DEVELOPMENT AND CHARACTERIZATION OF BREAST TUMOR SPECIFIC SURFACE-FUNCTIONALIZED NANOFORMULATION(S)

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In the field of cancer-specific formulations, nanoparticles are one of the biggest revolutions that have changed the entire arena, as it gives advantages in the field of both cancer diagnosis and cancer therapy. In comparison to conventional delivery systems, nanoparticles offer unique advantages, such as, reduced toxicity, precise targeting and enhanced permeation and retention effect. Nanoparticles can be created using a wide range of materials, such as, polymer, protein, lipids and other inorganic or organic material. Success of Abraxane has already proved the unique nature of protein nanocarriers in the field of cancer therapy. Apart from albumin, protein-based nanoparticles can be prepared from many sources.

Whey proteins are the proteins obtained from dairy source, an amphiphilic substance, holds good nutritional and functional properties. Whey proteins hold the ability to bind chemical substances (active pharmaceutical ingredient). Upon converting these proteins into a nanoparticulate form, these molecules have illustrated core-shell structures, where the core is an ideal compartment for various constituents for entrapment. These can self-assemble themselves into different structures, such as, film, gels, and spheres. They can be transported through cell membrane.

Moreover, whey proteins itself contain many components, due to which they themselves can be used as a targeting agent. Moreover, to make them breast cancer specific, they were loaded with drugs recommended for breast cancer and surface modified with the ligands (N-acetyl cysteine and L-carnitine) which could make them breast cancer specific, so that better efficacy and lower toxicity can be attained. The two model anti-cancer drugs chosen were doxorubicin hydrochloride (for intravenous delivery) and exemestane (for oral delivery).

Doxorubicin hydrochloride is a potent drug for cancer treatment, which is given with cyclophosphamide at clinical level. However, the drug is reported to bring toxicity, which brings in the need to make further developments related to the drug. Doxorubicin loaded whey protein nanoparticles were formulated to encapsulate them within whey nanocarrier.

After the whey protein passed the compatibility test with doxorubicin hydrochloride using UV-visible spectroscopy and FTIR, whey proteins were attached to doxorubicin hydrochloride and were converted into nanoparticulate form using alcohol as a desolvating agent and genipin as a cross-linker. Genipin is a plant-based product and is reported to be non-toxic, in comparison to other cross-linkers, such as glutaraldehyde.

In an initial screening study, whey proteins were assessed, and a direct proportionality between the concentration of whey protein and size of nanoparticles were found, due to an increase in collision and aggregation with increase in whey protein concentration. For an optimized cross-linked nanoparticle, Box Behnken design was applied. The response surfaces were studied. The first parameter studied was size. Ethanol's role was folding of protein to form nanoparticles, which results

in a smaller size. Upon alcohol concentration changes, there is an initial increase in size, however, after a certain extent, hydrophobic chains interact with each other, which to increase in agglomeration, ultimately leading to increase in the size. Zeta potential was the second parameter assessed, as it is an indicator of the stability of the compound. Most of the formulations displayed negative zeta potential with magnitude >20, which was good, because zeta potential with positive charge is not considered as suitable for intravenous delivery. Entrapment efficiency was found to be increased with increase in the cross-linker amount. Any free drug was removed with the help of frequent washing of nanoparticles.

For optimization, both numerical (desirability) and graphical (overlay) criterion provided the same solution, where the final cross-linking time was 16 h, 1.5 times the genipin concentration and 1 time the alcohol level to aqueous solution (i.e., 50% alcohol). 16 h time was crucial for the formulation. Along with 16 h time, temperature was also crucial (i.e., 20-25º C). A little increase in cross-linking time might create too much cross-linking. The surface topography of the nanoparticles was studied by transmission electron microscopy, which indicated rough surface and small size.

A layer of amino acid derivative (N-acetyl cysteine; a targeting ligand) was added to the surface of nanoparticle. The justification behind choosing N-acetyl cysteine as a targeting ligand was the reported dependency of triple negative cancer cells on cysteine in some literature. The pathway responsible for the import of cysteine into the cancer cells is "cysteine/glutathione antipotor SLC7A11", which imports cysteine for the biosynthesis of glutathione.

After surface modification with N-acetyl cysteine, a marked change in colouration was observed in case of blank nanoformulation (blue to green colour). Increase in the sulphur content indicated addition of cysteine (which contains sulphur). After this, the lyophilized nanoparticles were redispersed in water and liquid FTIR was taken. FTIR results also indicated the modification of nanoparticle surface with N-acetyl cysteine. Interestingly, it was found that, upon lyophilization, the zeta potential of nanoparticles decreased markedly. However, there was also an increase in zeta potential magnitude after surface modification.

N-acetyl cysteine-modified nanoparticles gave 75.39 % release whereas non-modified nanoparticles gave only 33% release after 24 h, which is possibly because the N-acetyl cysteine (NAC) is hydrophilic in nature. Upon assessing the cytotoxicity by MTT assay, the NAC-modified nanoparticles were better than free drug. Both whey nanoparticles and NAC-modified whey nanoparticles were internalized by triple negative breast cancer cells (MDA-MB-231 cells), which was attributed to both size and targeting ability of the nanoparticles.

Since the nanoparticle may interact with RBCs owing to their size and physicochemical properties, an assay was carried out to assess hemolysis property of a nanoparticle. The hemolysis was found to be absent after treating the nanoparticles dispersed in phosphate buffered saline with erythrocyte suspension. Also, the nanoparticles did not exhibit any hemagglutination. Thus, the nanoparticles were expected to be safe for intravenous administration to the mice. In addition, nanoparticles were also tested for any sign of reaction to the mice after injection. Upon injecting the formulations into the tail vain of mice, there was no adverse reaction found.

The triple negative breast tumor in BALB/c mice were induced using 4T1 cells. 4T1 is a cell line of breast cancer and is derived from the mammary gland tissue of BALB/c strain mouse. An initial organ distribution proved the more distribution of doxorubicin in NAC-modified nanoparticles into the tumor in comparison to other tissue. This proved the tumor-specific nature of the nanoparticles. However, this study was just an initial screening study.

For in-vivo efficacy, the animals were administered at day 0, day 6 and day 12 at a dose of 10 mg/kg. The best regression of tumor was found in case of NAC-modified whey nanoparticles (NAC-WDox), followed by whey nanoparticles (WDox) followed by Doxorubicin injection. The reason behind best tumor effect was attributed to better targeting effect by modified nanoparticles.

However, to assess what happened inside of tumor, a histological section was carried out. First, the tumor sections were stained using haematoxylin and eosin, and were observed using different magnifications under a microscope. Under 20 X, damages were more clearly visible, and area was quantified using ImageJ software. The more damage was found in case of doxorubicin injection treated and NAC-modified nanoparticles. After this, the images were viewed at 40 X, and the images were quantified for damaged cells, healthy cells and mitotic cells using QuPath® software. There was no mitotic cell found in any of the images of tumor sections treated with NAC-modified nanoparticles. Highest number of mitotic cells and healthy cells were in untreated tumor sections.

The overall results indicated the significance of N-acetyl cysteine modification. Though WDox nanoparticles gave good results, but modified nanoparticles gave better results than nanoparticles. Moreover, NAC is known to strengthen TNFα initiated apoptosis cascade and could have potentiated the results.

After exploring the potential of breast tumor specific drug-loaded whey nanoparticles for intravenous delivery, the whey nanoparticles were further explored for oral delivery. The nanoparticles were expected to enhance the efficacy of a model drug exemestane.

Exemestane is an aromatase inhibitor, which have proved its efficacy over tamoxifen in advanced breast cancer patients. Exemestane is a hydrophobic drug, belonging to BCS-class IV. However, it can bind to protein, which in the nanoparticulate may pass barriers, making the majority of drug released into the tumour.

Exemestane-loaded whey protein nanoparticles were also formulated using desolvation method, where ethanol was used as a desolvating agent, and genipin was utilized as a cross-linker. In the presence of alcohol, the structure of protein changes. The protein binding of exemestane is reported to be 90%, which probably would have made it to bind with whey protein and render good entrapment efficiency of the nanoformulation. Where in the previous nanoparticles, size, and entrapment 60-70% was desired, here, in this formulation, higher drug content and higher entrapment efficiency was given more importance than size.

The optimization was carried out using response surface methodology by Box-Behnken design. The optimized nanoparticles contained 40% v/v alcohol, 2 % w/v whey protein and 1.5 % w/v genipin concentration. The morphology was observed by scanning electron microscopy. The surface morphology was found to be rough and round but were not a perfect sphere.

It was then surface-modified with L-carnitine. Instead of direct modification with L-carnitine, it was first modified with PEG6000 to give long circulation effect and to prevent any agglomeration of nanoparticles. Since the nanoparticle was meant for oral delivery, so a ligand of dual nature was selected, which can aid in transportation of nanoparticles via oral route, as well as it can aid in targeting of nanoparticles to the tumor.

L-carnitine utilizes OTCN transportors and $ATB^{0,+}$ for their transport. $ATB^{0,+}$ expression is reported in colon, lung and mammary gland. Its expression is also indicated in a breast cancer cell (MCF-7). Because of its expression in mammary gland, it was expected that it might reach mammary and mammary tumors via active targeting approach. OCTN2 is a carnitine homeostasis regulator, which also regulates the metabolism and cellular proliferation. Thus, this transporter has been suggested as a target for breast cancer. It is upregulated in estrogen-responsive mammary cancers. It’s version, SLC22A5/OCTN2 (a carnitine transporter with

high affinity) is strictly upregulated via transcription and translation. Estrogens can upregulate SLC22A5 expression, and the first intron of SLC22A5 comprises an estrogen responsive element. If the above route bypassed, then, L-carnitine is also an intestinal transporter. Surface modification with L-carnitine might aid in transportation of nanoparticle via OCTN2 to intestine, and then, to bloodstream. After the nanoparticle reaches bloodstream, it might reach to the desired target site. Even if the outer layers got dissolved in the middle (after intestinal transport), whey protein nanoparticles are yet expected to reach the target tissue, via passive targeting approach.

Thus, the nanoparticles were further modified with L-carnitine. The release of both whey nanoparticle and PEG6000/L-carnitine was found to follow Higuchi kinetic model. The model molecules were much smaller than whey proteins; constant diffusivity, diffusion through single dimension, and negligible swelling and dissolution may have made the nanoparticles follow Higuchi model. The topography revealed smoother surface of L-carnitine modified nanoparticles. The surface modification was evaluated by FTIR.

The in-vivo efficacy was evaluated using DMBA-induced albino Wistar rat model. The formulations were administered in suspension form. Highest survival (100%) was observed in L-carnitine modified nanoparticles (Car-WExe) treated groups after the completion of study.

First parameter assessed after sacrifice of the animals was carmine staining of the fresh mammary glands isolated from different treated groups. Higher number of nodes and branching were observed in case of toxic (only DMBA-treated) group. These ductal nodes are usually responsible for further branching, indicating higher angiogenesis and higher proliferation. The Car-WExe treated groups showed least number of branching of the mammary duct.

Haematoxylin and eosin (H&E) staining of transverse sections of mammary glands were observed to look for distortion in the structures of mammary glands. Highest amount of distortion was observed in toxic group. Least distortion was observed in Car-WExe treated group. The better efficacy was attributed to improved targeting ability.

In addition, oxidative stress parameters were also observed in tissue homogenates obtained from mammary glands of various treated groups. A marked improvement was found in malondialdehyde, reduced glutathione inhibition, catalase, and superoxide dismutase levels in Car-WExe treated groups.

In conclusion, the novel surface functionalized nanoparticles were developed that were breast-cancer specific. Whey protein nanoparticles acted as an excellent nanocarriers, which was able to incorporate both hydrophilic (doxorubicin hydrochloride) and hydrophobic (exemestane) drugs. The final particle size of both the nanoparticles were small and, were obtained as desired. Quality by design and Design of Experiments was also successfully applied to optimize the two nanoparticles. The shortcoming of doxorubicin hydrochloride (perfusion to all tissues and killing normal cells) were overcome, as observed via in-vitro cytotoxicity. The modified nanoparticles were also able to successfully destroy the tumor, as observed in H&E staining of tumor sections. L-carnitine/PEG modification was able to enhance the anti-cancer efficacy of exemestane.