CHAPTER THREE

Targeting Cancerous Cells

Effect of Marine Extracts and TK Inhibitors
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3.1. Introduction

3.1.1. Development of inhibitors to the biological pathways - marine compounds

Long before the discovery of specific inhibitors, marine/plant extracts were tried to kill cancer cells. The recent approval of eribulin mesylate, a synthetic derivative of halichondrin B isolated from the marine sponge *H. okadai*, for metastatic breast cancer by the FDA and the European Medecines Agency (EMEA) is proof of how marine sponges can act as a treasure chest of bioactive agents. The first group of compounds tested for inhibitory activity against oncoproteins came from naturally occurring sources. Herbimycin A is a natural antibiotic originally isolated as an agent that reversed the transformation phenotype of RSV infected rat kidney cells. It inhibits a wide range of tyrosine kinases including YES, FPS, ABL, ERBB, ROS, and RET without affecting serine/threonine kinases such as protein kinase C (PKC). Genistein is an isoflavone compound isolated from soybeans that acts as an ATP competitive inhibitor toward the catalytic domain of different tyrosine kinases but not serine/threonine kinases. Erbstatin was isolated from *Streptomyces* and shown to be a low-molecular weight substance that resembled tyrosine and blocked the kinase activity of different tyrosine kinases. These findings demonstrated that compounds with selectivity towards tyrosine kinases and not serine/threonine kinases could be generated. These naturally occurring compounds were also shown to inhibit Gag-Abl/BCR-ABL cellular transformation, suggesting that inhibition of deregulated ABL tyrosine kinase activity may be a viable therapeutic approach toward ABL-induced leukemias\(^{95,96}\).

3.1.2. Specific inhibitors

However, a low success rate, side effects and variable therapeutic responses studied in large number of patients led to development of specific inhibitors,
one such example is Gleevac. It was the first therapeutic inhibitor used in clinical trials selectively inhibiting the activity of Bcr-Abl, Abl and Kit expressed in CML and GISTs. The drug targets the Bcr-Abl gene product, a fusion tyrosine kinase by inhibiting its activity in CML cells. Imatinib has been shown to inhibit the binding of ATP to the Bcr-Abl tyrosine kinase and thus reduction in phosphorylated downstream molecules\textsuperscript{97}. The activity of the TK is measured by phosphorylation of downstream molecules such as pCrkl and Grb2. Tyr207 in Crkl is the Bcr-Abl phosphorylation site. Crkl has been shown to be phosphorylated in Bcr-Abl positive neutrophils but not the normal neutrophils. Biological outcome of Crkl phosphorylation have been associated with cell adhesion, cell migration and immune response. The long term analysis of clinical trials revealed that Imatinib is a potent inhibitor of Bcr-Abl and results in remission in most of the CML patients\textsuperscript{25}.

\subsection{Assessment of the cytotoxic properties of chemical inhibitors}

One way to determine if any drug/extract has an anticancer potential is to look at its ability to induce apoptosis in those cells. In development and aging, programmed cell death or apoptosis play critical role in homeostatic balance in maintaining the number of cells. Apoptosis is a controlled cell death phenomenon in which a cell undergoes multiple morphological and molecular changes that ultimately lead to cell death. Apoptosis has been induced as a consequence of physiological and pathological stimulus. The morphological features of apoptosis include cell shrinkage, membrane blebbing, chromosome condensation and nuclear fragmentation. The molecular mechanisms initiates with the DNA damage by chemotherapeutic agents or radiations followed by activation of caspases. The caspases once activated pushes the cells for cell death in a p53 dependent pathway. It has been reported that cancer cells over-expressed Bcl2, an anti-apoptotic molecule or reduced expression of Bax, an apoptotic molecule. Another way of finding the efficacy of these compounds is to assess the proliferation potential of the cells that are being subjected to the treatment. The cell cycle status is reflected by the DNA content in different stages of cell division. To recognize the cell cycle phase specificity and to
quantify apoptosis, propidium iodide (PI) dye binds to DNA in cells at all stages of the cell cycle, and the intensity with which a cell nucleus emits fluorescent light is directly proportional to its DNA content.96

3.1.4. Effect of inhibitors on development and differentiation

These cytotoxic compounds have profound effect on cellular properties such as differentiation, migration and quiescence. Given the fact that haematopoiesis remains one of the best characterized systems so far, chemical inhibitors have filled the gaps in the hematopoietic differentiation pathways.98 The wide range of hematopoietic differentiation involves generation of all the lineage effector cells from the HSC. The two important compartments of these effector cells are myeloid and erythroid committed cells. This differentiation equilibrium is maintained by the precise production of progenitors. The numbers of erythroid and myeloid cells in blood which are maintained within the normal range, often found deregulated in malignancies. One such example is frequent increase of myeloid cells in AML and CML. Though a myeloid phenotype is frequently seen in CML, erythroid and lymphoid nature remain poorly understood. Often, deregulation in their numbers represents a physiological or external stimulus. While most of the early stage patients undergoing therapy achieve back the normal cellular distribution, a fraction of late stage patients display erythroid progression in CML under therapeutic selection.99 The molecular understanding of this lineage shift remains poorly explained.

Thus in order to understand mechanisms contributing to lineage switches in the context of TKI resistance we assessed the cytotoxic effects of marine extracts and Imatinib on widely established cancer cell lines.

3.2. Objectives

This chapter focuses on the cytotoxic effects of natural compounds and specific inhibitors on cancerous cells. The chapter is designed to
• Assess the effect of marine extracts on apoptosis in HL60 and Hela cells by Hoechst staining,
• Inhibit the activity of Bcr-Abl assessed by pCrkl level by using Imatinib on K562 cells and
• Assess the proliferation potential of Bcr-Abl inhibited K562 cells by cell cycle and colony formation assay.

3.3. Results

3.3.1. Marine extracts induce apoptosis in human cancer cell lines

In our effort of screening for bioactive compounds from marine sponges collected from Mauritius, we investigated the cytotoxic properties of the ethyl acetate extract from the sponge Jaspis sp (JDE). Initial screening of this extract has confirmed its potent anti-proliferative effect on several types of human cancer cell lines. The constituents of the ethyl acetate extract of the marine sponge Jaspis sp. induced apoptosis of HL-60 cells (Figure 3.1). Cells treated with JDE and cultured up to 12 h produced no obvious morphological changes, but at 24 h, there was an increase in the percentage of cells with progressive nuclear shrinkage. At a concentration of 50 g/mL, cells exhibited characteristic morphological changes of apoptosis including membrane blebbing, chromatin condensation and formation of apoptotic bodies (Figure 3.1B). The control cells were normal with round and homogeneous nuclei (Figure 3.1A). Similarly, HeLa cells were treated with 25g/mL of fraction VLC and morphological assessment demonstrated that at 25g/mL VLC induced apoptosis (Figure 3.2C), while control cells demonstrated no cell death (Figure 3.2D).

3.3.2. Tyrosine kinase inhibitor affects proliferation of CML cell line

We learned from the earlier experiments that cancerous cells can be killed by using marine extracts. However, these compounds are also known to have non-specific effects on other cell types thus should be used with care. In our next attempt to target the cancerous cells in a specific manner, K562 cells, a widely used CML cell line expressing Bcr-Abl oncogene were targeted. Our choice of
Figure 3.1 Fluorescent micrographs of HL-60 cells treated with ethyl acetate extract of *Jaspis* sp. (JDE). HL-60 cells were treated with 1% DMSO (A) or 50 g/mL JDE for 24 h (B). Cells were harvested, stained with Hoechst 33258 and then visualized under an inverted fluorescence microscope (200×). The arrow points to cells showing apoptosis.

Figure 3.2 Effect of VLC (13-17) on cell morphology. Morphological and chromosomal changes were examined in HeLa cells incubated with VLC. C- control, D- 25 μg/mL. Control cells show round and homogeneous nuclei (C); apoptotic cells display condensation and fragmentation of nuclei in the VLC treated cells (D) indicated by the arrows.
Figure 3.3 Changes in the morphology of human cervical adenocarcinoma cell line (HeLa) cells treated with ethyl acetate extract (JDE) and hexane extract (JDH) of Jaspis sp. under a fluorescence microscope. Morphological changes in untreated cells (A), JDE-treated (B-5 μg/ml and C-25 μg/ml) and JDH-treated (D 25 μg/ml) cells were examined by staining with Hoechst 33258. Control cells showed round and homogeneous nuclei while apoptotic cells displayed condensation and fragmentation of nuclei in the treated cells (the arrow point).
K562 cells was based on the following criteria i) A Philadelphia chromosome rearrangement analogous to the majority of CML cases ii) lack of mutations in the rearranged locus after TK inhibitor treatment to enable addressing clinical resistance phenotypes which are TK mutation independent and iii) potential to induce distinct lineages i.e. erythroid versus myeloid in the presence of differentiation cues. A TK inhibitor which targets Bcr-Abl tyrosine kinase, Imatinib mesylate was used for the purpose. The efficiency of TK inhibition was monitored by expression of pCrkl level before and after Imatinib treatment at IC50 concentration that is 0.75 µM. pCrkl expression is decreased to 32% in Imatinib treated K562 cells compared to 52% in control K562 cells (Figure 3.4A and B). In order to verify that TKI did not induce clinically relevant mutations in Bcr-Abl kinase domain, Sanger sequencing was performed on cDNA transcribed of Bcr-Abl RNA from Imatinib treated and untreated K562 cells. The T315I domain of Bcr-Abl and sequencing histogram are shown in the figure. T315I mutation is one of the most frequent mutations seen in relapsed CML patients and associated with in vitro resistance of CML cells. The histogram revealed that T315I mutation is absent in TKI treated K562 cells (Figure 3.4C and D). To understand if the TKI inhibitor affects the cell cycle property, we assessed the cell cycle status of the K562 cells in the context of TKI resistance. The cell cycle analysis of K562 cells upon Imatinib treatment displayed 7.7% cells in G2M phase compared to 18.7% cells in control (Figure 3.5A-C).

3.3.3. Tyrosine kinase inhibitor Resistance is associated with increased erythroid percentage in K562 cells.

Long term analysis of CML in the clinical context has revealed an accumulation of erythroid cells. The mechanisms that lead to this process are currently poorly understood and we investigated the potential role of miRNA182. We assessed the lineage distribution of K562 cells in the context of TKI resistance by treating K562 cells with 0.75 µM concentration of Imatinib. The mean number of colonies of BFU-E were 61, 53 and 29, CFU-G were 10, 8 and 1, CFU-E were 16, 11 and 1 and total colony counts were 93, 72, and 33 in RPMI, DMSO and Imatinib treated K562 cells respectively (figure 3.6E). TK inhibitor
Figure 3.4 Effect of Imatinib on Bcr-Abl tyrosine kinase. (A and B) Expression of pCrkl in K562 cells after Imatinib treatment (B) compared to control (A). (C) A cartoon diagram shows the Bcr-Abl gene with kinase domain. The T315I sequence is shown as asterisk. (D) Sanger sequencing analysis shows absence of T315I mutation in Bcr-Abl gene of K562 cells after Imatinib treatment. The asterisk shows T315I mutation.
Figure 3.5 Effect of Imatinib on cell cycle profile of K562 cells. K562 cells were transiently treated with or without 0.75 μM of Imatinib (tyrosine kinase inhibitor) for 48 hrs. The imatinib resistant cells were generated by treating K562 cells with Imatinib. The cells were first exposed to 0.05 Mm concentration of Imatinib and gradually the concentration of the inhibitor was increased (upto 10 μM). The cells were then fixed and added with Hoechst reagent and analyzed in BD FACS Calibur. The cell cycle phase analysis was performed based on DNA content index parameter to determine G0/G1, S and G2/M phases. The percentage of cell distribution in each cell cycle phase was summarized and shown for K562 cells (A), 0.75 μM Imatinib (B) and TKI resistant K562 cells (C).
Figure 3.6 TKI resistance is associated with a shift in ME% of K562 cells. TK inhibitor treatment of K562 cells increases BFU-E in methyl-cellulose media (Figure 3.6B-D) compared with control (Figure 3.6A-C). Figures 2A and B and 2C and D are taken at 4X and 10X respectively. Images are taken at 4X and 10X magnifications. The total numbers of colonies per 5 fields are shown. The scale bar represents 100 μm. Colonies are counted at day 21 from the day of treatment (Figure 3.6E). Data is shown as number of colonies per 5 fields. Error bars show standard error of 4 independent experiments with p-values of the mean. *p < 0.05, **p < 0.01, and ***p < 0.001.
treatment decreased the total number of colonies more than 50%. However, there was a marginal increase in the percentage of erythroid colonies from 88% to 92.3% in K562 cells cultured in the presence of TK inhibitor compared with the control cells (figure 3.6F). There was a reduction seen in myeloid colonies compared to control. TK inhibitor treatment of K562 cells increases Burst Forming Units-Erythroid (BFU-E) in methyl-cellulose media compared with control. Figure 3.6A-D represents the images of methylcellulose colonies taken at 10X magnification.

3.4. Discussion and summary

Cancer cells have properties of evading apoptosis and increased proliferation\textsuperscript{101}. These two properties have been targeted by specific and non-specific compounds. The outcome of these compounds has been assessed in model organisms such as drosophila and mammalian cell lines. Human cell lines are an invaluable resource to study the mechanisms of disease progression. The cell line data has largely helped to understand the signalling mechanisms specific to cancer cells. The non-restricted expandable capacity allows one to screen and inhibit multiple molecules instantaneously. The understanding of cell line data has been further extended to mammalian systems such as mice and apes. Clearly, signalling mechanisms are conserved in cell lines and higher organisms. Thus, they are exploited to understand biological processes and find new therapeutics in disease settings.

In this chapter, apoptosis and proliferation potential of cancerous cells has been assessed using the inhibitors. The two different types of inhibitors used are non-specific compounds derived from marine extracts and Bcr-Abl inhibitor, Imatinib mesylate. Apoptosis or programmed cell death follows condensation of chromatin and nuclear fragmentation. These morphological features result in formation of apoptotic bodies. Apoptosis can be measured by microscopic analysis of fragmented nuclei and Annexin V staining which binds to phosphatidyl serine flipped from inner cell membrane to the outer surface in apoptotic cells. The effect of marine extracts shows apoptosis in HL60 and HeLa
cells while control cells do not display any sign of apoptosis. Our screen data with marine extracts implicates their use in treating cancers by detailed analysis of the signalling mechanisms they are targeting.

Next, we specifically targeted CML cells using TKI, Imatinib mesylate and tried to understand the TKI resistance mechanisms independent of Philadelphia chromosome mutations. Previously, longer term analysis of CML patients revealed a successful inhibition of Bcr-Abl that results in remission in most of the cases\textsuperscript{102}. However, a small set of patients relapse to the current available drugs. The mechanisms underlying are poorly characterized and requires better model systems. Interestingly, the absence of mutations in the kinase domain in the surviving K562 cells suggests that intrinsic cellular mechanisms might be responsible for resistance towards TKI. We detected a decrease in the levels of pCrkl, a measure to the Bcr-Abl activity. These data are consistent with earlier findings that mutations arise in CML cells independent to the selection pressure. The possibility of activation of other kinases such as Src and Lyn has not been denied here. The cell cycle analysis and colony formation assay revealed that Bcr-Abl inhibition leads to decrease in proliferation potential of CML cells. One of the striking observations from our initial experiments of lineage determination shows that TK inhibition marginally shifts the myeloid erythroid ratio. There was a marginal increase in erythroid cell number. Myelopoiesis and erythropoiesis has been mostly understood in terms of transcription factors implicated in lineage choices such as GATA1, Pu1 and Cebp\textsuperscript{a}\textsuperscript{14}. In various hematological malignancies, differentiation deregulation has been seen. Of particular interest was detection of erythroid differentiation in the late stage CML patients under therapeutic selection. The underlying mechanisms are poorly defined. This laid the very foundation of our next experiments. The molecular mechanism that we have identified is discussed in the next chapters.