**ABSTRACT SUMMARY**

The transcription factor p53 is a potent tumor suppressor that protects cells from neoplastic transformation. Among the various signaling pathways regulating p53 activity, proteasomal degradation via ubiquitination through murin double minute 2 (MDM2) (an E3 ubiquitin ligase) is the most common way for p53 degradation. Nutlin-3a, a MDM2 antagonist, restores p53 function by inhibiting p53-MDM2 interaction and is thus an appealing candidate for the treatment of cancer retaining wild-type p53. However, proteasomal degradation of p53 via other ubiquitin ligase like Pirh-2 and COP-1 is a major impediment to the success of nutlin-3a mediated p53 activation. To overcome above limitation, co-delivery of nutlin-3a with a herbal proteasomal inhibitor curcumin in targeted nanoparticles (NPs) was explored in the present investigation. Results suggest that, both nutlin-3a and curcumin inhibited proteasome activity dose dependently in U87 cell line and combined treatment resulted in augmented proteasome inhibitory activity. Further, EGFR antibody targeted dual drug loaded NPs showed superior inhibition compared to native drug and unconjugated drug loaded NPs. In vitro studies demonstrated an augmented therapeutic efficacy of targeted dual drug loaded NPs over other formulations. Our results suggest that, such an approach could be potentially useful for cancer therapy.

**INTRODUCTION**

Various elements of proliferative and/or survival signaling pathways are deregulated in most human malignancies. One such potential target is deregulated p53 pathway. p53 is one of the most frequently mutated proteins in human tumors. Even in cancer retaining wild-type p53, its function is effectively inhibited by another oncoprotein called murine double minute 2 (MDM2), an E3 ubiquitin ligase that binds with p53 and sequester it for proteasomal degradation. Hence, strategies that suppress MDM2-mediated inactivation of p53 may be effective for the treatment of p53 wild type tumors and therefore has been the focus of many efforts in anticancer therapy in recent years. Recently developed anticancer drug nutlin-3a, a small molecule antagonist of MDM2 has shown greater potentiality in inhibiting MDM2-p53 interaction; however its clinical relevance is hinder by proteasomal degradation of p53 via other ubiquitin ligase like Pirh-2 and COP-1. Hence, co-delivery of nutlin-3a with a proteasome inhibitor represents a promising approach to potentiate nutlin-3a activity. In this setting drug combinations have played a prominent role in the treatment of cancer. Administration of a combination of agents hitting different targets and displaying different toxicity profiles can improve the therapeutic index either in the form of better efficacy and reduced toxicity. Curcumin a natural polyphenolic compound has been recently documented to have proteasome inhibitory effect with no or minimal side effect. With this background, we hypothesize that, by encapsulating nutlin-3a and proteasome inhibitor curcumin in polymeric nanoparticles prepared from biocompatible and biodegradable polymers like PLGA, we can overcome the shortcomings associated with native nutlin-3a and can improve the therapeutic index to many fold. Additionally, to increase the therapeutic efficacy of the dual drug loaded NPs; a targeted tumor-specific strategy is warranted. In this regard, EGFR receptor mediated targeting of antibody functionalized dual drug loaded NPs may further enhance the therapeutic value in tumor like glioblastoma that are currently most difficult to treat by classical therapeutic modalities. Thus, the objective of the present study was to evaluate the therapeutic efficacy of EGFR targeted nutlin-3a and curcumin co-loaded NPs (E-Nut-Cur-NPs) in activation of p53 pathway through inhibition of p53-MDM2 interaction and proteasome complex in glioblastoma (Scheme 1).

**EXPERIMENTAL METHODS**

Drug loaded NPs were prepared by oil-in-water single emulsion solvent evaporation method. Surface of NPs were functionalized with EGFR antibody cetuximab by EDC-NHS chemistry and conjugation efficiency was estimated by Bradford assay. The physiochemical properties of NPs were investigated by FTIR, dynamic light scattering (DLS), transmission electron microscope, AFM and SEM. Drug encapsulation efficiency and in vitro release profile was determined by RP–HPLC. Proteasome inhibition in U87 glioblastoma cell line following treatment with curcumin/nutlin-3a (single/combined) in native or nanoformulation was investigated by enzymatic assay and westernblot analysis. Further, cellular uptake of targeted drug loaded NPs compared to native drug and unconjugated counter part was studied by confocal microscopy and flow cytometry. In vitro cytotoxicity of targeted dual drug loaded NPs over other formulation was evaluated by MTT assay.
RESULTS AND DISCUSSION

Physicochemical characterization of the formulated NPs showed that they were of in nanometer range (260 nm) with negative zeta potential (~25 mV) as confirmed by DLs (Figure 1A) and further validated by TEM (Figure 1B). AFM analysis revealed that, NPs are of smooth and spherical surface (Figure 1C). The release profile of single drugs (Figure 1D) or drugs in combination (data not shown) from NPs exhibited a biphasic drug release pattern that was characterized by an initial rapid release followed by a slow and continuous release phase over 15 days.

Proteasome activity assay using Suc-Leu-Leu-Val-Tyr-AMC fluorogenic substrate was performed and the result indicates that, nutlin-3a and curcumin can effectively inhibit proteasome activity dose dependently (Figure 2). Further, combination of both the drugs resulted in augmented proteasome inhibition compared to native counterpart (Figure 2).

Figure 2. U87 cells were incubated with various treatments for 24 hrs and chymotrypsin like protease activity was measured using fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC

Cytotoxicity assay elucidate that, E-Nut-NPs and E-Cur-NPs exhibited significantly higher cytotoxicity (lower IC_{50}) compared to native drugs or unconjugated drug loaded NPs in U87 cells (Table 1). It is noteworthy that, cells treated with drugs in combination (nutlin-3a and curcumin either in solution or unconjugated/EGFR conjugated NPs) showed augmented cytotoxicity, compared to single drug treatments.

Table 1. Cytotoxicity effect of various formulations for 3 days was assayed by MTT assay and inhibitory concentration for 50 % cell death (IC_{50}) was calculated.

CONCLUSION

Our study reveals the fact that EGFR targeted dual drug loaded NPs in U87 cells treated with drugs in combination (nutlin-3a and curcumin) showed augmented cytotoxicity, compared to single drug treatments.

REFERENCES


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