SUMMARY
&
CONCLUSION
In terms of cancer treatment, there are serious limitations in chemotherapy, namely the lack of selectivity of active ingredients and the development of resistance by cancer cells to these chemicals [Setzer et al., 2003]. Current chemotherapeutic agents destroy both cancerous and non-cancerous cells. Thus, there is an urgent need to find new chemical agents that can differentiate between normal and cancerous cells in order to selectively kill the cancerous cells and drug resistant tumor with reduced toxicity [Tian et al., 2006].

In recent times to address the unmet need of adequate cancer therapies efforts are made to study various classes of chemical compounds with potent anticancer properties. Targeted cancer therapy has now been rapidly expanding and small organic molecules are being exploited for this purpose. Quinolones (camptothecin, topotecan and irinotecan), diterpenoids (Paclitaxel and docetaxel), combretastatin (CA-4) and aryltetraline (etoposide) are few chemical classes which had shown excellent anticancer properties. Recently, epothilones are emerging as future potential anti-tumor agents.

Naphthyridine class is also being exploited in cancer chemotherapy. There is evidence that its antitumor activity is due to the intercalation between the base pairs of DNA and interference with normal functioning of the enzyme topoisomerase II which is involved in the breaking and releasing of DNA strands (Baez et al., 1983).

Recently, 1, 8-naphthyridine derivatives have been explored in cancer chemotherapy and one compound of this class SNS-595, a derivative of 1, 8-naphthyridine is in second phase of clinical trials and acts as a cell cycle modulator. SNS-595 is a replication-dependent DNA-damaging agent that intercalates DNA and inhibits topoisomerase II, resulting in double-stranded DNA breaks, irreversible G2 arrest, and rapid apoptosis. The Topoisomerase II-associated DNA intercalation and DNA damage produced by SNS-595 are highly selective and show selectivity for proliferating cells. SNS-595 is a naphthyridine-derived small molecule that exists as a zwitterion-containing carboxylic acid and amine functional groups. It is currently under clinical investigation in acute myeloid leukemia and ovarian cancer. (FDA news, 2006; Tomita et al., 2002; Tsuzuki et al., 2004).
In light of the tremendous potential and interest generated with respect to the chemistry and the pharmacological properties of these compounds, the research was undertaken in an effort to explore the potential of these 1, 8-naphthyridine derivatives. No. of derivatives were synthesized by making changes at C-3 position (Refer section Naphthyridine derivatives-structure & code) and screened using a human tumor cell lines. Potent derivatives were selected and further studies were carried out to determine the potential of these derivatives for the development as anticancer drugs.

**Evaluation of Purity, characterization and in vitro cytotoxicity of Naphthyridine derivatives and selection of pure and potent derivatives**

At the early stage of drug screening i.e. at the stage of hit selection, hundreds of synthesized molecules undergo *in vitro* biological screening. In order to avoid false positive from *in vitro* screening, the purity and confirmation of these synthesized molecules is very important. High impurity containing compounds may lead to false results due to biological activity of impurity so assessing the purity of chemical compound libraries is critical in the discovery phase, especially prior to evaluating the biological activity of potential NCEs during hit identification and lead optimization.

In the present investigation, total 48 derivatives were taken which were synthesized by carrying out modification at C-3 carboxamide acid with different amino acid derivatives and with cycloalkyl, aryl, hetro aryl and tertiary amine derivatives with and without substitution at C-6 and C-7 positions. (Derivatives provided by medicinal chemistry division, Dabur Research Foundation)
In order to eliminate false positive results in biological screening and to ensure that the only pure, authentic derivatives were taken for further development, purity estimation and characterization of Naphthyridine derivatives were performed. Two gradient chromatographic methods and a generic LC/MS method was developed which were found capable of estimating the purity and characterization of all 48 derivatives. The derivatives which showed purity of more than 95.0% were selected for characterization study. Out of the total 48 derivatives, 40 derivatives had shown purity of more than 95.0%. Purity of all the derivatives found to lie from 95.0% to 100.0%. Eight derivatives were excluded from the study at this stage because of poor purity (<95.0%).

The selected 40 derivatives which were then tested for characterization study and were found in conformation of their structure based on molecular weight determination. These studies ensured that the derivatives selected were the authentic pure derivatives.

All the 40 derivatives selected from purity and characterization studies were screened for cytotoxicity in the panel of six cancer cell lines. Selection of cell lines were based on the previously reported cytotoxicity of 1, 8-naphthyridine carboxamide against various cancer cell lines (Hoch et al., 2009; Banti et al. 2009), where the anticancer activity of the derivatives is reported in breast, ovarian, colon, lung, gastric, and melanoma cell lines. All the 40 derivatives were tested on panel of six cancer cell lines which included PA1 (ovary), DU145 (prostate), KB (oral), SW620 (colon), HBL100 (breast), A549 (lungs). Naphthyridine derivatives were screened using two screens: cytotoxicity screen and cancer specificity screen (effect of derivative on normal cell lines)

Out of the total 40 derivatives, 13 derivatives had not shown any anticancer activity in any of these cell lines. 15 derivatives which showed acceptable cytotoxicity (Mean IC$_{50}$ less than 7.0µg/ml & IC$_{50}$ less than 10.0µg/ml on at least three individual cell lines) were subjected to specificity screen. 10 derivatives showed acceptable specificity. It was observed that C-3’ aryl substituted (5059, 6113), C-3’ heteroaryl substituted (5016), dihalo (5058, 5079, 6090) and mono halo substituted (6089) derivatives had excellent cytotoxicity.
5059 showed potent cytotoxicity with IC\textsubscript{50} of 2.8 µM in prostate (DU-145). 5016 showed a very potent cytotoxicity with IC\textsubscript{50} of 0.43 and 1.2 µM on ovary (PA-1) and colon (SW620) respectively. Naphthyridine derivative 5063 showed broad spectrum activity with IC\textsubscript{50} <4µM on three cancer cell lines (DU145, SW620, HBL100). 6089 and 6090 had shown high cytotoxicity on ovary cancer cell lines with IC\textsubscript{50} of 0.54 and 1.1 µM respectively. 6113 showed high and broad spectrum cytotoxicity against ovarian, prostate, oral and colon cancer cell lines with IC50 values of 0.6, 0.55, 1.23 and 1.39 µM with good safety index.

From the purity, characterization and cytotoxicity studies, derivatives 5016, 6090, 6113, 5063, 6089, 6088, 5059, 6023, 5079 and 5059 were found to be suitable for the next stage of studies i.e. ADME studies

**Evaluation of Physicochemical, ADME and preliminary in vivo pharmacokinetic properties of selected potent derivatives and screening of suitable derivatives**

Evaluation of physicochemical and in vitro ADME properties was performed for these potent derivatives in order to reduce the number of compounds that consume precious secondary screening resources and may fail in ADME properties in the final stage before preclinical testing. The derivatives were evaluated on following physicochemical and in vitro ADME properties.

1) Solubility  
2) Permeability  
3) Metabolic stability  
4) Plasma protein binding  
5) Plasma stability  
6) Partition coefficient determination  
7) Enzyme inhibition

Aqueous solubility (kinetic solubility) of these derivatives was determined by DMSO back precipitation method. The selected derivatives showed poor to moderate solubility. Only two derivatives, 5016 and 5059 showed the moderate aqueous solubility of 202.36
µM and 148.75µM respectively. Rest of the derivatives showed poor aqueous solubility (< 82 µM).

Permeability of these derivatives was determined by using PAMPA assay. It was found that the short listed compounds had moderate to poor permeability (Log Pe < -5.0). The permeability of the derivatives was found from Log Pe -4.9 to -6.08. The derivative 5016 showed the highest permeability (Log Pe -4.9). Based on permeability, the short listed compounds were ranked as: 5016 > 5059 > 5079 > 5063 > 5058 > 6090 > 6113 > 6088 > 6089 > 6023.

Partition coefficient i.e. Log P of the selected derivatives was determined using water/octanol phase separation method. Log P values of most of the Naphthyridine derivatives was found to be higher (>5.0). Derivatives 5016 and 5079 showed the Log P values around 4.0 which suggested that these two derivatives had good hydrophilic and lipophilic balance while the other derivatives had high lipophilic character.

The results of solubility, permeability and Log P suggests that though these derivatives had potent cytotoxicity but the poor aqueous solubility, permeability and Log P values of most of these derivatives could result into the poor oral bioavailability and distribution in the body which in turn could affect the in vivo efficacy of these derivatives. Few derivatives like 5016, 5059, 5063 and 5079 may provide some plasma exposure on oral administration. For i.v administration, these derivatives could face formulation preparation issues because of poor physicochemical properties.

ADME properties like metabolic stability, plasma protein binding and plasma stability are the ADME properties which decides the fate of the compound once it enter the systemic circulation. Metabolic stability of these compounds was evaluated using pooled liver microsome preparations comprising of a pool of 22 human livers. The data of metabolic stability showed that the derivatives 5059 5016, 5079 and 5063 were stable and remain un-metabolized. All these compounds showed 100% metabolic stability towards human liver microsomes. Derivatives 6088 metabolized to some extent (about 15%),
while the derivatives 5058, 6090, 6113, 6089, 6023 were found to extensively metabolized. Derivative 6090 was found to metabolize by more than 93.0% while 5058, 6113, 6089 and 6023 were metabolized by more than 75%

Plasma protein binding of these selected derivatives was assessed using ultrafiltration method. All the naphthyridine derivative showed very high protein binding (>97 %) in human plasma accept compound 6088 which showed relatively low binding (~92 %) as compared to other derivatives.

Stability of the selected derivatives was evaluated in human plasma by incubation the derivatives with human plasma at 37°C for 60 mins. None of the derivatives showed any instability in plasma. All the derivatives were found to be stable in human plasma.

The results of these important ADME properties (metabolic stability, plasma stability and plasma protein binding) suggested that due to metabolic instability, the derivatives 5058, 6090, 6113, 6089, 6023 can not be further developed as drugs as these would extensively metabolise in clinical situation which would ultimately results in loss of efficacy. Plasma stability and plasma protein binding results suggested that these properties had not any significant effect on its selection for further development. However, the high protein binding of these derivatives suggested that the free drug concentration of these compounds will be less; therefore high doses need to be administered for sustained activity.

Assessment of CYP inhibition potential of the compounds at early stage has become vary important now a day, especially in the cancer therapy where the combination therapy is very common. These studies asses the potential of drug-drug interaction of the compound if given together with other compounds. The effect of short listed derivatives was determined on five most common CYP enzyme isoforms (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4). The assay tests the effect of a test compound when incubated along with CYP enzyme isoforms and the substrate .The derivatives were
tested for CYP inhibition at 10 µM. None of the derivatives showed inhibition of any of the selected CYPs by greater than 50%. The derivatives showed inhibition in the range of 33.32% to 3.0% across all the CYPs. In conclusion, it may be said that although naphthyridine derivatives do not have any significant effect on CYP enzymes at selected concentrations, further studies may be carried out for these enzyme systems, before considering short listed compounds for combination chemotherapy. The results are reproduced in a CYP inhibition assays using fluorescent substrates. Results with CYP inhibition studies suggested that these derivatives could be used as in combination therapy with known anticancer drugs.

Based on the evaluation of physicochemical and in vitro ADME data four compounds 5059, 5016, 5063 and 5079 were selected for preliminary in vivo PK study. The preliminary in vivo PK study was conducted in Wistar rat. The plasma concentration – time data following i.v administration showed plasma levels of compound 5079 and 5016 while the other two derivatives 5059 and 5063 showed no detectable levels. Further investigation of the derivatives 5059 & 5063 suggested that these derivatives form the glutathione conjugate on reaching systemic circulation. Between the two compounds 5059 and 5016, obtained from the in vitro ADME and preliminary PK study, compound 5016 showed slightly better pharmacokinetics as compared to 5079. Both the compounds showed similar Co value while the AUC and half life values of 5016 were found higher than 5079, suggesting that the extent of exposure of 5016 is better than the 5079.

Above findings suggested that 5016 can be considered as the LEAD compound and subjected to the detailed in vivo pharmacokinetic and preliminary efficacy study.
Evaluation of in vivo pharmacokinetics and preliminary efficacy of LEAD compound 5016.

Despite that valuable insight is obtained from in vitro ADME (absorption, metabolism, distribution and excretion) screening assays, in vivo drug exposure is still emphasized by drug discovery teams when making decisions about molecules. In vivo animal PK studies provide a reality check which guides the medicinal chemists to optimize the chemical structure of compounds. In vivo animal PK information also assists pharmacologists to design effectively in vivo efficacy studies and accurately interpret pharmacodynamic (PD) observations. There is no substitute for actual in vivo data in assessing pharmacokinetic profiles of drug candidates.

The in vivo pharmacokinetic study of the lead molecule 5016 was conducted to asses the bioavailability, dose linearity and gender effect. Following set of pharmacokinetic study was conducted in order to evaluate complete pharmacokinetic profile of 5016.

a) Bioavailability study.
   1) Pharmacokinetic study of 5016 in male Wistar rat at dose of 5.0 mg/kg by intravenous route.
   2) Pharmacokinetic study of 5016 in male Wistar rat at dose of 10.0 mg/kg by oral route.

b) Dose linearity study
   1) Pharmacokinetic study of 5016 in male Wistar rat at dose of 2.5 mg/kg by intravenous route.
   2) Pharmacokinetic study of 5016 in male Wistar rat at dose of 10.0 mg/kg by intravenous route.

c) Gender effect study
   1) Pharmacokinetic study of 5016 in female Wistar rat at dose of 5.0 mg/kg by intravenous route.
Non compartmental analysis of the time concentration data of compound 5016, following oral and intravenous administration of dose 5.0 mg/kg, was performed using WinNonlin v5.0.1 and \( C_{\text{max}} \), \( C_0 \), \( T_{\text{max}} \), AUC and half life was calculated in order to determine the oral bioavailability. The pharmacokinetic data of oral and i.v study suggested that the oral bioavailability of 5016 is very poor. The calculated oral bioavailability of 5016 was found to be only 2.0%. Bioavailability data suggested that preferred route for other preclinical studies of 5016 including pharmacokinetic should be intravenous route. The poor oral bioavailability was expected as it showed only moderate solubility and permeability in the \textit{in vitro} experiments.

Derivative 5016 showed excellent dose linearity across the doses of 2.5 mg/kg, 5.0 mg/kg and 10.0 mg/kg following i.v administration. The \( C_0 \), Cmax and AUC of 5016 excellent dose linearity across the three doses. Half life values at the three doses were also found to be not significantly different.

No significant variation was observed in the pharmacokinetic parameters in female Wistar rat as compared to male Wistar rat data. The data showed that there is no gender related effect on the pharmacokinetics of 5016.

The findings from the \textit{in vivo} pharmacokinetic suggested that the 5016 have acceptable pharmacokinetic properties of a suitable drug candidate.

\textit{Preliminary in vivo efficacy evaluation}

The LEAD derivative 5016 showed acceptable \textit{in vitro} ADME and \textit{in vivo} pharmacokinetics. It also showed the excellent in vitro cytotoxicity in PA1 (ovary) cell line. These finding suggests that it should show the \textit{in vivo} efficacy. In order to validate this; a preliminary efficacy study was conducted in ovarian xenograft. Co-solvent formulation of 5016 was dosed continuously for 14 days in ovarian xenograft at an early stage (tumor volume \( \approx 100 \text{ mm}^3 \)), by intravenous route along with vehicle control group. All the animals were observed daily for 21 days for tumor reduction and body weight.
Data showed that 5016 inhibited the growth of tumor volume in comparison with vehicle control. Tested dose level of 5 mg/kg did not cause significant body weight loss and mortality during dosing period which indicates there was no test item related systemic toxicity. 5016 showed marginal efficacy at dose of 5.0 mg/kg in ovarian xenograft.

From the conducted studies, it can be concluded that 5016 have the optimum potency and acceptable pharmacokinetic properties to become a potential anticancer drug. At the moment, for detailed pre-clinical development, derivative 5016 could be subjected to formulation development, detailed efficacy and toxicity studies.

**Future scope of work**

Present investigation provided the selection of suitable derivative from the no. of synthesized naphthyridine derivatives. This investigation reduced the time of the discovery and development of naphthyridine derivative as anti cancer drugs considerably using suitable set of studies. Though the lead compound showed the acceptable pharmacokinetics and potent *in vitro* cytotoxicity which was later validated in the study itself in the form of marginal efficacy, a detailed set of studies needs to be further conducted in order to develop this as drug. The future scope of the work pertaining to this is summarized below

1) Detailed toxicological studies including the genotoxicity reproductive toxicology studies need to be performed in order to generate data on the potential short and long term toxicities.
2) More detailed *in vivo* efficacy study with various study design pertaining to doses and dose regime need to be performed.
3) Though the detailed pharmacokinetic is established in this work. The extrapolation of this pharmacokinetic behavior in non-rodents needs to be established.
4) Few other important ADME studies like tissue distribution studies, in vivo metabolism studies and excretion studies needs to be performed in order to understand the behavior of the molecule *in vivo*.

5) Since short listed derivatives, including 5016, have moderate solubility and permeability, better formulation strategies like liposomes, nanoparticles and pegylation could be used to make these molecules more soluble and improve the pharmacokinetic profile.