ABSTRACT

Despite various discoveries in cancer research, new approaches are required for major improvement of therapeutic agents. Therefore, new broad-spectrum cytotoxic analogs and target specific agents are being continuously synthesized worldwide in order to find anticancer agents with lesser toxicity and more potency as well as efficacy.

Consequently, there is an increasing trend to optimize pharmacokinetics, enhance antitumor activity and reduce toxicity. The main objective of our research effort to select novel betulinic acid derivative(s) with drug-like properties after in vitro assays as a filter and the selected candidates are subjected to various aspect of in vivo pharmacokinetic evaluation (ADME). These insights are expected to help in identifying potent anticancer agent with improved pharmacokinetic and better safety profile versus existing anticancer drugs in the market.

Primary in-vitro screening was performed using a diverse panel of various cancer cell lines like colon (PTC), ovary (PA1), lung (A549), pancreas (MIAPaCa), prostate (DU145), and human leukemia (K562) cells. Potent derivatives were selected which shows cytotoxicity activity, specificity and purity. In this thesis, about more than 500 (five hundred) novel derivatives with modifications in C2, C3, C20, and C28 position of betulinic acid were screened and 25 molecules with better activity profile, as compared to betulinic acid have been identified. The five short listed derivatives were dihydrobetulinic acid derivatives with free carboxylic acid group at C-28 (R3) and functionalized at C-2 and C-3 (R2) with the following functional groups i.e. 4012 (5’-Chloro-2, 3-didehydroindolo), 4015 (2, 3-didehydroindolo amide linkage), 829 (4-fluorophenyl-hydrazono), 807 (benzoyl-hydrazono) and 1065 (2, 4, difluoro-benzylidene-amino).

Based on good in vitro anti-cancer activity, specificity, purity and established SAR, selected molecules were evaluated using in vitro ADME screening assay for lead optimization.

The in vitro ADME studies of five short listed derivatives were tested using solubility, Log P & Log D, permeability, metabolic stability, plasma protein binding, plasma stability and cytochrome P450 inhibition. All five short listed derivatives were found to have poor solubility (less than 0.1 µg/mL) and permeability (log Pe < -5.0) with high protein binding (% binding > 98%). However derivatives 4012 and 4015 were found to possessed good metabolic stability and did not inhibit key CYP450 enzymes. We found
that derivative 4012 and 4015 had more or less comparable ADME characteristics and taken up for further development work.

After in vitro ADME evaluation, potential drug likeness short listed LEAD molecules 4012 and 4015 were subjected to in vivo preliminary pharmacokinetic (PK) screening in wistar rats each, at 10 mg/kg dose (body weight). PK screening parameters concluded that more favorable pharmacokinetic was seen with 4012 compared to 4015. Hence, novel betulinic acid derivative 5'–Chloro-2,3-didehydroindolo [2’,3’: 2,3] betulinic acid (DRF-4012) was selected as a LEAD molecule for further in vivo development.

The in vivo efficacy study was carried out to determine the anti-tumor potential of potent compound 4012 in sensitive and selective PTC (Colon) cell line. At 10 mg/kg tested dose level of 4012, significantly inhibition of tumor growth volume was observed compared with vehicle control group. No significant weight loss and mortality was observed during this period which indicates no or minimal 4012 related toxicity.

Full in vivo pharmacokinetic evaluation of DRF-4012 like single dose PK, absolute bioavailability, dose range finding, gender effect, excretion, tumor uptake, biodistribution and metabolism studies were performed.

Single dose PK data suggested that DRF-4012 is widely distributed and has as moderate clearance, high volume of distribution and long elimination half life in rats. Excellent linear relationship between the i.v. administered dose and initial concentration (C₀) ($r^2=0.98$) and/or area under the curve (AUC) ($r^2=0.98$) were observed at 2, 5 and 10 mg/kg doses. It exhibited poor absolute bioavailability in rats. Gender difference has no effect on pharmacokinetic of DRF-4012. WinNonlin calculated PK parameters in tumor concluded that DRF-4012 has long elimination half life, high mean residence time (MRT$_{last}$) and apparent volume of distribution with low clearance. As DRF-4012 has a long elimination half life and resulted nanoparticle formulation leads to pro-longed circulation time in blood and enhance accumulation by tumors.

Biodistribution and excretion studies in human tumor-bearing nude mice were performed for DRF-4012 nanoparticle (30 mg/kg body weight) after i.v. injection, to understand the target efficiency, assessment of off-target accumulation and prediction of potential sites of adverse reactions for safe biomedical application. After 0.5 h, tumor showed the second highest concentration, which was nearly half of the liver. After 4 and 24 h, the
highest concentration of DRF-4012 was found in tumor indicating its retention in tumor site for a longer time. Excretion studies revealed that very low amount of unchanged DRF-4012 was observed in urine and primarily excreted through fecal route. This study may be useful to explain the manner in which DRF-4012 can inhibit tumor growth without apparent toxicity.

As very low amount of unchanged DRF-4012 was observed in urine, so it is necessary to carry out further studies to determine the fate of compound in the body.

During metabolite characterization, it is concluded that DRF-4012 was recovered as unchanged form in feces and urine. Minor hydroxy metabolites are obtained in urine with major glucuronide metabolite was observed in feces (due to presence of free carboxyl group) and excreted as phase-II elimination reaction.

From the above studies it can be concluded that betulinic acid derivative DRF-4012 have the potential to be developed as therapeutics for the treatment of various cancers when administered by systemic route.

Being a natural product derived molecule with ready availability of starting material, high purity, yield of synthesis and low toxicity in animal these molecules are promising anticancer agents. These findings have made betulinic acid and its derivatives attractive candidates for the clinical treatment of various forms of cancer.

**Keywords:** Betulinic acid; DRF-4012; HPLC; Pharmacokinetic; Rat plasma; LC/MS; Biodistribution study; Excretion study; liquid-liquid extraction