volume (1ml) was replaced with fresh release media. To quantify drug release pattern, samples were diluted in 1:2 ratio of methanol. US spectrophotometer was used to obtain results. Finally, graph was plotted between cumulative drug release (%) as a function of time. As shown in graph, after 24 h only 24% of the formulation was released while 50% of the drug solution was observed in release media. 100% of drug solution was release in 48 h while only 79% ± 0.004 of the formulation was calculated in release media at end of 240 h period.(figure 5) This confirmed the sustained release action of HON-MPEG-PCL micelles.

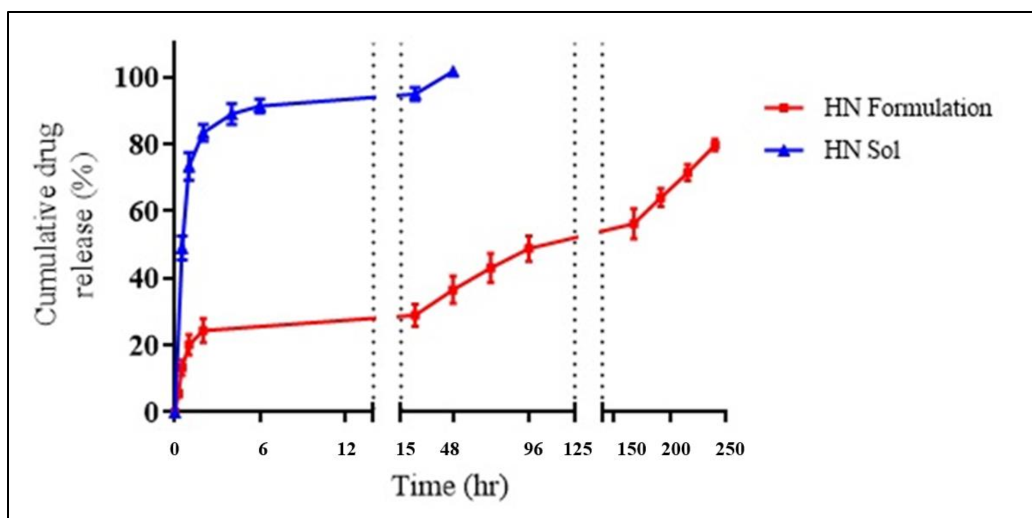

Figure 5: comparison of cumulative drug release percentage of HON MPEG-PCL Micelles and HON drug solution for period of 144 h (10 days) at 37° in release media (i.e.) Phosphate buffer saline (pH 7.4) + 0.1 % (v/v) Tween-80 (mean± SD, n=3)

Cell line Studies:

Cytotoxicity Study

The cytotoxicity of the HON-MPEG-PCL formulation was evaluated using MTT assay performed on Human Epithelial Retinal Pigment (ARPE-19) cell line. Cell were treated in triplicate with serum-free media having varying concentrations of Honokiol drug solution, Blank micelles, Honokiol MPEG-PCL micelles (0.001,0.01,0.1,1,10,20 µM) for the period of 24 h. Results obtained were analyzed by taking negative control as 100% cell viable. Blank micelles were 88% cell viable at highest concentration of 20 µM. For all concentrations of micelles formulation, cell viability was calculated to be above 80% showing formulation to be biocompatible. Lowest concentration (0.001 µm) of HON-MPEG-MPCL micelles showed cell viability of 97% ± 0.028 while highest concentration (20 µm) of formulation was 84% ±0.024 cell viable. Honokiol drug solution showed concentration dependent decrease in cell viability with 20 µm concentration showed only 18% ±0.001 cell viability. The low cell viability with higher concentrations of drug

solution showed that drug is toxic to cells while Blank micelles and HON-MPEG-PCL micelles were cell compatible with cell viability greater than 80% at higher concentrations as shown in figure 6.

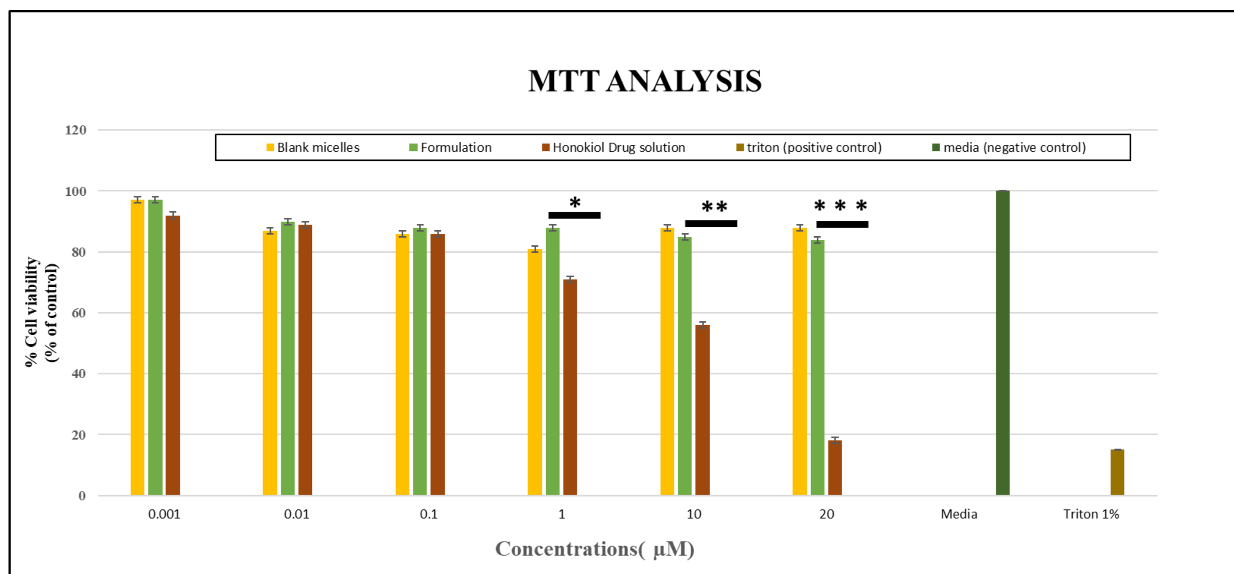

Figure 6: Cell viability plots of ARPE-19 cells after treatment with HON drug solution, HON-MPEG-PCL micelles, and blank micelles (mean ± SD, n=3*p < 0.05 **p<0.01 ***p<0.001)

Cellular uptake:

The cellular uptake of prepared formulation was checked to understand the micelles interaction with cells. This study helped to evaluate the effect of size and shape on micelles uptake by cells and surface characteristics of micelles. This experiment was performed by preparing fluorescent emitting formulation. The Florescent loaded micelles were prepared using the same protocol as Hon-MPEG-PCL micelles except micelles were loaded with fluorescent dye instead of drug. ARPE-19 cells were treated with these fluorescent containing micelles for the period of 20 mins. As shown in figure, the cell uptake increased in time dependent manners. DAPI was used to stain nucleus Blue color, Cell membrane was stained with cell mask dye exhibiting red florescence.

Green florescence represents cytoplasm of cell membrane .Micelles are taken by the cytoplasm of the micelles as shown in figure 7

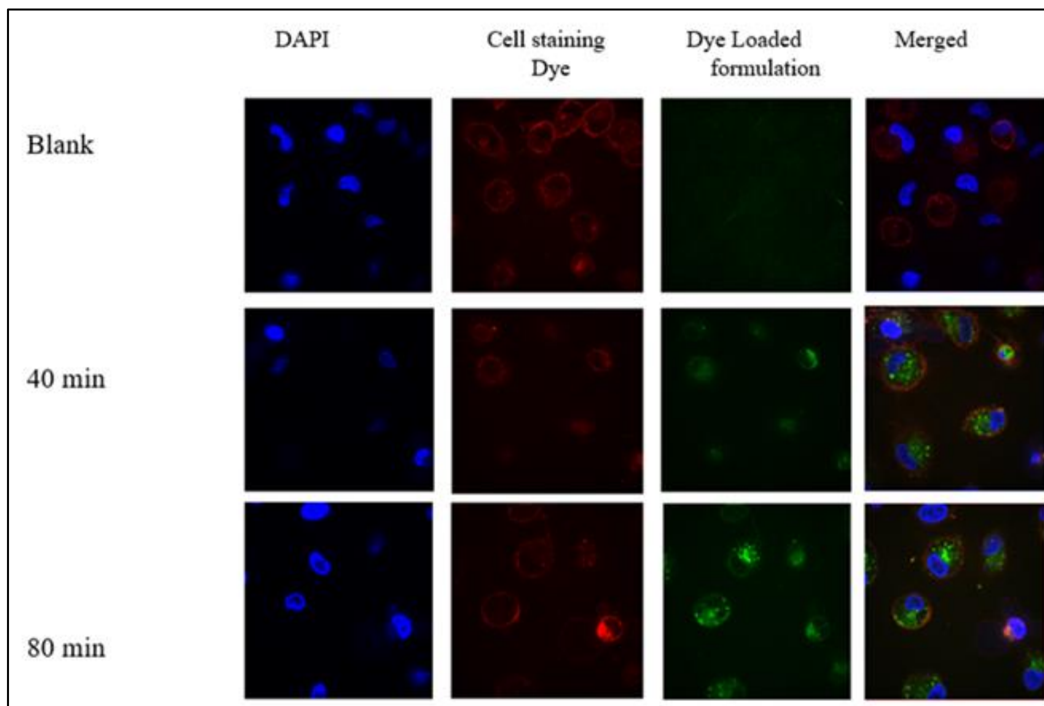

Figure 7 : Cellular uptake of dye loaded MPEG-PCL Micelles (37°c) in ARPE-19 Cells at 40 min & 80 min observed using confocal microscopy

Anti- HIF ELISA

The effect of Honokiol drug solution and HON MPEG- PCL micelles on HIF expression was studies using ELISA technique. Human HIF ELISA ready to use ELISA Kit was used to for this purpose. Samples were taken at 24 h, 48 h, 72 h & 96 h and percentage decrease in HIF expression was calculated by taking control as 100%. Results showed that for first 24 h, honokiol drug solution was able to suppress 70 % ± 0.04 of HIF expression more than formulation which suppressed 63% ±0.002 while at 96 h time point, formulation performed better with 83%± 0.05 reduction ($p < 0.01$) in HIF level. This can be explained by initial burst of free drug, but its effect decreased overtime which can be regarded to drug metabolism. On contrary, formulation showed

a sustained release effect, and its effect increased over a period of 96 h ($p < 0.01$) as shown in figure 8

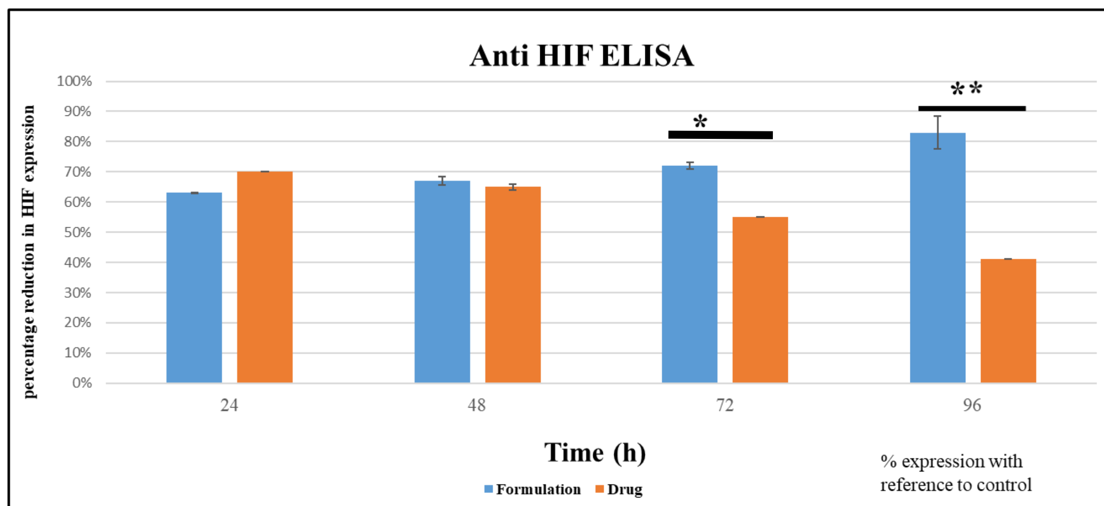

Figure 8 : % decrease in HIF expression in ARPE-19 cells treated with HON drug solution and HON MPEG PCL Micelles for different time point determined using ELISA assay method (mean ± SD, n =4) *$p<0.05$, **$p<0.01$.

Anti-VEGF ELISA:

The Anti – VEGF ELISA was further performed to check the effectiveness of HON-MPEG-PCL micelles to downregulate expression of VEGF. By taking into consideration the physiological role of HIF to increase expression of pro-angiogenic factors including VEGF. Its downregulation on treatment with HON-MPEG-PCL micelles in ARPE-19 cells shown in figure 7 was expected to decrease level of VEGF expression as well. To analyze this, the effect of HON-MPEG-PCL micelles to decrease expression of VEGF was compared with HON drug solution in Human Retinal pigment epithelial cells (ARPE-19) after treating cells for 24h, 48h, 72h, 96h. % reduction in VEGF expression was calculated by taking control as 100%. Honokiol MPEG-PCL micelles (10 μM) was able to reduce 46% ± 0.05 expression of VEGF as compare to honokiol drug formulation which decreased VEGF expression by only 13% ±0.004 ($P < 0.01$) after 96 h of

treatment (figure 8). This showed that formulation was successfully able to downregulate expression of VEGF responsible of choroidal neovascularization (CNV)

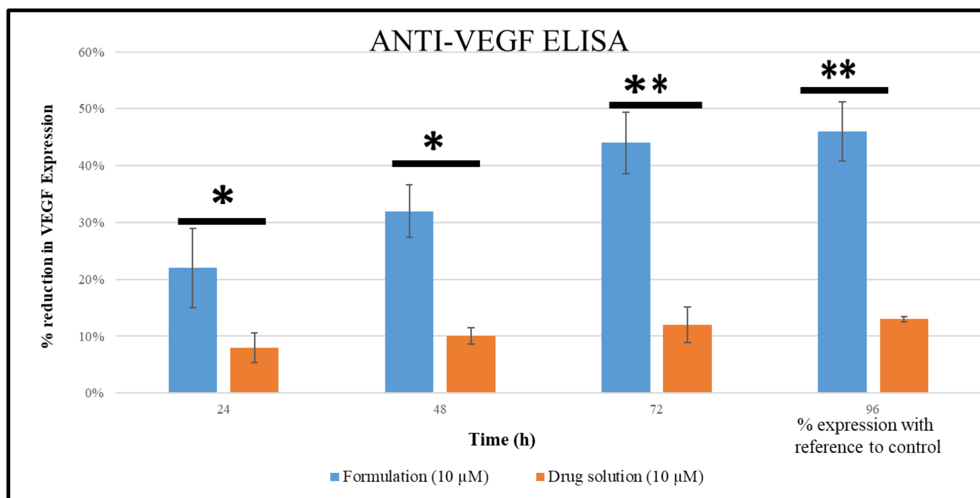

Figure 9 : % decrease in VEGF expression in ARPE-19 cells treated with HON drug solution and HON MPEG PCL Micelles for different time point determined using ELISA assay method (mean ± SD, n =4) *P<0.05 **p<0.01.

DISCUSSION

Currently, the most employed treatment for AMD includes the use of Anti-VEGF agents to counter choroidal neo-vascularization (CNV) which is the underlying cause of AMD. These treatments are expected to work by selectively inhibiting the binding of VEGF to its receptor. However, research has shown that only VEGF downregulation is insufficient to treat AMD(Kelly et al., 2003) . Therefore, in this study both important factors playing key role in pro-angiogenesis VEGF and HIF are targeted. Studies regarding systemic delivery of these agents have shown limit clinical efficiency as side effects such as proteinuria & thromboembolism have been reported. Therefore, the local delivery of these agents is preferred to overcome these side effects (Bhatt et al., 2019). However, there are some clinical challenges associated with this route of delivery as well such as the need for repetitive administration that is associated with retinal detachment, hemorrhage, edema etc. This study, therefore, was aimed to develop a sustained drug delivery system. This was achieved by loading of the drug in a polymer that has shown sustained release action. MPEG-PCL polymer is an FDA approved bio-compatible and bio-degradable system. (Streets et al., 2020). Honokiol used in a traditional herbal medicine has demonstrated Anti-HIF activity and Anti-VEGF activity. The Hon-MPEG-PCL micelles were prepared by solvent evaporation method (figure 1) and in-vitro studies were performed to check efficacy of these micelles by characterizing them and performing cell line studies on Human Pigment retinal epithelial (ARPE-19) cells. Size of micelles were calculated to be 30.8±0.8 nm and PDI value was

found to be less than 1 (0.19 ± 0.0004) which showed the uniformity and monotonous distribution of micelles (figure 3). TEM images obtained further showed the micelles to be spherical in shape. Compatibility of micelles formed were tested by running DSC. Appearance of characteristic peaks of drug and polymer in the formulation shows the incompatibility of both components. However, on successful entrapment of drug in polymer these peaks disappear. The thermal analysis performed on the formulation confirmed the encapsulation of honokiol into the polymer successfully with no individual peaks of drug and polymer. (figure 4). To corelate in vivo drug release pattern of formulation, in vitro drug release experiment was performed. Release media for this study was prepared by adding 0.1% v/v tween-80 solution to create sink conditions. The experiment was conducted at pH 7.4 and comparison of formulation and drug solution's release pattern was studied. 100% of the drug was released within 48 h showing no extended-release pattern while only 79% ± 0.0004 of the formulation was calculated in release media after 144 h. This showed that formulation had capability to act in sustained release manner while initial burst of drug release in first 24 h at site of action would result in most percentage of the drug metabolized without providing any clinically significant effect.(figure 5). The MTT analysis performed to check the safety of formulation MPEG-PCL micelles showed the formulation (Hon-MPEG-PCL micelles) to be less cytotoxic as compare to Honokiol drug solution with cell viability calculated to be more than 80% at higher doses of 10 μM ($P< 0.05$) and 20 μM ($P <0.01$). The low cytotoxic effect of formulation can be attributed to the usage of FDA approved PEG-PCL polymer that forms the outer matrix of formulation. Sustained release effect of this formulation further exposes cells to the small increment of total dose at a time that explains the better safety profile of formulation as compare to drug solution(figure 6). The cellular uptake profile of the formulation was further studied by analyzing the samples in confocal microscopy. The uptake of formulation by cells can

be explained by interaction of the formulation with receptors. The time dependent increase of green florescence observed in images (figure 6) further provide explanation for sustained release action of formulation.(figure 7). The pathophysiology of AMD details the role of HIF. Presence of hypoxic conditions due to increase accumulation of free radicals and decrease ability of ARPE cells to get rid of these free radical accumulated debris results in activation of HIF which further favors the increase expression of pro-angiogenic factor including VEGF. Its upregulation results in Choroidal neo-vascularization causing blindness. Thus, the Honokiol formulation was aimed to decrease the expression of HIF which was further expected to decrease VEGF expression. This effect was studied by performing HIF and VEGF ELISA analysis. Human Retinal pigment epithelial cells (ARPE-19) cells were treated for time of 24h, 48h,72h & 96h with different treatment groups at a concentration of 10 μM. In the case of HIF ELISA, initially drug solution was able to suppress HIF expression better than formulation however at later timepoints formulation performed better than drug solution with 83% ± 0.05 reduction in HIF expression $p<0.01$ (fig 8). Wearing off the drug effect can be explained by the rapid metabolism of drug in comparison with formulation. Anti – VEGF ELISA further performed showed downregulation of VEGF expression as well (Figure 9). Formulation was able to significantly reduce the expression of VEGF as compare to drug solution $p<0.01$. This showed the dual effect of formulation in downregulating two important pro-angiogenic factors (HIF & VEGF) playing major role in pathophysiology of the disease.

CONCLUSION

Honokiol-MPEG-PCL micelles were developed and characterized successfully. In-Vitro studies were performed on human retinal pigment epithelial cells (ARPE-19) to check the effectiveness of prepared formulation. The two main aims of formulating Honokiol -MPEG-PCL micelles were successfully achieved. The formulation was able to show sustained release action that can help to overcome patient compliance issues associated with need of multiple intravitreal injections to treat AMD. Finally, the formulation showed potential to serve dual purpose of downregulating expression of HIF and VEGF, two important genetic factors involved in underlying pathology of AMD.

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