Chapter 1

Introduction

Cancer is one of the most important disorder causing morbidity and mortality all over the world. Amongst several cancer types, glioma is grade IV neuroepithelial tumours, accounts for 80% of all primary central nervous system (CNS) tumours [1]. Glioma is diffusely penetrating throughout the brain and extends far beyond the original tumour mass. As a consequence of such metastatic nature, unfortunately, every last tumour cell cannot be removed surgically. Despite combination treatment of surgery, radiotherapy and chemotherapy, glioma is insidious/destructive and is associated with poor prognosis, frequent recurrence and extremely high lethality [2]. Surgical resections of brain tumours led to a median survival of 3 months [3]. Additional radiotherapy and chemotherapy successfully prolong median survival of the patient up to a year [4]. Therefore, modern research is still trying to find new drug molecules and novel therapeutic delivery systems to improve the lifetime of glioma patients.

Trans resveratrol (3,5,4'-trihydroxystilbene) (RSV) is a natural non-flavonoid polyphenolic compound abundantly present in grapes, red wine, peanuts, berries and in several materials of normal human diet. RSV was first isolated from the roots of *Veratrum grandiflorum O. Loes* (white hellebore) in 1940 and latter from the roots of *Polygonum cuspidatum* (Japanese knotweed) in 1963 [5, 6]. RSV is existing in *cis* and *trans* forms. Among them, *trans* form of RSV was proved for several desirable biological actions such as cardioprotection, preventing platelet aggregation, vasodilation,

prolongation of lifespan and cancer prevention [7]. Recently, RSV has been proved for its anticancer potential against glioma [8-10].

Several molecular mechanisms have been proved for chemopreventive and chemotherapeutic potential of RSV against glioma [11]. RSV initiated p53-dependent apoptosis in glioma cells through essential binding to plasma membrane integrin αVβ3 [12]. RSV induced both dose-dependent and time-dependent apoptosis in human glioma U251 and U87 cells by suppressing cyclin D1 expression in G0/G1 growth phase [9]. In C6 glioma cell lines, RSV increased the expression of caspase-3 mRNA and caspase-3 activation, thereby inhibits cell growth [8]. Cancer cells of solid tumours stimulate the formation of new blood vessels for providing nutrients and oxygen to tumour cells for the development of tumour. Malignant gliomas are vascular tumours that produce an important mediator of angiogenesis called vascular endothelial growth factor (VEGF). RSV suppressed VEGF expression in rat RT-2 glioma cells and inhibited the proliferation of human umbilical vein endothelial cells. Intra peritoneal administration of RSV to Fischer 344 rats implanted with RT-2 glioma cells showed anti tumour and anti angiogenesis efficacy. Survival rate of animals were significantly increased [13]. RSV is also reported for suppression of tumour invasion by inhibiting matrix metalloproteinases (MMPs) which is a key factor involved in the degradation of extracellular matrix during invasion [10]. Thus, RSV exerts cell cycle arrest by anti angiogenesis and reduction of tumour invasion mechanisms. Though RSV showed strong efficacy against glioma cells and associated with several desirable pharmacological effects, its therapeutic applications are limited because of short biological half life, rapid metabolism and elimination.

Plasma half life ($t_{1/2}$) of RSV after oral administration was found to be only 15 minutes [14]. RSV undergoes glucuronidation by glucuronosyl transferase to form *trans*-resveratrol-C/O-diglucuronides and sulphonation by sulfotransferase to form *trans*-resveratrol-3-sulfate and *trans*-resveratrol-disulfates [15]. About 22 - 44% of the administered dose or 31 - 63% of glucuronic acid conjugates and sulfate conjugates were excreted with in 12 h in urine [15, 16]. Intravenous administration of RSV also showed a short $t_{1/2}$ ranging from 7.8 to 33 minutes while varying the dose [17, 18]. Short half life and rapid metabolism of RSV requires higher dose and frequent administration for achieving therapeutic effect. Several attempts such as complexation with β-cyclodextrins, biodegradable polymeric nanoparticles, solid lipid nanoparticles, polymeric lipid-core nanocapsules and β-cyclodextrin nanosponges have been attempted in milieu of improving bioavailability and decreasing intensive metabolism [15]. However, prolongation of systemic circulation has not yet been focused to improve the therapeutic potential of RSV. Moreover, major obstacle for glioma chemotherapy is the blood brain barrier (BBB). Therefore, brain targeting efficacy is also essential to attain the chemopreventive and anticancer potential of RSV against glioma.

D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS) is an amphiphilic molecule useful in chemotherapeutic delivery of drugs via nanoparticles as emulsifier, stabilizer, bioavailability enhancer, solubiliser, additive, P-glycoprotein (P-gp) inhibitor. TPGS having hydrophilic liphophilic balance (HLB) value 13.2 and a relatively low critical micelle concentration (CMC) of 0.02% w/w. Therefore, TPGS is suitable to serve as an effective surfactant to emulsify hydrophobic molecules and stabilize nanoparticles. TPGS is approved by FDA as pharmaceutically safe adjuvant. The co-administration of TPGS

has been shown to inhibit P-glycoprotein mediated multi-drug resistance and increase oral bioavailability of anticancer drugs. Nanoparticles made up of copolymers of TPGS were widely investigated for cancer diagnosis and therapy [19-22]. Moreover, TPGS was proved to enhance drug encapsulation efficiency, cellular uptake, *in vitro* cytotoxicity in cancer cells and prolonged systemic circulation of nanoparticles. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE PEG 2000) is also one of the crucial molecule frequently reported for the preparation of conventional stealth nanoparticles. DSPE PEG 2000 coated nanoparticles were well proved for long circulation and bioavailability enhancement of many therapeutic molecules.

In view of the above facts, we aimed in our research to formulate RSV loaded TPGS and DSPE PEG 2000 coated nanoformulations (solid lipid nanoparticles, PLGA: TPGS blend nanoparticles, core-shell polymer-lipid hybrid nanoparticles and liposomes) to prolong the systemic circulation and brain targeted delivery of RSV after intravenous (*i.v.*) administration in rats. These nanoformulations were prepared sequentially to overcome their shortcoming in order to achieve the objective of effective treatment of glioma by prolonging systemic circulation and increasing the brain accumulation. Systematic nanoparticulate characterizations were carried out extensively to evaluate the nanoformulations. *In vitro* cytotoxicity of nanoformulations against C6 glioma cell lines and cellular internalization of coumarin 6 loaded nanoformulations were carried out to demonstrate the efficacy against glioma. Further, pharmacokinetics, passive brain targeting potential and haemocompatibility were also carried out to prove the prolonged systemic circulation, passive brain targeting potential and safety up on *i.v.* administration.

As RSV is highly hydrophobic and poorly water soluble in nature (solubility = 30 mg/L; log P = 3.06), we aimed to formulate solid lipid nanoparticles (SLN). SLN have been proved to be the potential drug delivery systems for brain targeting through passive mechanisms. SLNs are made up of lipids present in human body and several foodstuffs. SLNs are highly suitable for brain targeting due to their small diameter, spherical shape and favourable zeta potential, prolonged drug release, stability, rapid cellular uptake (5-10 min), etc [23]. SLNs were also proved as safe and potential carrier for parentral administration of chemotherapeutic agents [24]. The evaluation of prepared SLN indicated that brain accumulation of RSV can be enhanced with multiple dosing in a day which will be patient unfriendly. This prompted us to think of polymeric blend nanoparticles as these are shown to have prolonged systemic circulation with higher plasma half life [25-29]. Polymeric nanoparticles are capable of opening tight junctions of BBB, effectively mask barrier limiting characteristics of drug molecules, sustaining drug release, prolonging the systemic circulation and protecting against enzymatic degradation. In recent literature, PLGA:Poloxamer blend nanoparticles were reported to provide protective environment and prevent structural alteration of encapsulated molecules [30]. In addition to blend nature, poloxamer molecules are occupying surface of the nanopaticles and thereby render hydrophilic surface. Addition of hydrophilic substances in blend nanoparticles significantly reduces opsonisation/uptake by reticulo endothelial system (RES) and thereby prolongs the systemic circulation. Therefore, in our subsequent attempt, we developed RSV loaded PLGA: TPGS blend nanoparticles (RSV-PLGA-BNPs) for prolonged systemic circulation by improving biological half life along with higher accumulation in the brain. Since PLGA:DSPE PEG 2000 blend nanoparticles

were unstable, they were not focussed in our study. RSV-PLGA-BNPs showed significantly higher half life, mean residence time and prolonged systemic circulation in comparison to SLN formulations but with significantly lesser the brain accumulation. The higher lipophilic nature of SLN might be the reason for higher brain accumulation. Blend nanoparticles favour prolonged systemic circulation and SLNs support higher brain accumulation.

Core-shell polymer-lipid hybrid nanoparticles (HNPs) are novel and robust drug delivery platform for high drug entrapment, tunable and sustained drug release, excellent serum stability, prolonged systemic circulation and differential targeting of cells or tissues. Therefore, we aimed to investigate RSV loaded TPGS and DSPE PEG 2000 coated core-shell polymer-lipid hybrid nanoparticles to improve the brain accumulation and to prolong the systemic circulation of RSV. In the present design, HNPs comprised of three distinct functional components viz polymeric core, surrounding lipid layer and hydrophilic shell. The hydrophobic polymeric core comprised of poly (lactic-co-glycolic acid) (PLGA) encapsulates poorly water soluble RSV (causes efficient entrapment). The surrounding lipid layer comprised of phosphatidylcholine promotes the retention of RSV molecules (further improving entrapment efficiency, higher drug loading and sustained drug release). The hydrophilic shell comprised of TPGS or DSPE PEG 2000 avoids adsorption of plasma proteins (opsonisation) and RES uptake (causes prolonged systemic circulation and higher treatment efficacy). As per the expectations, stay in the systemic circulation was prolonged and brain accumulation was improved. However, brain distribution of both HNPs was still found to be lesser in comparison to SLNs. Liposomes are the excellent carriers to deliver the drug across BBB [32, 33]. Moreover, liposomal

formulations are currently manufactured in large scale and marketed for chemotherapeutic applications. Passive targeting through enhanced permeation and retention (EPR) effect and active targeting toward cell surface receptors on cancer cells by ligand conjugation are possible using liposomes. PEGylated liposomes are the second generation vesicular carriers that are attempted for improving pharmacokinetics and long circulation of its therapeutic pay loads. Steric stabilisation of nanocarriers reduces interaction and adsorption of plasma proteins and thereby decreases the uptake of liposomes by RES. Reduction of macrophage uptake or opsonisation ultimately favours prolonged systemic circulation with an overall impact of reduced dose and frequency of administration [34-36]. Moreover, the liposomal entrapped RSV will not readily available for metabolism. Therefore, vigorous glucuronidation and sulphonation of RSV will be delayed until exposed to the metabolizing enzymes [37]. This prompted us to investigate liposomes for improving the brain distribution as well as prolonged systemic circulation. Therefore, we subsequently developed TPGS and DSPE PEG 2000 coated RSV liposomes to improve the biological half life and prolonged systemic circulation of RSV after *i.v.* administration in rats. Among the prepared nanoformulations, liposomes showed lower particle size, higher entrapment efficiency, higher area under the curve, improved plasma half life, lower clearance, lower liver distribution and higher brain accumulation.