CHAPTER 6

DISCUSSION
DISCUSSION:

6.1 To decipher mechanisms of Mcl-1 overexpression in oral cancers-

Oral cancer is the eighth common cancer worldwide and one of the three most fatal cancers in males of the Indian subcontinent [2]. Despite recent advances in surgical treatment and radio/chemo therapy, the long term survival of oral cancer patients has not changed significantly [3]. Therefore, it is an urgent need to improve the early detection of oral carcinomas and in depth study to elucidate the mechanisms involved in the development and progression of oral cancer [6]. The treatment outcome depends primarily on early detection therefore; characterization of identifiable molecular markers will help in the early diagnosis and treatment of oral cancer.

Oral squamous cell carcinomas (OSCCs) have repeatedly been linked to apoptotic dysregulation [7]. Bcl-2 and related pro- and anti-apoptotic proteins are important mitochondrial apoptosis pathway regulators and play a critical role in regulating cell survival [68]. Mcl-1 is an anti-apoptotic member of the Bcl-2 gene family have been shown to be overexpressed and involved in progression of variety if solid and nonsolid malignancies [14]. Earlier studies from our lab already have demonstrated over-expression of Mcl-1 in human oral carcinomas and oral cell lines [19, 20].

In the present study we therefore wanted to evaluate the mechanism of Mcl-1 overexpression namely the occurrence of genomic alteration in Mcl-1 gene in oral tumors and correlate the alterations if any with the expression of Mcl-1 isoforms and clinico-pathological parameters of oral cancer patients. Our studies revealed the presence of 18bp polymorphic repeats in the promoter region of 22% oral tumors, which may contribute to the Mcl-1 overexpression in these tumors. Interestingly, a study by Moshynska et al, had demonstrated that the presence of 6 and/or 18 nucleotide insertions in the Mcl-1 promoter correlated with increased RNA and protein levels of Mcl-1 & clinical outcome in CLL patients [157]. The presence of these insertions was also shown to be associated with poor overall survival and
disease-specific survival, suggesting the potential use of the Mcl-1 promoter insertions as a prognostic factor.

Although there are a few reports on the polymorphic insertions in Mcl-1 promoter and their clinical significance, to the best of our knowledge there are no such studies in oral cancer. The present study is the first one evaluating the effect of Mcl-1 promoter alterations on the expression of Mcl-1 isoforms and its clinical significance in oral cancer patients. We analyzed 40 oral cancer patients, 11 oral cell lines and 25 healthy volunteers, utilizing PCR sequencing of Mcl-1 promoter to detect the presence of these two insertions. Neither normal nor oral cancer cell lines showed presence of 18 nucleotide repeats, however only two cancer cell lines (SCC15 & SCC40) showed presence of 6 bp repeats. Interestingly, the 18-nt polymorphic insertions were present in 9/40 tumors (22%) & also in 8/40 healthy volunteers (32%), indicating that these represent hereditary polymorphisms rather than somatic mutations as reported from studies in B-CLL & acute lymphoblastic leukemia (ALL) patients [158, 159]. Our studies did not show any correlation between the presence/absence of 6/18-nt repeats and Mcl-1 gene expression (neither mRNA nor protein) as reported by Saxena et al [160]. Similar to our findings, several other studies on CML & AML patients and healthy volunteers revealed no significant correlation between 6/18-nt repeats and Mcl-1 expression [161, 162].

Further, we assessed the clinical significance of 6/18-nt repeats i.e with overall survival & prognosis of OSCC patients. Unlike studies from Moshyanska et al and Saxena et al, we did not find any correlation between polymorphic insertions of Mcl-1 & clinical outcome of oral cancer patients. The overall survival of patients was independent of the presence or absence of Mcl-1 promoter polymorphisms, indicating that Mcl-1 promoter polymorphisms may not be useful to predict outcome/prognosis of OSCC patients. In support of our findings, several correspondence letters/reports as Tobin et al, are available [159, 163, 164] indicating that the Mcl-1 insertions represent hereditary polymorphisms rather than somatic mutations that probably do not predispose to CLL and are not associated with prognosis.
In contrast, studies by Reed et al & Moshynska et al, demonstrated association between these promoter insertions with high Mcl-1 mRNA and protein levels. Indicating that these insertions represent somatic alterations and not hereditary polymorphisms [165, 166]. Our studies revealed that the presence or absence of 18bp promoter polymorphism did not affect Mcl-1 expression and was also independent of status of tissue i.e. normal or tumor. Thus, polymorphic insertions in Mcl-1 promoter appear to be the hereditary in nature and not somatic in studies of Indian population. Therefore, the presence of promoter insertions appears be evidently insufficient to reliably drive high levels of Mcl-1 expression in oral cancers. Although, studies by Saxena et al demonstrated that polymorphic insertions were associated with increased promoter activity & Sp1/Sp3 binding sites [160]. Although, our studies found additional binding sites (SP1, CAP, ADR1, ADR2 & ADR4) due to the presence of 18-nt polymorphic repeats in promoter region, no significant effect was observed on Mcl-1 mRNA / protein of oral tumors. Recently, Mcl-1 promoter variants were shown to increase transcriptional activity of Mcl-1 and correlated with reduced risk of lung cancer in nonsmokers, thereby suggesting a dominant anti-proliferative function of Mcl-1 against its anti-apoptosis effect [167]. Thus from our studies indicates, presence or absence of promoter polymorphisms does not appear to influence Mcl-1 expression & therefore may not be the possible mechanism, responsible for Mcl-1 overexpression in oral tumors. However, evidence for the biological effect of Mcl-1 promoter polymorphisms on gene expression and the significance of Mcl-1 promoter polymorphisms in oral cancer needs to be analyzed in a larger cohort.
6.2 Role of Mcl-1 isoforms in pathogenesis of oral cancer:

6.2.1 Expression of Mcl-1 in Oral Submucous Fibrosis versus normal mucosa:

In the present study we studied the expression of Mcl-1 protein in OSF samples versus normal mucosa, to determine whether Mcl-1 expression has a possible role early in oral carcinogenesis. Our study revealed high expression of Mcl-1 protein in OSF as compared to normal, indicating upregulation of Mcl-1 in OSF tissues. The mean Mcl-1 expression was significantly higher in OSF than normal’s. The Mcl-1 staining pattern was observed to be homogenous with cytoplasmic distribution.

The molecular mechanism leading to the upregulation of Mcl-1 in OSF is not known. However, Mcl-1 is known to be upregulated through multiple transcriptional and post transcriptional mechanisms. Therefore, alterations in mechanisms of such highly regulated proteins may be responsible of their upregulation. Several genetic alteration have shown to be responsible for Mcl-1 up regulation as discussed earlier [160]. Mcl-1 was originally identified as a gene upregulated early in differentiation of human myeloid cells [92].

The Mcl-1 protein expression may be an important early event in initiation and progression of OSCC. Interestingly, Mcl-1 gene undergo alternative splicing and produces functionally distinct isoforms namely anti-apoptotic Mcl-1L & pro-apoptotic Mcl-1L & Mcl-1ES. Therefore a detailed study, in larger cohort of premalignant samples is needed in order to point out the role of Mcl-1 isoforms in progression of human oral cancers. However, Mcl-1 isoform analysis by qRT-PCR for fresh premalignant samples were not available, hence we compared the relative levels of Mcl-1 isoforms in oral tumors versus the normals.
6.2.2 To examine the levels of Mcl-1 isoforms in oral cell lines & tumors from different subsites and their correlation with clinico-pathological parameters-

In the present study, we assessed the expression Mcl-1L isoforms (Mcl-1L, Mcl-1S & Mcl-1ES) in oral tumors, to determine whether they would be useful as prognostic markers in oral cancer patients. Our studies demonstrate significant high expression of anti-apoptotic Mcl-1L at both mRNA & protein level in majority of oral tumors (64%) versus adjacent normal tissues & in cancer cell lines versus normal cell lines. The observed high expression of Mcl-1 in oral tumors is consistent with reported overexpression of Mcl-1 in hepatocellular carcinomas, cervix cancer, pancreatic cancer, non-small cell lung cancer, testicular germ cell tumors and melanomas [168-171]. The up-regulation of Mcl-1L may be associated with the pathogenesis of oral cancer [149].

Mcl-1 overexpression also appears to be a key factor in the resistance of some cancer types to conventional treatment. An altered expression pattern of Mcl-1 has been reported in association with progression of colorectal cancer [172]. Sieghart et al, have reported overexpression of Mcl-1 protein in human hepatocellular carcinoma tissues and its potential as a molecular drug target in HCC [168]. Moreover, Mcl-1 down-regulation is known to promote apoptosis in cancer cells, suggesting that Mcl-1 can potentially act as a therapeutic target in the treatment of several human malignancies [14].

Dysregulation of apoptosis regulating genes may play a key role in the development and progression of several human malignancies. Overexpression of anti-apoptotic Mcl-1L may represent an important mechanism in the development & progression of oral cancer. Hence, we evaluated the correlation between Mcl-1 isoform expression and clinico-pathological parameters in oral cancer patients. The anti-apoptotic Mcl-1L expression was found to be significantly associated with tumor size (p = 0.013) and lymph node positivity (p = 0.020)
A comparison of Kaplan–Meier survival curves of low and high expressers of Mcl-1L mRNA showed that high Mcl-1L expression was significantly associated with poor overall survival ($p = 0.002$). However, the only other study analyzing Mcl-1 isoforms & their clinical significance in clear cell renal carcinoma by Kempkensteffen et al, has shown results in contrast to ours, wherein downregulation of Mcl-1L was associated with aggressive phenotypes in clear cell renal carcinoma [173]. The high expression of Mcl-1 and its association with poor prognosis has also been reported in cervical, ovarian and gastric and breast cancers, however to the best of our knowledge, there are no report delineating the prognostic significance of Mcl-1 isoforms in human oral cancer. This is the first study demonstrating the correlation of high Mcl-1L levels with poor overall survival and its possible use as an independent prognostic marker for oral cancer patients.

Several possible mechanisms can lead to the high Mcl-1 mRNA levels. Mcl-1 is also regulated at the post-transcriptional level by micro RNAs through a mir29 binding in the 3′UTR of Mcl-1 mRNA [117]. Interestingly, the expression of mir29 was also found to be decreased in malignant cholangiocytes, favoring the increased levels of Mcl-1, which might be a possible reason for the observed high levels of Mcl-1 mRNA in oral tumors. Mcl-1 protein has a rapid turnover and possesses a short half-life of about one to 3 hrs. It possess the PEST domain responsible for ubiquitin dependent degradation by the 26S proteosomal machinery [174]. Another reason could be due to loss of S159/T163 phosphorylation sites essential for its detection & degradation, reported to be crucial in nicotine mediated Mcl-1 activation and chemoresistance [175]. Though earlier studies form our lab has shown high Mcl-1 protein in OSC versus normals, no statistically significant correlation was found between Mcl-1 protein expression & clinico-pathological parameters of oral cancer patients [19].
Also, it is known that the short isoform Mcl-1S only binds to Mcl-1L possibly neutralizing its anti-apoptotic function [176]. Interestingly, the anti-apoptotic Mcl-1L was the only predominantly overexpressed variant as compared to both pro-apoptotic Mcl-1S & Mcl-1ES variants indicating that Mcl-1L isoform alone may contribute in the progression of oral cancer. The Mcl-1L not only binds to Mcl-1S but is also known to heterodimerise with pro-apoptotic Bax & Bak etc. preventing the release of cytochrome-c and thereby evading the induction of apoptosis and providing short term survival [94]. Therefore, we looked at the relative ratios of Mcl-1L / Mcl-1S isoforms, which revealed a significant/positive correlation with the poor overall survival of patients, supporting Mcl-1L expression as an independent prognostic factor for oral cancer patients. Our findings are supported by the previous reports in gastric and cervical cancers demonstrating the association between high Mcl-1 expression with tumor size, histological grade, lymph node involvement, metastasis & poor clinical outcome [16, 17]. Also several other studies in breast cancer, ovarian cancer and various hematological malignancies have shown prognostic significance of high Mcl-1 protein expression [140, 177, 178]. However, the studies evaluating the association of the Mcl-1 isoform expression with clinico-pathological parameters are rare.

This is the first study indicating that Mcl-1L expression might be an independent prognostic marker for human oral cancer. Taken together, we have shown that Mcl-1L splice variant was overexpressed in oral cancer cell lines & tumor tissues compared with normal immortalized cell lines and noncancerous tissues. The high Mcl-1L mRNA was correlated with tumor size, nodal involvement & poor overall survival of oral cancer patients. Thus, high Mcl-1L expression was positively correlated with poor prognosis of oral cancer patients. These findings suggest that Mcl-1 may play an important role as a pro-survival factor and could be a potential therapeutic target in oral cancer. However, as cellular expression of Mcl-1 is tightly regulated via multiple mechanisms, further studies are necessary to elucidate the molecular mechanisms and role in the pathogenesis of oral cancer.
6.3 Role of Mcl-1L in radioresistance and/or chemoresistance:

6.3.1 Role of Mcl-1 in Radioresistance:

In the present study we demonstrate the effect of anti-apoptotic Mcl-1L expression on the radiosensitivity of oral cancer cells. So far limited information is available on the role of Mcl-1 in radiation response of tumor cells. To our knowledge this is the first study to report a time course expression of Mcl-1 isoforms post-IR and effect of Mcl-1L knockdown on radiosentitzation of oral cancer cell lines using siRNA strategy. Our studies demonstrated, an inverse correlation of Mcl-1 expression with cellular apoptosis and a synergistic effect of Mcl-1L knockdown along with IR on cell viability and clonogenic survival thereby enhancing the radiosensitivity of OSCC cells.

Various growth factors and cellular stresses like radiation and cytotoxic agents are known to upregulate Mcl-1 levels, thereby enhancing short term viability [174]. Our earlier studies had demonstrated, higher expression of Mcl-1L transcript and its association with poor disease free survival in patients treated with definitive radiotherapy [179, 180]. In the present study two tongue cancer (AW8507 & AW13516) and an immortalized oral (FBM) cell line were used due to their differing radiosensitivities and based on their $D_0$ values, both AW8507 & AW13516 were demonstrated to be relatively more radioresistant than FBM. Therefore, to evaluate the association of Mcl-1L with radioresistance if any, we evaluated the expression of Mcl-1 isoforms in radioresistant AW8507 & AW13516 as compared to radiosensitive FBM. Our studies revealed higher expression of Mcl-1L at both mRNA and protein level in relatively more radioresistant AW8507 & AW13516 cell line versus FBM, indicating a possible association of anti-apoptotic Mcl-1L splice variant with radioresistance.

Several possible mechanisms can lead to the high Mcl-1 levels in oral cancer cell lines post IR. Mcl-1 is known to be rapidly induced at the transcriptional level and its mRNA has a short half-life [174]. MCL-1 is also regulated at the post-transcriptional level by micro RNAs through a mir29 binding in the 3’UTR of Mcl-1 mRNA [117]. Interestingly, the expression of
mir29 was also found to be decreased in malignant cholangiocytes, favoring the increased levels of Mcl-1, which indicates the possible reason for the observed high levels of Mcl-1 mRNA in our cancer cells lines and also responsible for its immediate degradation within few hours post IR.

Mcl-1, a PEST domain containing protein is also known to undergo ubiquitin-dependent degradation by the 26S proteosome and possesses a short half-life of 1 to 3 hrs & is rapidly downregulated during apoptosis [174]. Notably, the BH3 domains of E3 ligases (MULE/LASU1) specifically interacts with the hydrophobic BH3 binding pocket of Mcl-1 and not with other anti-apoptotic Bcl-2 family members [181] and are responsible for the constitutive turnover of Mcl-1. Such ubiquitin mediated degradation of Mcl-1 has been shown to be essential for the initiation of apoptosis, following UV damage [100]. Hence it was interesting to study the time course expression profile of Mcl-1 isoforms and other bcl-2 family members in the above cell lines post-IR. Also, Mcl-1 protein is known to be phosphorylated by GSK-3β at Ser159, located within the PEST domain, resulting in a significant decrease in the protein half-life and leading to initiation of apoptosis [121]. Such, alterations in the phosphorylation of Mcl-1 protein by GSK3 may also contribute to the elevation of Mcl-1 levels.

Moreover, it is known that the short isoform Mcl-1S only binds to Mcl-1L possibly neutralizing its anti-apoptotic function [176]. No significant alterations in levels of Mcl-1S & Mcl-1ES were observed post IR, indicating that the predominantly overexpressed Mcl-1L isoform may contribute significantly in the development of radioresistance. Mcl-1L not only binds to Mcl-1S but is also known to heterodimerise with pro-apoptotic Bax, Bak etc. preventing the release of cytochrome-c and subsequent apoptosis [94]. We observed a downregulation of pro-apoptotic Bax & Bak proteins in AW8507 post-IR, coinciding with decreased apoptosis, while in contrast the radiosensitive FBM showed an increase in Bax & Bak protein levels. High expression of anti-apoptotic Bcl-xl was also observed in AW8507 & AW13516 cells. Bcl-xl which has already been shown to be associated with radiosensitivity of
colon cancer cells [182]. Thus, high expression of known radioresistant factors Bcl-xl & Bcl-2 and of Mcl-1L in more radioresistant AW8507 & AW13516 versus FBM may indicate their possible contribution to their radioresistant character.

We assessed the ratios of Mcl-1L/Mcl-1S, Mcl-1L/Bax, Bcl-xl/Bax, wherein radioresistant AW8507 & AW13516 showed higher ratios as compared to that in FBM indicating predominance of anti-apoptosis which may contribute to radioresistance. We are the first to elucidate the comparative levels of Mcl-1 isoforms and their association with radioresistance in oral cell lines. The prolonged high expression of Mcl-1L observed in AW8507 & AW13516 could possibly be due to the Mcl-1 protein stabilization via binding with other proteins. Another reason could be its enhanced half-life due to S159/T163 phosphorylation post-IR, reported to be crucial in nicotine mediated Mcl-1 activation and chemo resistance [175].

We observed a significant reduction in apoptosis post-IR which coincided with the high levels of Mcl-1L, indicating a possible association of Mcl-1L expression with radiation response of AW8507 & AW3516 cells. The immunofluorescence staining of Mcl-1 indicated a peri-nuclear accumulation & nuclear localization post IR in more radioresistant AW8507 cell line. Such peri-nuclear accumulation of Mcl-1 has also been observed earlier in polymorphonuclear leukocytes post-etoposide treatment [132]. In AW8507, the observed nuclear and perinuclear accumulation of Mcl-1 may possibly help in cell survival to lower doses of DNA damaging agents. A similar regulatory role for Mcl-1 (snMcl-1), perhaps acting as an adaptor protein in controlling the ATR-mediated regulation of DNA damage checkpoint kinase Chk1 phosphorylation and activation has been reported, placing Mcl-1 at the interface of apoptosis and cell cycle regulation [183]. Mcl-1 has been shown to regulate cell cycle by binding to proteins like CDK1 & PCNA [184] possibly explaining the observed nuclear localization of Mcl-1. High expression of anti-apoptotic Mcl-1L and Bcl-xl proteins and reduced pro-apoptotic proteins like Bak & Bax together may possibly contribute in lowering the sensitivity of AW8507 & AW3516 cells to IR.
The downregulation of Mcl-1L alone leads to the induction of apoptosis, in both AW8507 & AW13516 cells. Interestingly, the combination of Mcl-1L siRNA plus IR induced significantly higher apoptosis as compared to siRNA or IR-alone in both oral cell lines. Notably, the expression of closely related Bcl-xL, a known radioresistant factor was not altered. However, the expression of pro-apoptotic Bax protein correlated with the increased apoptosis on Mcl-1L knockdown. This overexpression of Bax, may activate the intrinsic apoptotic pathway resulting in increased cell death. To address the fact that the induction of apoptosis may not necessarily lead to long-term response to radiotherapy we performed the clonogenic assay which demonstrated that combination of IR and Mcl-1L downregulation synergistically reduced clonogenic survival as compared to each treatment alone. Our studies demonstrate that Mcl-1L downregulation potentially enhanced radiosensitivity of AW8507 & AW13516 cells in vitro.

Complex interactions occur between Bcl-2 family proteins especially Bak & Bax, where Mcl-1 plays a crucial role in engaging and maintaining pro-apoptotic Bak in an inactive state and accumulates H2AX and ATM proteins to activate DNA repair pathways suggesting that elimination of cellular Mcl-1 may be essential for initiating apoptotic pathways [99]. Overexpression and nuclear accumulation of Mcl-1 in AW8507 may occur due to a protein called IEX-1 which has been shown to interact specifically and timely with Mcl-1 controlling its accumulation and nuclear translocation in response to DNA damage and contribute in the activation of DNA repair pathway by Chk1 activation and G2 checkpoint arrest [185]. The high expression of Mcl-1L in radioresistant sublines developed by fractionated ionizing radiation provides a direct evidence for the role of Mcl-1L in radioresistance of OSCC cells. Thus, the combination of radiotherapy and Mcl-1L downregulation has the potential to improve the response rate of treatment-resistant oral cancer cells.
6.3.2 Role of Mcl-1 in Chemoresistance:

Cisplatin is widely used for chemotherapy of many malignancies, especially of oral squamous cell carcinoma (OSCC). However, the effectiveness of Cisplatin in the treatment of recurrent/metastatic tumors is limited because of acquired or intrinsic resistance [186]. The pro-survival Bcl-2 family members are one of the important factors contributing to the intrinsic resistance to chemotherapy [187]. Interestingly, high Mcl-1 expression has been linked with resistance to Cisplatin in ovarian cancers [188] and high Bcl-xl confers multi drug resistance in several squamous cell carcinoma cell lines [189]. Notably, both Bcl-xL and MCL-1 were known to constitute pertinent targets in ovarian cancers & mesothelioma cells and their concomitant inhibition was sufficient to induce apoptosis [190, 191]. However, to the best of our knowledge no reports are available about the role of both these molecules in oral cancers. Therefore, in present study we investigated the role of anti-apoptotic Bcl-xl and Mcl-1 proteins in chemoresistance of OSCC cells using siRNA mediated knockdown and BH3 mimetic small molecule inhibitor (Obatoclax).

Earlier studies from our lab have demonstrated high expression of both Mcl-1 & Bcl-xl proteins in oral cell lines & tumors. Further, Mcl-1 & Bcl-xl were also shown to be useful as prognostic marker and a predictor of complete tumor response in oral cancer patients respectively [20, 192]. Therefore, it was of interest to study the role of these proteins in chemoresistance of OSCC if any. Our studies indicate, a time dependent depletion of Mcl-1 after Cisplatin treatment in AW8507 cell line, which is consistent with earlier studies in renal tubular epithelial cells where Mcl-1 expression is rapidly declined at the posttranslational level in response to Cisplatin [193]. Further, knockdown of both Bcl-xl & Mcl-1 expression using siRNA approach could successfully downregulate Bcl-xl for more than 96 hrs but not Mcl-1. Unlike Bcl-xl, Mcl-1 has a rapid turnover; therefore transient knockdown with siRNA could deplete its levels only upto 48 hrs. In order to overcome this effect a stable system where, Mcl-1 expression was downregulated via an inducible pTRIPZ system transduced in AW8507 cells.
Doxycyclin induced expression of Mcl-1 shRNA has successfully provided a stable & long term knockdown of Mcl-1. Mcl-1 overexpression has been reported in a variety of human hematopoietic cancers, lymphoid cancers including multiple myelomas. Further, it is also suggested that Mcl-1 overexpression could be a resistance mechanism to conventional cancer therapies in solid tumors [14].

Our results demonstrate induction of cell death post knockdown of both Mcl-1 & Bcl-xl in AW8507 cells. Interestingly, combined depletion of both Mcl-1 & Bcl-xl proteins and Cisplatin treatment significantly reduced cell viability and proliferation compared to any treatment alone, indicating that Mcl-1 & Bcl-xl expression is crucial for survival of oral cancer cells. Bcl-xl and Mcl-1 appear to be important targets in oral squamous carcinomas and using small BH3-mimetics molecules we could induce apoptosis in such cells. In this regard, Obatoclax is a promising BH3-mimetics as it exerts both the conventional BH3-mimetic effect and has the proven ability to overcome resistance generated by Mcl-1 overexpression (unlike other BH3 mimetic like ABT737) [141]. Our results indicate that Obatoclax treatment can successfully sensitize oral cancer cells to Cisplatin treatment by decreasing proliferation and inducing cell death. Thus, BH3-mimetic Obatoclax has the potential use in the treatment of oral cancer. The development of multitargeted therapies directed against Bcl-xl and Mcl-1 constitutes a major challenge for the therapeutic care of chemo resistant oral cancers. However, selective small molecule inhibitors like Obatoclax, which specifically overcome Mcl-1 mediated resistance, is already in phase2 clinical trials [194] and may have important therapeutic implications, when used in combination with chemo/radiotherapy in treatment of oral cancer patients.