Development of resistance to Imatinib, a tyrosine kinase inhibitor, in chronic myeloid leukemia cell line-K562: Role of COX-2 and MDR1

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Development of resistance to Imatinib, a tyrosine kinase inhibitor, in chronic myeloid leukemia cell line-K562: Role of COX-2 and MDR1"** has been carried out by me under the supervision of Prof. P. Reddanna and this has not been submitted for any degree or diploma of any other university earlier.

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CERTIFICATE

This is to certify that **Ms**. **MK Aruna Sree** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend her thesis ***"Development of resistance to Imatinib, a tyrosine kinase inhibitor, in chronic myeloid leukemia cell line-K562: Role of COX-2 and MDR1"*** for submission for the degree of Doctor of Philosophy of this University.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

µM	**:**	**micro molar**
^{0}C	**:**	**degree centigrade/ degree celsius**
AA	**:**	**arachidonic acid**
ATP	**:**	**adenosine triphosphate**
BCIP	**:**	**5-bromo-4-chloro-3-indolyl phosphate**
bp	**:**	**base pair**
COX	**:**	**cyclooxygenase**
cpm	**:**	**counts per minute**
C-terminal	**:**	**carboxy terminal**
DAPI	**:**	**diamino phenyl indole**
DNA	**:**	**deoxy ribonucleic acid**
EDTA	**:**	**ethylene diamine tetra acetic acid**
FACS	**:**	**fluorescence activated cell sorter**
FBS	**:**	**fetal bovine serum**
g	**:**	**gram**
h	**:**	**hour(s)**
kb	**:**	**kilobase pair**
kDa	**:**	**kilodalton**
l	**:**	**litre**
mg	**:**	**milligram**
min	**:**	**minutes**
ml	**:**	**milliliter**
mM	**:**	**millimolar**

MTT	**:**	**3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide**
NBT	**:**	**nitroblue tetrazolium**
nm	**:**	**nanometers**
NSAIDs	**:**	**non-steroidal anti-inflammatory drugs**
N-terminal	**:**	**amino terminal**
OD	**:**	**optical density**
PAGE	**:**	**polyacrylamide gel electrophoresis**
PARP	**:**	**poly(ADP-ribose) polymerase**
PBS	**:**	**phosphate buffered saline**
PCR	**:**	**polymerase chain reaction**
PKC	**:**	**protein kinase C**
pmole	**:**	**picomole**
rpm	**:**	**revolutions per minute**
SDS	**:**	**sodium dodecyl sulfate**
TEMED	**:**	**N,N,N',N'-tetramethylene diamine**
Tris	**:**	**tris-(Hydroxymethyl) aminoethane**
UV	**:**	**ultraviolet**

1.1 Leukemia

Leukemias are cancers of the immature blood cells that grow in the bone marrow and tend to accumulate in large numbers in the bloodstream. Recurring chromosomal abnormalities have been identified as one of the causes for the development of leukemias. At present, more than 500 recurring cytogenetic abnormalities have been reported in hematological malignancies. In lymphoid leukemias, chromosomal translocations frequently lead to the transcriptional activation of proto-oncogenes by bringing their coding regions in the vicinity of immunoglobulin or T-cell receptor gene-regulating elements, thus leading to their inappropriate expression (Kuppers and Dalla-favena, 2001). In addition to proto-oncogene transcriptional activation, chromosomal translocations might cause gene fusions leading to leukemias.

1.2 Types of leukemia

Leukemia, the term coined by Virchow is a cancer of white blood cells. Leukemia occurs when a white blood cell, whose development is frozen, continues to duplicate itself. The resulting progeny of cells are all in the same stage of development and bear the distinctive hallmarks of the type of ancestral white blood cell that gave rise to them. Based on this understanding, by 1900 leukemia was no

longer seen as a single disease. Instead it was imagined akin to a tree with two main limbs that inturn have two primary branches, all of which reflect from what type of cell the leukemia originates (Fig.1). One limb, myelogenous leukemia, has its hallmark in the blood and bone marrow either a predominance of immature myeloblasts (acute myeloid leukemia-AML) or mature myeloid cells (chronic myeloid leukemia-CML). With the other limb, lymphocytic leukemia, the blood and bone marrow is over populated by either precursor B or T cells (acute lymphocytic leukemia-ALL) or mature B or T cells (chronic lymphocytic leukemia-CLL).

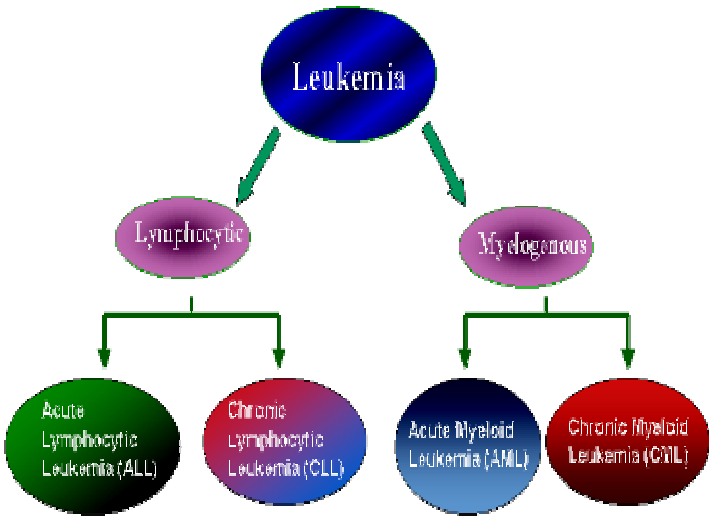


Fig. 1: Types of Leukemia.

Despite their divergent origins, all types of leukemias share the same set of symptoms, just as fever and congestion can be the symptoms for a number of different microbial infections. When people develop leukemia, their abnormal white blood cells crowd out or hamper the functioning of their red blood cells, fostering the tiredness and paleness that are the hallmarks of anemia. These white blood cells also do not effectively fight infections, which can be fatal. A lack of functioning cells that clot blood platelets makes people with leukemia prone to life-threatening bleeding episodes. Leukemia cells that congregate at various spots in the body can also spur a variety of other symptoms, including bone or joint pain, or enlarged organs. Untreated acute leukemia progresses rapidly to death, whereas untreated chronic leukemia can be long lasting, although just as deadly in the long run. Although leukemia afflicts both adults and children, by the 1920's it was recognized that the disease mostly attacks children. In 1930 it was considered a relatively rare disease. But by 1960, statistics collected in Great Britain revealed that leukemia had become the second leading cause of death in children.

1.3 Chronic myelogenous leukemia (CML)

Chronic myelogenous leukemia (CML) was described in 1845 by Hughes Bennet, a physician from Edinburgh who thought that the disease was an infection (Bennett, 1845). Rudolf Virchow, who published a similar case, only a few weeks

later, postulated that the disease was noninfectious and later coined the term leukemia (Virchow, 1845). The leukemic cells originated from the bone marrow were recognized by Neumann in 1870 (Neumann, 1870). In 1960, Nowell and Hungerford (1960), two Philadelphia researchers noted that an abnormally small chromosome was consistently present in the cells of CML patients, and this chromosome was subsequently called the Philadelphia (Ph) chromosome (Fig. 2). This was the first time that a chromosomal abnormality had been associated with a malignant disease. In 1973, Janet Rowley recognized that the Ph chromosome was indeed the product of a reciprocal translocation between the long arms of chromosomes 9 and 22, the t(9;22)(q34;q11).

1.3.1. Clinical Features and Phases of CML

The clinical hallmarks of CML are leukocytosis, a left shift in the differential count, and splenomegaly. Importantly, the disease is not restricted to the myeloid compartment, since the Philadelphia chromosome is regularly demonstrable in megakaryocytes and erythroid precursor cells. Thus, high platelet counts are frequent, but for unknown reasons, erythrocytosis is rarely seen. CML runs a three-phased course. During the initial *chronic phase*, there is gross expansion of the myeloid cell compartment, but the cells still retain the capacity to differentiate and function normally. Symptoms in the chronic phase are generally mild and many

patients are asymptomatic, being diagnosed by routine blood sampling (Cervantes et al., 1999). After an average of 4 to 5 years, the disease typically progresses to *accelerated* phase, characterized by the appearance of more immature cells in the blood, frequent constitutional symptoms, and a less favorable response to therapy. The diagnostic criteria for accelerated phase are not universal, reflecting that disease progression from chronic to accelerated phase is a continuous process rather than a single step. Although the duration of accelerated phase varies from weeks to years, the disease inexorably progresses to the final stage of blast crisis, where immature cells dominate and survival is measured in weeks to months.

1.3.2 The Philadelphia Chromosome and the molecular basis of chromosome translocation

The Philadelphia chromosome is a shortened chromosome 22 that results from the reciprocal exchange of DNA between the long arms of chromosomes 9 and 22; breaks occur at positions q34 and q11 (t(q;22) (q34;q11)). The leukemic cells of more than 90% of CML patients contain the Ph chromosome, and an additional 5% have a cytogenetically silent translocation. The remaining 5% have truly Ph-negative CML, which constitutes a separate disease entity. In current usage, CML refers to Ph-positive CML only.

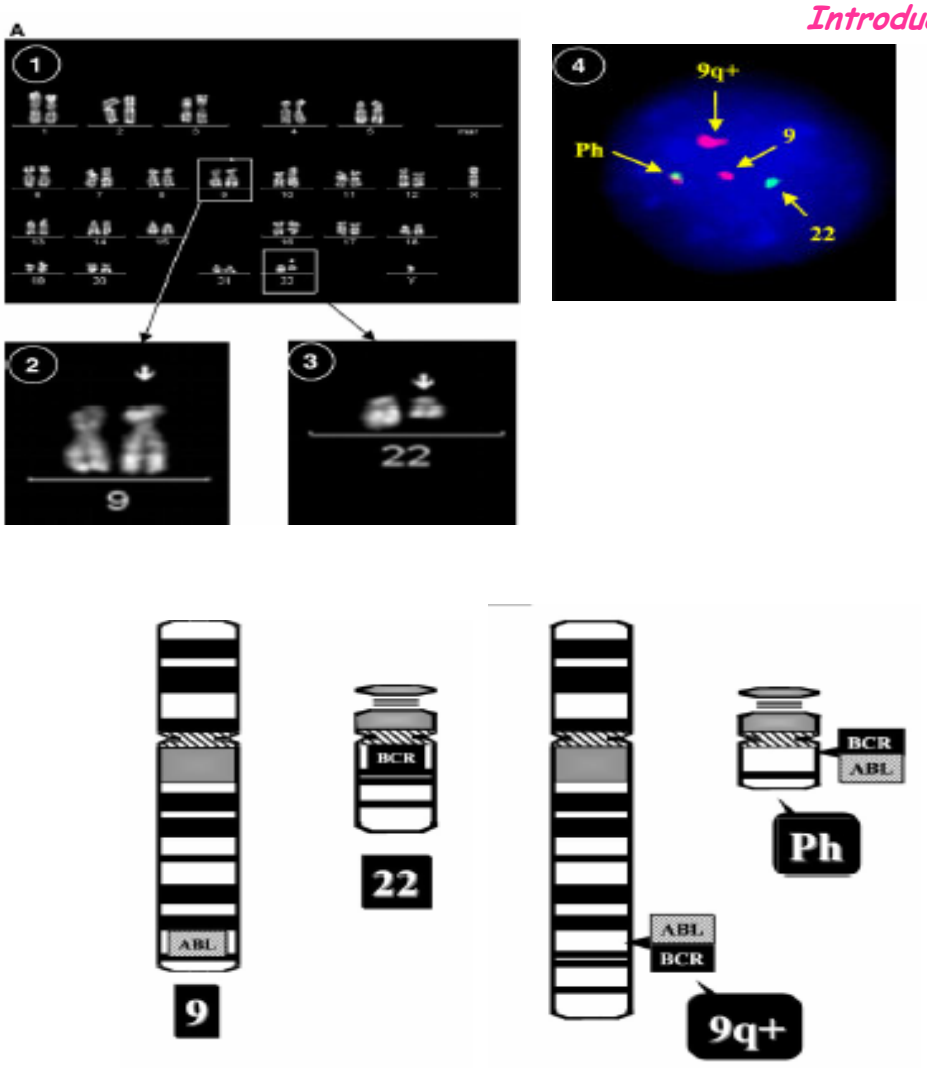


Fig. 2: Molecular genetics of CML.

A. 1. Karyotype of a Philadelphia-positive CML patient. 2. Derivative chromosome 9 (arrow). 3. Philadelphia chromosome (arrow). 4. FISH analysis of BCR (green signal), ABL (red signal) and BCR-ABL fusion gene (yellow signal) in the interphase nucleus. B. Schematic representation of the Philadelphia chromosome (Pharmacol Rev 2003; 55:401–423).

The molecular genetics of the Philadelphia chromosome translocation is now known (Kurzrock et al., 1987; Kurzrock et al., 1988; Kurzrock et al., 1987; Sokal et al., 1988; Sawyers 1999; Groffen et al., 1984) (Fig. 2). The t(9;22) anomaly leads to an exchange of DNA between chromosomes 9 and 22. The 3' part of the *ABL* gene is moved from chromosome 9 to chromosome 22 and is juxtaposed to the proximal segment of the disrupted *BCR* gene on chromosome 22. The result is a chimeric *BCR-ABL* gene. The breaks in the *BCR* gene on chromosome 22 vary. In CML, they most often occur centrally, that is, between exons 12 and 16 (also known as exons b1 to b5), in a region designated as the major breakpoint cluster region (M-BCR). However, in a small subset of patients, a more distal region (between exons 19 and 20; the micro-BCR) is disrupted. In contrast, in ALL, about 50% of patients with Philadelphia chromosome–positive disease have breaks within the central M-BCR; the remainder has more proximal breaks, just distal to the first exon of *BCR* (in the minor-BCR [m-BCR]). As a result of these variable breakpoints as well as promiscuous alternative splicing between *BCR* and *ABL* exons, different amounts of DNA from *BCR* are joined to *ABL* exons 2 to 11 (Laurent et al., 2001) (Fig. 3). Therefore, breaks in m-BCR join only the first exon of *BCR* to the entire

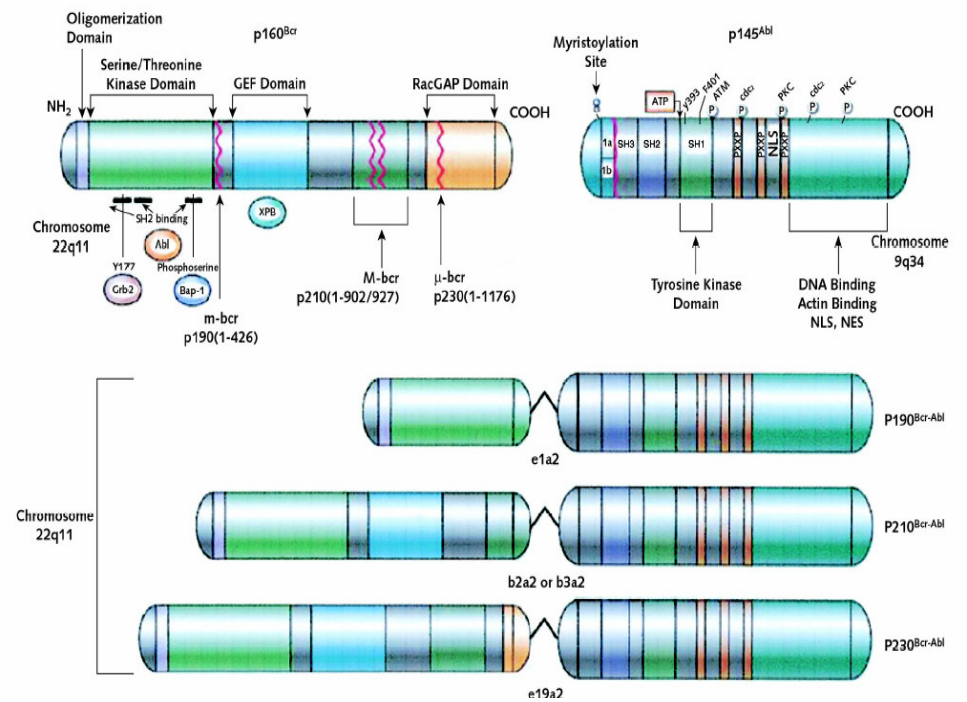


Fig. 3: The normal BCR, ABL proteins and various BCR-ABL counterparts.

Functional sites in the BCR protein include a serine and threonine kinase domain in exon 1, a central guanine exchange factor (GEF) domain, and a carboxy-terminal guanosine triphosphatase–activating protein (GAP) domain. Src homology-2 (SH2)–binding sites are also present in exon 1. The BCR-associated protein (Bap-1) interacts with the more distal of these sites. Growth factor receptor–bound protein 2 (Grb-2) associates with the proximal SH2-binding site containing a phosphotyrosine in position 177. ABL interacts with the second and third SH2 binding sites. The GEF domain interacts with the xeroderma pigmentosum B (XPB) DNA repair protein. The normal ABL protein contains three SH domains near the N-terminal. The tyrosine at position 393 (Y393) is the major site of autophosphorylation within the kinase domain. Phenylalanine 401 (F401) is highly conserved in protein tyrosine kinases containing SH3 domains. The central area of the protein has proline-rich regions (PXXP) capable of binding to SH3 domains and a nuclear localization signal (NLS). The carboxy-terminus contains DNA as well as G- and F-actin–binding domains, a nuclear export signal (NES), and nuclear localization signals. The phosphorylation sites by Atm, cdc2, and protein kinase C (PKC) are depicted. At the bottom of the figure, various BCR-ABL proteins and their junction breakpoints are shown. Ragged red lines indicate breakpoints in BCR and ABL (Ann Intern Med 2003; 138: 819-830).

ABL gene from exon 2 to the end of the gene (e1–a2 junction), breaks in M-BCR join all of *BCR* up to exons 13 or 14 (also known as exon b2 or b3 of M-BCR) to *ABL* (again, the entire gene from exon 2 to the end) (b2–a2 or b3–a2 junction), and breaks in micro-BCR join all of *BCR* up to exon 19 to *ABL* (exons 2 to 11) (e19–a2 junction). As a result, BCR-ABL proteins are sized at 190, 210, and 230 kDa, respectively (Fig. 3). Hence, the smallest BCR-ABL protein (p190BCR-ABL) contains less of BCR than does the larger BCR-ABL protein p210BCR-ABL; p230BCR-ABL contains a still larger segment of BCR. All harbor the same amount of ABL. Subtle differences in the biological effects of the various BCR-ABL proteins may be crucial to disease phenotype.

1.3.3 The ABL and BCR genes

1.3.3.1 THE ABL GENE

Forms of ABL Viral and Cellular ABL

The cellular *ABL* gene is the human homologue of the viral *ABL* (v-*ABL*) oncogene carried by the Abelson murine leukemia virus (Abelson and Rabstein, 1970; Rosenberg and Witte, 1988). Viral *ABL* originates from cellular *ABL* (c-*ABL*). Presumably, at some point in evolution, the Abelson murine leukemia virus incorporated the mammalian *ABL* gene (Rosenberg and Witte, 1988).

ABL Protein

Human ABL is a ubiquitously expressed 145-kDa protein with two isoforms (Rosenberg and Witte, 1988). In hematopoietic cells, steady state levels of ABL decrease with myeloid maturation (Wetzler et al., 1993). ABL functions as a nonreceptor tyrosine kinase enzyme (Kharbanda et al., 1998; Cheng et al., 2002; Sawyers et al., 1994) (Fig. 3).

Subcellular Location of ABL

While BCR-ABL is found exclusively in cytoplasm, surprisingly (for a tyrosine kinase enzyme), ABL can shuttle between the nucleus, where it can bind DNA, and the cytoplasm, where it binds the actin cytoskeleton (Van Etten, 1999). In primary human hematopoietic cells (Wetzler et al., 1993) and neurons (Koleske et al., 1999), ABL is more cytoplasmic than nuclear.

Biology of ABL

Cytoplasmic functions of ABL include signaling and cytoskeletal molding; nuclear ABL has been implicated in regulation of the cell cycle (Van Etten, 1999) and in genotoxicity (Wang, 1998). ABL also has DNA binding capacity of uncertain significance.

ABL Tyrosine Kinase Enzymatic Activity

Tyrosine kinases are enzymes that phosphorylate (add a phosphate group) to a tyrosine in a substrate. They have a catalytic domain, which promotes the transfer of the terminal phosphoryl group from adenosine triphosphate (ATP) to a tyrosine amino group acceptor in a substrate (or they may autophosphorylate). Normal ABL phosphorylation is tightly controlled (Van Etten, 1999), probably by motifs in the *N*-terminal. Loss of this region (as occurs in the formation of *BCR-ABL*) results in high constitutive kinase enzymatic activity, a key factor in the oncogenic potential of transforming ABL proteins (Pluk et al., 2002; Van Etten et al., 1995; Dai and Pendergast, 1995).

Other Properties: Impact on Cytoskeleton, Cell Cycle, and DNA Repair

ABL influences the cytoskeleton locally, and, in turn, ABL kinase activity is modified by outside-in cellular signals (Kain and Klemke, 2001; Van Etten et al., 1994; Cheng et al., 2002; Lewis et al., 1996). Most cytoplasmic ABL is associated with filamentous actin, a building block of the cellular cytoskeleton (Van Etten et al., 1994). ABL also interacts with cell-cycle regulatory genes at several checkpoints, thereby affecting cellular proliferation (Sawyers et al., 1994; Van Etten, 1999). Both positive and negative regulatory effects have been reported, depending on the cell-cycle phase studied. ABL has DNA-binding activity, which may be involved in

initiating transcription of DNA to RNA, in DNA damage response, and in meiotic processes (Miao and Wang, 1996, Yuan et al., 1997). A role for ABL in DNA repair has been suggested by its interaction with other molecules involved in this process, such as the ATM gene product. Mutation of the ATM gene product causes ataxia telangiectasia, a disorder characterized by hypersensitivity to radiation damage (Kharbanda et al., 1997; Yuan et al.,1998).

1.3.3.2 THE BCR GENE

BCR is situated on the long arm of chromosome 22 (22q11). It is translated into two major proteins that have molecular weights of 160 and 130 kDa (Stam et al., 1987) (Fig. 3). Similar to the situation with ABL, BCR protein levels decrease with myeloid maturation in hematopoietic cells (Wetzler et al., 1993).

Subcellular Location of BCR

Like the ABL protein, the normal BCR protein resides in both the cytoplasmic and nuclear compartments (Wetzler et al., 1993; Laurent et al., 2000; Wetzler et al., 1995). In the nucleus, BCR associates with condensed DNA in both interphase and metaphase (Wetzler et al., 1995).

Biology of BCR

The *BCR* gene is a complicated molecule with many different functional motifs. It is implicated in the two major signaling pathways in eukaryotes

(phosphorylation and guanosine triphosphate [GTP] binding) (Muller et al., 1991; Voncken et al., 1995; Sadowski et al., 1986; Pendergast et al., 1991). The first exon of the *BCR* gene is pivotal to oncogenesis. It is the one exon of *BCR* included in all known BCR-ABL fusion proteins (Muller et al., 1991; Arlinghaus, 1998). BCR has serine and threonine kinase enzymatic activity in its first exon. It can phosphorylate itself as well as key substrates and, hence, propagates cellular signals. Several Src homology-2 (SH2)–binding domains are also in the first exon of *BCR.* SH2 domains are highly conserved, noncatalytic regions of 100 amino acids that bind SH2-binding sites consisting of 3 to 5 amino acids, including a phosphotyrosine. This interaction is important in the assembly of signal transduction complexes (Sadowski et al., 1986).

BCR also interacts with or has homology to G proteins at multiple levels (Diekmann et al., 1991; Ron et al., 1991). These proteins are essential players in intracellular signaling, cytoskeletal organization, cell growth, and normal development. G proteins cycle between an inactive guanosine diphosphate (GDP)–bound state and an active GTP-bound state. Homeostasis within this process is regulated by guanosine triphosphatase (GTPase)–activating proteins (which turn off G proteins) and guanine nucleotide exchange factors (which turn on G proteins). BCR has both GTPase-activating protein and guanine nucleotide exchange factor

functions, suggesting a dichotomous role for this molecule in G protein–associated signaling pathways. BCR (and p210BCR-ABL) interact with the xeroderma pigmentosum gene product (Maru et al., 1999; Takeda et al., 1999). Xeroderma pigmentosum is an inherited disorder whose hallmark is increased sensitivity to sunlight coupled with a defect in the DNA damage response process. Therefore, BCR may also participate in DNA repair.

Association of BCR with Normal ABL and with BCR-ABL

BCR binds to SH2 domains of normal ABL and can form complexes with BCR-ABL (Campbell et al., 1990). The result of interaction between BCR and BCR-ABL may be functional feedback regulation (Arlinghaus, 1998).

1.3.3.3 THE BIOLOGY OF BCR-ABL

p210 BCR-ABL and p190BCR-ABL (Fig. 3) are pleiotropic molecules with many qualitatively similar activities; their differences are still being unraveled. Studies suggest that not only is p210BCR-ABL critical to the development of the chronic phase of CML, but its effect on the DNA repair process may also be responsible for genomic instability and, hence, disease progression.

Kinase Activation

Tyrosine kinase enzymatic activity is central to cellular signaling and growth, and constitutively elevated kinase activity has been associated with transformation in

several systems. The ABL protein is a nonreceptor tyrosine kinase whose enzymatic activity is under close physiologic control (Konopka et al., 1984). In contrast, BCR-ABL proteins are constitutively active tyrosine kinases. The degree of transforming activity of BCR-ABL correlates with the degree of tyrosine kinase activity (Lugo et al., 1990). p190 BCR-ABL, which has higher tyrosine kinase activity, is therefore associated with the development of the more aggressive acute leukemia phenotype, while p210BCR-ABL plays a role in the more indolent chronic leukemia phenotype.

1.3.4 Conventional Treatment Options for Chronic Myelogenous Leukemia

1.3.4.1 Assessment of Response to Therapy

As with any other disease, the ultimate measure of the efficacy of a therapy is survival. However, for a disease with a relatively long course such as CML, surrogate markers are often used to allow for an earlier assessment of efficacy. From historical data, it has been estimated that the median survival of CML patients without any treatment is between 2 and 3 years. Three levels of disease control can be defined in CML. Complete hematological response (CHR) is defined as the normalization of the blood counts and the white cell differential as well as the disappearance of all symptoms and signs of disease. Complete cytogenetic response (CCR) means that no Ph-positive metaphases are detectable using

classical cytogenetics, with at least 20 metaphases available for analysis. Major cytogenetic response (MCR) is the presence of less than 35% Ph-positive metaphases. Molecular remission implies that no BCR-ABL transcripts

are detectable by RT-PCR. This assay is far less standardized than the other tests, and its sensitivity varies greatly between laboratories. However, there is a general consensus that PCR negativity requires a level of sensitivity that allows for detection of one BCR-ABL positive cell in 105 to 106 normal cells (Bose et al., 1998). There is good evidence that achievement of a major cytogenetic response on interferon-α therapy predicts improved survival, unless the patient belongs to the high risk group of patients (Hehlmann et al., 1994; Italian Cooperative Study Group on CML, 1994). Thus, cytogenetic response instead of survival is frequently used as a surrogate marker to assess efficacy. It must be stressed that such endpoints have been validated only in interferon-treated patients.

1.3.4.2 Conventional Cytotoxic Drugs

The first effective treatment for CML was Fowler's solution, which was widely used in the 19th century and contains arsenic as the active component. Recent *in vitro* studies have confirmed activity of arsenicals against CML cells, and the agent may see a comeback in the future (La Rosee et al., 2002a). With the advent of radiotherapy, splenic irradiation became popular in the 1920s and 1930s. It offered

symptomatic relief but probably did not prolong life. The first synthetic compound with activity in CML was busulfan, an alkylating agent. Busulfan is extremely toxic to stem cells, which may explain why it is particularly effective in the stem cell disease CML; it was the first therapeutic modality that offered a definitive survival benefit, although no randomized study was carried out. Interestingly, there are anecdotal cases of long-term remissions after high-dose busulfan (Djaldetti et al., 1966). Although superseded by more effective and less toxic alternatives, busulfan is still used in preparative regimens for allogeneic stem cell transplantation. The next effective drug to be introduced for CML was hydrea. Compared with busulfan, hydrea does not cause prolonged cytopenias, since it primarily targets the more mature myeloid cells. It also has a far more benign nonhematological toxicity profile than busulfan. A survival advantage for hydrea over busulfan was shown in a controlled randomized trial (Hehlmann et al., 1994). Another drug with significant single agent activity in CML is cytarabine, although it never became widely used. Neither busulfan, hydrea, nor cytarabine produced cytogenetic remissions in a significant number of cases. The 1970s saw a number of trials using acute leukemia-type multiagent chemotherapy. In contrast to conventional chemotherapy, a proportion of patients achieved some degree of Ph-negative hematopoiesis (Kantarjian et al., 1985). However, as a rule, these were transient responses. Given

the very considerable toxicity of polychemotherapy in CML, this approach was abandoned for patients in chronic phase.

1.3.4.3 Interferon-α

At the beginning of the 1980s, interferon-α was introduced as a therapy for CML. In contrast to other drug treatments, interferon-α produced sustained cytogenetic responses in up to one-third of patients (Talpaz et al., 1991). The initial single center results were subsequently confirmed in randomized trials that demonstrated a survival advantage for interferon-α over hydrea and busulfan (Hehlmann et al., 1994; Italian Cooperative Study Group on CML, 1994). A large randomized trial suggested that the combination of interferon-α and cytarabine is superior to interferon alone (Guilhot et al., 1997), a finding that was not confirmed in a subsequent study (Baccarani et al., 2002). The cytogenetic remissions induced by interferon are durable in a proportion of patients, sometimes even after discontinuation of the agent (Bonifazi et al., 2001). Although, with RT-PCR, BCR-ABL mRNA is still detectable, these long-lasting remissions amount to a biological although not molecular cure of the disease.

1.3.4.4 Allogeneic Stem Cell Transplantation

As of now, allografting is the only treatment capable of disease eradication, with the majority of patients achieving RT-PCR negativity (Savage and Goldman,

1997). Long-term disease-free survival is in the range of 50 to 80% in most studies (Savage and Goldman, 1997; Hansen et al., 1998). However, allografting is limited to patients who have a suitable donor and are medically fit to undergo the procedure, which involves high-dose chemotherapy and total body irradiation. Less toxic (nonmyeloablative) transplant regimens make allografting an option for patients who do not qualify for a conventional transplant (Mc- Sweeney et al., 2001; Or et al., 2003). It is not yet clear if the durability of remissions with nonmyeloablative regimens equals that of conventional transplants, and transplant-related complications remain a problem (Bornhauser et al., 2001). Although no randomized trials have been conducted, there is no doubt that decisively more patients are long-term survivors after an allograft than with nontransplant therapies.

1.3.5. Imatinib

1.3.5.1 Development of ABL-Specific Tyrosine Kinase Inhibitors

Given that the BCR-ABL protein has deregulated tyrosine kinase activity, it was logical to search for specific pharmacological inhibitors. In 1993, Anafi and colleagues (1993) reported a tyrphostin, related to erbstatin that inhibited the PTK activity of BCR-ABL and suggested that it might be possible to design specific compounds for the treatment of ABL-associated human leukemias (Anafi et al., 1993). In a more extensive analysis of number of tyrphostins, the compounds

AG568, AG957 and AG1112 proved to be the most specific agents. Growth inhibition of the CML cell line K562 occurred at micro molar concentrations and was associated with inhibition of BCR-ABL tyrosine kinase activity (Kaur et al., 1994). Tyrphostins are competitive toward ATP or substrate, or both (Kovalenko et al., 1997). Although active *in vitro*, tyrphostins have not been developed for clinical use. Another compound with activity toward BCR-ABL is herbimycin A, an antibiotic derived from *Streptomyces hygroscopicus*. Its efficacy in inhibiting transforming tyrosine kinases was recognized as early as 1988 (Uehara et al., 1988). Herbimycin was originally thought to inhibit BCR-ABL PTK (Okabe et al., 1992), but it was subsequently shown that its mode of action is the acceleration of BCR-ABL protein degradation (Shiotsu et al., 2000). Selective inhibition of primary CML cells was also shown for genistein, a flavonoid (Carlo Stella et al., 1996). In 1995 and 1996, Buchdunger and colleagues reported the synthesis of a series of compounds that exhibited specific inhibitory activity against the platelet derived growth factor receptor (PDGF-R) (Buchdunger et al., 1995) and ABL (Buchdunger et al., 1996). These compounds emerged from a high-throughput screen of chemical libraries with the goal of identifying kinase inhibitors. From this time-consuming approach, a lead compound of the 2-phenylaminopyrimidine class was identified. This lead compound had weak inhibitory activity against both serine/threonine and tyrosine kinases, but

served as a starting point for the synthesis of other related compounds. Of these compounds, which were found to possess inhibitory activity toward ABL, CGP57148 (STI571, now imatinib mesylate, Gleevec, Glivec) (Fig. 4) emerged as the lead compound for clinical development. Studies demonstrated that imatinib had extremely potent and specific *in vitro* and *in vivo* activity against BCR-ABL-transformed cells (Druker et al., 1996), results that were independently confirmed by other groups (Deininger et al., 1997; Gambacorti-Passerini et al., 1997). The success of imatinib in chronic-phase CML led to the approval of the drug as frontline therapy in 2002. Patients diagnosed in chronic phase are initiated on a regimen of oral imatinib at 400 mg/day. This regimen is maintained if patients achieve hematologic response after 3 months of therapy, cytogenetic response after 6 months, major cytogenetic response (Philadelphia chromosome–positive cells reduced to <35%) after 12 months, or complete cytogenetic response after 18 months. Imatinib was particularly effective in newly diagnosed chronic-phase CML, in which the complete hematologic response rate was greater than 90%, and the complete cytogenetic response rate was between 70% and 80% (O'Brien et al., 2003). The drug was also generally well-tolerated. Adverse events were typically mild or moderate; common events included superficial edema, nausea, and muscle cramps (Kantarjian et al, 2002).

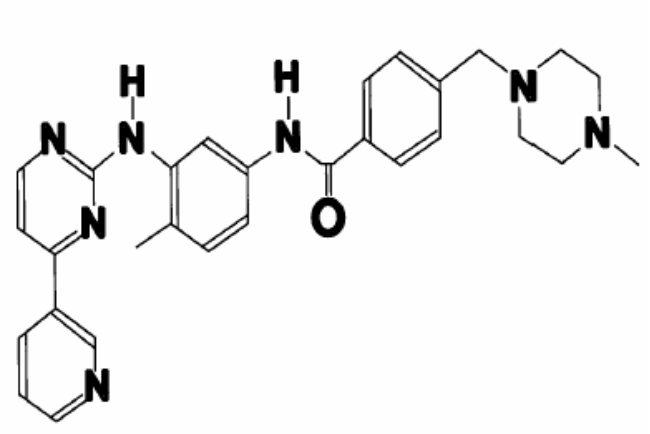


Fig. 4. Structure of Imatinib.

1.3.5.2 Mechanism of Action

Imatinib functions as a competitive inhibitor of ATP binding, with a *K*i value of 85 nM (Buchdunger et al., 1995). The crystal structure of the catalytic domain of the ABL kinase in complex with an imatinib analog (Schindler et al., 2000) and with imatinib (Nagar et al., 2002) has been solved (Fig. 5). The most important finding of these studies is that the compound binds to the inactive conformation of ABL, contacting 21 amino acid residues (Nagar et al., 2002). By exploiting the distinct inactive conformation of the A-loop of ABL, imatinib is able to achieve its high specificity. No major structural rearrangements are required for imatinib to bind to the A-loop. In contrast, there is an induced-fit mechanism for binding to occur in the N-lobe, which normally accommodates the phosphate groups of ATP and is

therefore referred to as the P-loop. The P-loop is a glycine-rich and highly flexible structure, which folds down upon binding of imatinib, resulting in increased surface complementarity. This change in position is stabilized by a newly formed hydrogen bond between Tyr-253 and Asn-322 (Schindler et al., 2000). A consequence of the induced fit is the formation of a hydrophobic cage that surrounds imatinib, engaging van der Waals interactions with residues Tyr-253, Leu-370, and Phe-382 (Nagar et al., 2002). Moreover, imatinib forms a number of hydrogen bonds with the kinase domain. Methionine 318 [which normally binds N1 of ATP (Schindler et al., 2000), threonine 315, methionine 290, glutamine 286, lysine 270, and asparagine 381, together with water molecules, form a network of hydrogen bonds around the imatinib

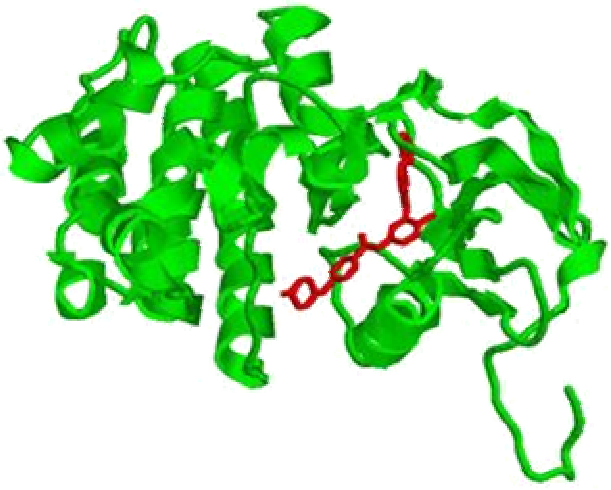


Fig. 5. Imatinib in the kinase domain of ABL.

molecule. Given this extremely tight fit, it is not surprising that changes of single amino acids can affect the binding of imatinib (Fig. 6). For example, threonine 315 is replaced by methionine in the insulin receptor kinase (Schindler et al., 2000), which is completely insensitive to imatinib (Nagar et al., 2002). In agreement with these predictions, ABL phosphorylated on tyrosine 393 is much less sensitive to imatinib, since phosphorylation of tyrosine 393 stabilizes the active, open conformation of the A-loop (Schindler et al., 2000; Roumiantsev et al., 2002), to which imatinib does not bind. BCR-ABL is constitutively tyrosine-phosphorylated and will thus be in a conformation that is unable to bind imatinib. From experiments with BCR-ABL-positive cell lines treated with imatinib, it is known that exposure to the compound results in inhibition of kinase activity within minutes. This indicates that there is rapid turnover between the phosphorylated form (constituting the bulk of the protein) and the unphosphorylated form, which is capable of binding imatinib.

1.3.5.3 Resistance to Imatinib

Several mechanisms have been proposed to underlie the development of imatinib resistance in CML, including *BCR-ABL* gene mutations (Gorre et al., 2001; Gorre et al., 2002); over expression and amplification of the *BCR-ABL* gene locus (Gorre et al., 2001; Hochhaus et al., 2002); activation of BCR-ABL–independent

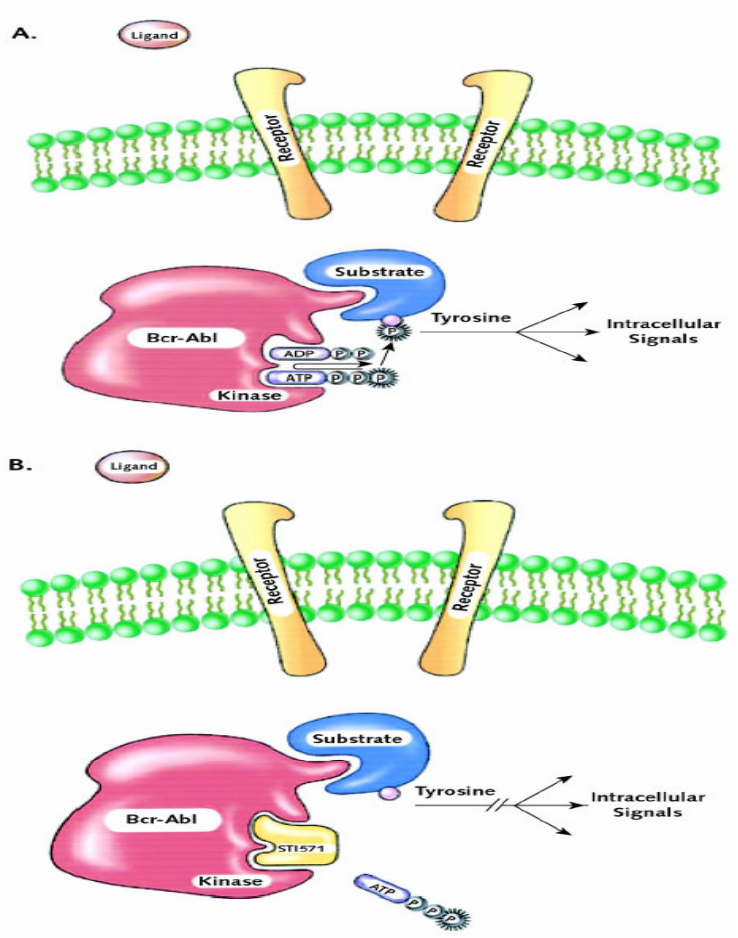


Fig. 6: Mechanism of action of Imatinib.

A. *The BCR-ABL tyrosine phosphokinase enzyme is constitutively active. Adenosine triphosphate (ATP) is an energy molecule used to drive BCR- ABL enzymatic function. The enzyme's tyrosine kinase function is carried out at the kinase pocket. BCR-ABL binds ATP and transfers phosphate from ATP to tyrosine residues on its substrates, thereby transmitting intracellular signals independently of ligand binding to growth factor receptors, such as that for interleukin-3.* ***B.*** *When imatinib mesylate (STI571) occupies the kinase pocket, it blocks the action of ATP, thereby suppressing phosphorylation of downstream effector molecules. ADP = Adenosine diphosphate (Ann Intern Med. 2003; 138:819-830).*

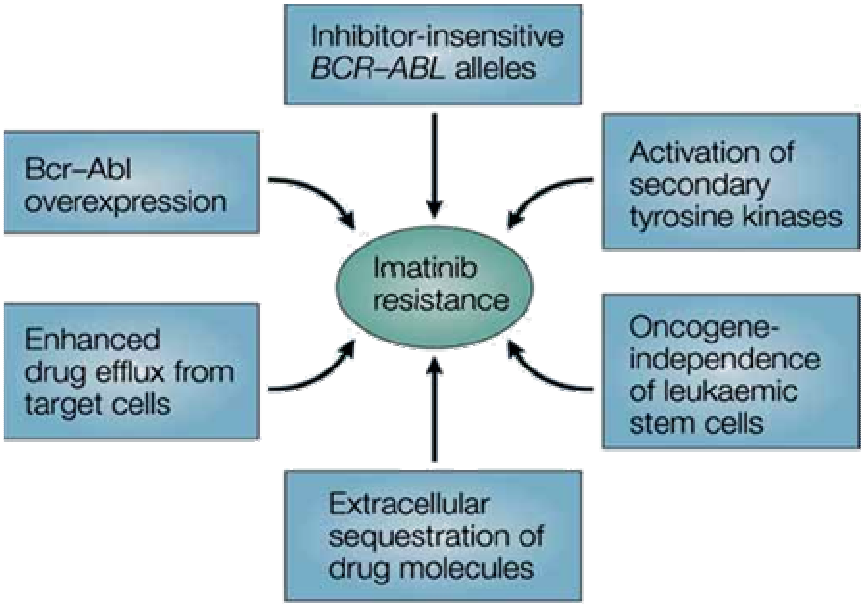


Fig. 7: Potential mechanisms of resistance to imatinib.

pathways, such as members of the Src kinase family (Donato et al., 2003); binding of imatinib to serum α-1 acid glycoprotein (Gambacorti-Passerini et al., 2000); and increased drug efflux through the multidrug resistance gene (Thomas et al., 2004; Illmer et al., 2004) (Fig. 7).

***BCR-ABL* gene mutations**

Of the proposed mechanisms, a common cause of imatinib resistance seems to be point mutations in the ABL kinase domain (Gorre et al., 2001), which preclude

the binding of imatinib (Fig. 8). These mutations have been characterized into 2 groups. The first group includes mutations that impede contact between BCR-ABL and imatinib (Azam et al., 2003). Substitution of any 1 of approximately 20 ABL kinase domain residues involved in imatinib binding could result in a reduced affinity for imatinib binding or in steric inhibition of binding. The second group includes mutations that alter the spatial conformation of the protein (Azam et al., 2003). The BCR-ABL structure contains 2 flexible loop structures, the adenosine triphosphate–binding phosphate loop and the activation loop, which have specific arrangements in the inactive conformation of BCR-ABL that stabilize the structure (Shah et al., 2002). Mutations in these loops destabilize their arrangement such that the kinase domain cannot assume the inactive conformation required for imatinib binding (Schindler et al., 2000; Shah et al., 2002; Corbin et al., 2003). *In vitro* screening has now identified a more comprehensive set of 50 BCR-ABL mutations associated with imatinib resistance (Azam et al., 2003). There are conflicting data regarding potential differences in the prognostic significance of these mutations in terms of time to progression and survival (Hochhaus et al., 2002; Shah et al., 2002; Branford et al., 2003). Branford and colleagues (Branford et al., 2003) showed that in patients with late chronic-phase (that is, chronic-phase CML diagnosis ≥12 months) and

accelerated-phase CML, a specific subgroup of mutations in the phosphate loop is associated with a poor prognosis in terms of survival.

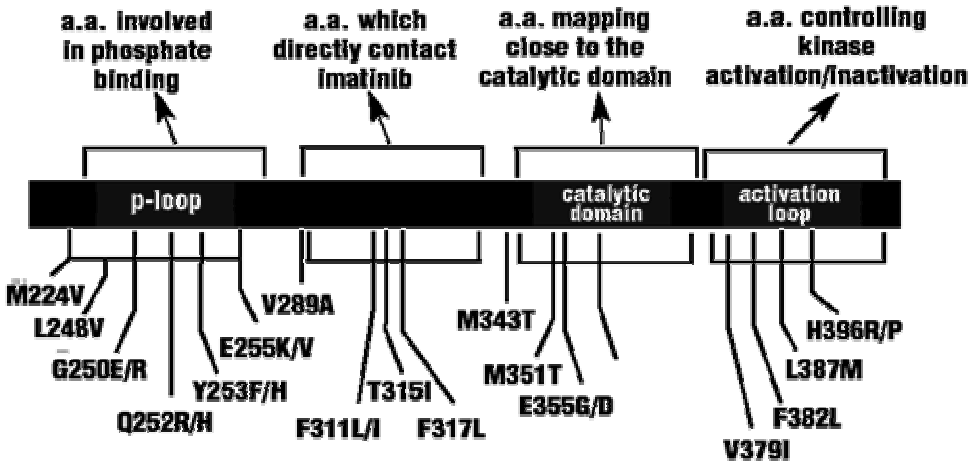


Fig. 8: Distribution of reported point mutations within the ABL kinase.

Activation of Src Kinases

Increasing evidence suggests that Src family kinases may play a role in the development of treatment resistance and in disease progression. *In vitro* data with kinase-defective Src mutants and Src kinase inhibitors show that Src family kinases mediate the oncogenic signaling of BCR-ABL (Lionberger et al., 2000; Warmuth et al., 2003; Wilson et al., 2002). Experiments in mouse models indicate that BCR-ABL and Src kinases are required to induce an ALL phenotype akin to lymphoid blast

crisis CML and Philadelphia chromosome–positive ALL (Hu et al., 2004) and that blockade of Src kinase signaling prevents CML progression to lymphoid blast crisis (Li et al., 2006). BCR-ABL–positive CML cells cultured in the continuous presence of imatinib or those obtained from patients who have disease progression while receiving imatinib therapy have a decrease in BCR-ABL protein or mRNA levels and a corresponding increase in the activity of Src family kinases (Donato et al., 2003). The role of Src kinases in drug-resistant BCR-ABL cells has been further supported by the decreased survival and proliferation of drug-resistant BCR-ABL cells after inhibition of Lyn expression by RNA interference (Ptasznik et al., 2004). The inability of imatinib to inhibit Src kinases directly (Druker et al., 1996) further corroborates their potential role in the development of imatinib resistance. *In vitro* experiments with cells from imatinib-sensitive patients showed that imatinib inhibits Src family kinase activation through its effect on BCR-ABL (Donato et al., 2003). However, in multiple specimens from imatinib-resistant patients, imatinib inhibition of BCR-ABL had no effect on Src family kinase activation, which indicates that their activity in these patients had become uncoupled from BCR-ABL regulation (Donato et al., 2003). In animal models, loss of BCR-ABL–mediated inhibition of Lyn substantially impaired the antitumor activity of imatinib, which was recovered by the addition of an inhibitor of BCR-ABL and Src family kinase. These data support the hypothesis that

increased Src kinase activity may reduce the dependence of leukemic cells on BCR-ABL kinase activity in some patients with imatinib-resistant CML. In these patients, inhibition of BCR-ABL and Src family kinase is required for apoptosis (Donato et al., 2003; Dai et al., 2004). Studies investigating the pathways by which Src kinases may mediate disease progression and resistance remain elusive.

Increased MDR1 expression

Multi-drug resistance (MDR1) gene, also known as ABCB1 gene, is a term that describes the cross-resistance of cells against a range of drugs with different structures and targets. This gene encodes an efflux transporter P-glycoprotein (P-gp) that limits a wide variety of drugs from penetrating cells and depositing them into the extracellular space (Xie et al., 1999; Wang et al., 2004). P-glycoprotein is expressed at relatively high levels not only in tumor cells, where it confers multiple drug resistance, but also in many normal tissues, such as intestine, liver, kidney, blood-brain-barrier, and placenta (Thiebaut et al., 1987; Lotsch, 2001; Mizuno et al., 2003; Sun et al., 2004).

Intestinal drug efflux by P-glycoprotein is widely recognized as a major determinant for low or variable oral absorption and bioavailability of several drugs (Cummins et al., 2003). It has been discovered that P- glycoprotein plays a role in drug disposition including absorption, distribution, metabolism, and excretion

(Mizuno et al., 2003; Sun et al., 2004). Its expression could be an obstacle to adequate drug therapies (Choo et al., 2000). Besides the drug pharmacokinetics alteration, the presence of this protein has been related with increased susceptibility to diseases in human beings (Mealey, 2004).

Increased expression of MDR1 has been observed in imatinib-resistant Lama-84 cells, and resistance was partially reversible by verapamil, a P-glycoprotein (PGP) inhibitor (Mahon et al., 2000). Several other studies have since reported on the role of PGP as a transporter of imatinib in cell lines, indicating that MDR1 expression confers resistance (Mahon et al., 2003; Hamada et al., 2003; Widmer et al., 2003; Hegedus et al., 2003; Dai et al., 2003), although this was not universally confirmed (Ferrao et al., 2003).

Amplification and over expression of BCR-ABL.

In a study by Gorre et al. (2001), fluorescence *in situ* hybridization demonstrated amplification of *BCR-ABL* at the genomic level in 3 of 9 patients with resistant disease. In a subsequent report, an increase in the level of BCR-ABL mRNA was seen in 4 of 37 patients (Hochhaus et al., 2002). Both mechanisms are thought to lead to increased expression of BCR-ABL protein, although, due to the difficulties in analyzing BCR-ABL protein in clinical specimens, this has not yet been formally demonstrated.

Other mechanisms of resistance

In addition, despite its impressive therapeutic efficacy in halting disease progression in the early stages of CML, imatinib treatment does not seem to eradicate the actual cause of the disease — quiescent Ph+ stem cells. In contrast to the mature, differentiated leukemia cells, quiescent Ph+ stem-cell progenitors seem to tolerate the inhibition of BCR–ABL kinase activity and are therefore not forced into apoptosis on exposure to imatinib (Graham et al., 2002). Subsequent proliferation of stem-cell clones is therefore likely to result in the reappearance of leukemia on cessation of imatinib therapy. Although this does not account for a resistance mechanism per se, it nevertheless necessitates a life-long continuation of imatinib therapy.

1.3.5.4 Strategies to overcome resistance

Reactivation of BCR-ABL signaling is an almost universal finding in patients with imatinib resistance. This implies that the cells continue to depend on the specific signaling output of the genetic event that initiated the malignant transformation in the first place, and that BCR-ABL remains the best therapeutic target (Gorre et al., 2002).

(i) Dose escalation of imatinib

Retrospective data suggest that dose escalation can overcome hematologic or cytogenetic resistance in some patients (Kantarjian et al., 2003), although these responses may not be maintained (Marin et al., 2003). An important consideration is the specific type of mutation. Dose escalation is likely to be effective in the case of mutants with a low or moderate level resistance to imatinib, such as H386P, but not in highly resistant mutants such as T315I or E255K (Corbin et al., 2003).

(ii) Combination with conventional cytotoxic drugs

Many drugs have been tested for synergism with imatinib (La Rosee et al., 2002). The use of different cell lines and different models of data analysis may explain why the results are not always consistent among the various studies. Altogether, it appears that most combinations are synergistic or at least additive. Since one of the pillars of BCR-ABL–mediated malignant transformation is inhibition of apoptosis (Bedi et al., 1994), this is consistent with the concept that inhibition of BCR-ABL function resensitizes the cells to the induction of apoptosis by conventional agents. Naturally, drugs with established activity in CML have attracted particular attention. Cytarabine, homoharringtonine, and IFN are synergistic *in vitro*, and combinations with imatinib are currently tested clinical trials. Promising results have also been obtained for decitabine, a novel hypomethylating agent with activity

in CML blast crisis (Sacchi et al., 1999). In contrast, the data are conflicting for hydroxyurea (Topaly et al., 2001; Kano et al., 2001; Thiesing et al., 2000), while methotrexate and topotecan are antagonistic in combination with imatinib. Given the requirement for many dose points, drug combination assessments are difficult to perform in primary cells. Thus, most of the data are based on cell lines, although in a strict sense, any combination would have to be tested in primary cells confirm that the differential between CML and normal cells afforded by imatinib is maintained.

(iii) Alternative inhibitors of ABL

Several compounds with inhibitory activity toward ABL kinase nanomolar concentrations have been identified. These compounds are less specific and have activity against SRC family kinases as well as various other targets.

(iv) Combinations with other signal transduction inhibitors

Extensive *in vitro* studies have tested specific inhibitors of signal transduction pathways downstream of BCR-ABL alone and in combination with imatinib. In most cases, synergistic or additive effects were demonstrable, in some instances also in imatinib-resistant cell lines. Several compounds, including farnesyl transferase inhibitors (FTIs), mammalian target of rapamycin (mTOR) inhibitors (rapamycin, RAD001), and flavopiridol, an inhibitor of cyclin-dependent kinases, are in early clinical development, alone or in combination with imatinib. Although there is

rationale for these agents, BCR-ABL itself remains the best target, as resistance to imatinib is almost universally associated with reactivation of BCR-ABL kinase activity, and it is doubtful whether disrupting individual pathways downstream of BCR-ABL will be effective. Studies in mice with homozygous deletions of IL-3, GM-CSF (Li et al., 2001), or STAT5 (Sexl et al., 2000) revealed that BCR-ABL remained fully leukemogenic, indicating that targeting one of these pathways would not be sufficient. Another important lesson from preclinical studies is that synergism between imatinib and other agents depend on residual sensitivity of the leukemic cells to imatinib (La Rosee et al., 2004). Thus, combinations of imatinib with decitabine, arsenic trioxide, or FTIs are synergistic in resistant cell lines with over expression of BCR-ABL but not in lines expressing the T315I mutant (La Rosee et al., 2004; Peters et al., 2001; Hoover et al., 2002). This emphasizes the need to establish the mechanism of resistance to allow for a rational choice of salvage therapy.

1.4 Cyclooxygenases

Elevated concentrations of prostaglandins have been reported in a wide range of malignancies for more than 25 years (Jaffe, 1974; Jung, et al., 1985; Lupulescu, 1996; Bennett et al., 1977; Bennett, 1986; Rigas, 1993). Prostaglandins and other bioactive prostanoids (e.g., thromboxanes, prostacyclins) are derived from

arachidonic acid (AA), a 20-carbon, polyunsaturated fatty acid that is liberated from the cell membrane by phospholipase A2 in response to exogenous stimuli (e.g., inflammation or mitogens). Once hydrolyzed from the lipid bilayer, AA is sequentially metabolized by the bifunctional COX enzyme to PGG_2 (*via* COX's cyclooxygenase activity), and then to PGH_2 (*via* COX's peroxidase activity (Fig. 9). PGH_2 is subsequently transformed by cell-specific synthases into a wide array of bioactive prostanoids including PGE_2, $PGF_{2\alpha}$, PGD_2, prostacyclin, and thromboxane A_2. The types and amounts of prostanoids produced by this enzymatic activity depend on cell-specific synthases. Each prostanoid mediates discrete biologic activities, which in turn facilitate diverse physiologic and pathophysiologic responses (Kerin et al., 1991).

COX activity results from at least two enzymatic isoforms, termed COX-1 and COX-2 (Smith et al., 1996; Williams and DuBois, 1996; Herschman, 1996); a third COX isoform has been hypothesized, but not yet demonstrated (Willoughby et al., 2000). Both COX-1 and COX-2 are homodimeric, glucosylated, heme containing proteins with two catalytic sites. Both isoforms have high structural identity but are different in substrate and inhibitor selectivity (Smith *et al.,* 1996), also in their intracellular localization. The gene for COX-1 resides on human chromosome 9 (Funk et al., 1991) while the COX-2 gene is on chromosome 1 (Kosaka et al., 1994).

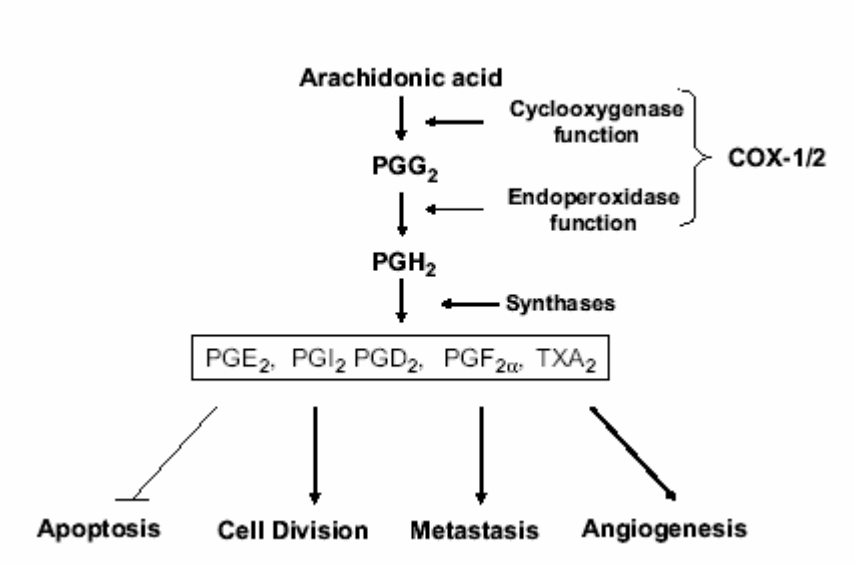


Fig. 9: The COX pathway.

Unlike COX-1 enzyme, COX-2 has valine at position 523 instead of isolueucine. The difference between valine and isoleucine is of a single methyl group. This substitution allows COX-2 inhibitors to access the secondary internal side pocket of the molecule that is obstructed by isoleucine in the COX-1 isoform (Kurumbail *et al.,*1996).

COX-1 mRNA and protein are constitutively expressed at relatively constant levels in nearly all cell types. Prostanoids formed by COX-1 are thought to mediate a variety of homeostatic functions including gastric mucosal cytoprotection, renal vasodilation, macrophage differentiation, and platelet aggregation (Crofford, 1997;

Crofford et al., 2000). In contrast, COX-2 is an early response gene that is constitutively expressed in relatively few cell types, but can be markedly induced in response to proinflammatory cytokines such as tumor necrosis factor and interleukin-1 (Ristimaki et al., 1994; Chen et al., 1994), growth factors (i.e., epidermal growth factor, platelet derived growth factor, transforming growth factors a and b) (Tsuji et al., 2001), hormones, mitogens (i.e., lipopolysaccarides), phorbol esters (i.e., TPA), interferon-α (Bostrom et al., 2001), and several other chemical or mechanical stimuli (Komhoff et al., 2000; Mohammed et al., 1999; Hwang et al., 1998). Initially it was thought that COX-2 was not constitutively expressed in any tissue. However, recent work has demonstrated constitutive expression of COX-2 in a variety of non-inflammatory tissues, including kidney, brain, pancreatic islets, bone, testis, tracheal epithelium and ovary (DuBios *et al.,* 1998; Neeraja *et al.,* 2003). COX-2 appears to play a pivotal role in inflammation, and probably contributes significantly to neoplastic progression as well.

Many publications have reported COX-2 over expression in a wide range of human neoplasias at both preinvasive and invasive stages of tumor progression. Indeed, COX-2 mRNA, protein, or both are elevated relative to the normal tissues from which they are derived in human neoplasias (Grösch et al., 2006; Mazhar et al., 2005; Anderson et al., 2002).

Over expression of COX-2 RNA and protein does not necessarily mean that COX-2 plays a critical role in carcinogenesis. The pathophysiologic significance of COX-2 over expression, however, has been demonstrated by its association with poorly differentiated cancers in some studies (Komhoff et al., 2000; Ryu et al., 2000; Joki et al., 2000; Madaan et al., 2000) and with well-differentiated cancers in others (Soslow et al., 2000; Bae et al., 2001; ; Rahman et al., 2001). More importantly, COX-2 over expression is positively correlated with poor prognostic features such as tumor size (Uefuji et al., 2001), invasive and metastatic properties of CRC tumor cells (Ohno et al., 2001; Tomozawa et al., 2000; Hull, et al., 2000), and even poor survival (Gaffney et al., 2001; Shono et al., 2001; Sheehan et al., 1999). In certain organs COX-2 over expression is associated with distinct histologies. In the lung, COX-2 levels are consistently elevated in adenocarcinomas, but much less frequently so in squamous cell and small cell cancers, which suggest an association between increased COX-2 expression and the development of adenocarcinomas and possibly invasive/metastatic phenotypes (Ochiai, et al., 1999; Hida et al., 1998). High concentrations of COX-2 are also found in central nervous system malignancies, correlating with increased tumor aggressiveness and reduced survival (Deininger et al., 1999; Joki et al., 2000). Thus, although our knowledge remains

incomplete, correlative data support the hypothesis that COX – particularly COX-2 – contributes to carcinogenesis and its progression.

1.5 NSAIDs and COX-2 inhibitors

Non-steroidal anti-inflammatory drugs, usually abbreviated to NSAIDs, are drugs with analgesic, antipyretic and anti-inflammatory effects - they reduce pain, fever and inflammation. Discovered initially in 1853, acetylsalicylic acid (aspirin, ASA) was introduced to the medical profession in 1897 by the Bayer Company in Elberfeld, Germany (Stevenson, 1968). Today, ASA and other NSAIDs are among the most widely used drugs in the world. Part of the popularity of NSAIDs is that, unlike opioids, they do not produce sedation or respiratory depression and have a very low addiction rate. Despite diverse chemical structures, NSAIDs as a class share clinical effects that may be considered beneficial or harmful, depending on the intent with which they are administered. For example, NSAIDs are commonly used to reduce pain, fever, and inflammation. NSAIDs also promote renal salt retention with the unwanted side effects of hypertension and edema in certain patients, but improved electrolyte homeostasis in children with salt-losing nephropathies. NSAIDs inhibit platelet aggregation, increasing hemorrhagic tendencies in certain patients, while reducing the risk of cardiovascular thrombotic events in others. Other double-edged effects include reduced uterine tonicity, which is advantageous in the setting

of a premature labor but deleterious in other contexts; and the induction of apoptosis, which may prevent neoplastic progression, but may cause gastric irritation and ulceration as well. Most NSAIDs act as non-selective inhibitors of the cyclooxygenase, inhibiting both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoenzymes (Bjorkman, 1998). John Vane elucidated this mechanism of action of NSAIDs in the year 1971.

COX-1 inhibition likely accounts for the more worrisome side effects, whereas COX-2 inhibition relates primarily to anti-inflammatory properties (Vane et al., 1998; Crofford, 1997). For example, one of main functions of COX-1 is protecting the stomach lining by preventing the stomach mucosa from being eroded by its own acid. When non-selective COX-1/COX-2 inhibitors (such as aspirin, ibuprofen, and naproxen) lower stomach prostaglandin levels, these protective effects are lost and ulcers of the stomach or duodenum and potentially internal bleeding can result. The discovery of COX-2 in 1991 by Daniel L. Simmons at Brigham Young University raised the hope of developing an effective NSAID without the gastric problems characteristic of these agents. It was thought that selective inhibition of COX-2 would result in anti-inflammatory action without disrupting gastroprotective prostaglandins.

In an effort to improve the TI of NSAIDs, a new class of agents, Coxibs that selectively inhibit COX-2 have been developed (Moore and Simmons; 2000). Classic

non-selective NSAIDs such as ASA occupy both COX-1 and COX-2 binding sites, whereas COX-2 selective inhibitors preferentially bind COX-2. Indeed, novel COX-2 inhibitors are up to 1000 times more likely to suppress COX-2 than COX-1 (Li, 1996; Herschman, 1996). COX-2 selective inhibitor celecoxib (Celebrex^{TM}) is widely marketed for rheumatologic indications. Several other compounds – such as etoricoxib, etodolac, meloxicam, and nimesulide –exhibit approximately equal selectivity for COX-1 and COX-2 (Riendeau, 2001; Ogino, 2000; van Ryn and Pairet; 1999; Okajima et al., 1998).

1.6 Celecoxib

The IUPAC name of celecoxib is 4-[5-(4-methylphenyl)-3-(trifluoromethyl) pyrazol-1-yl] benzenesulfonamide (Fig. 10) and its chemical formula is $C_{17}H_{14}F_3N_3O_2S$ (MW 381.38). Celecoxib is a highly specific inhibitor of COX-2, approximately 7.6 times more selective for COX-2 inhibition over COX-1. This was the first COX-2 inhibitor approved for the use in U.S, for relief of signs and symptoms of rheumatoid arthritis and osteoporosis in adults. In addition to analgesic, antipyretic and anti-inflammatory activity, it has chemopreventive properties against colon cancer. Celecoxib is the only NSAID that has been approved by the FDA (in December 1999) for adjuvant treatment of patients with familial adenomatous polyposis. Since the introduction of celecoxib in 1998, more than 3000 studies have

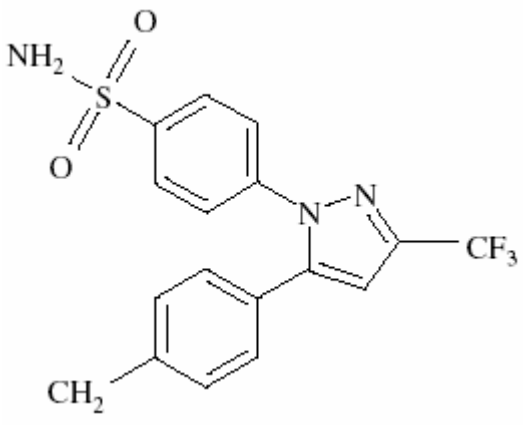


Fig. 10: Chemical structure of Celecoxib.

investigated the molecular targets and clinical effects of the drug. Anticarcinogenic effects of celecoxib have been demonstrated in many types of cancers including non-small-cell lung cancer (Chen et al., 2007), gastric cancer (Zhu et al., 2007), lung cancer (Harris et al., 2007), prostate cancer (Anai et al., 2007), bladder cancer (Zhang J, et al., 2007), chronic myeloid leukemia (Zhang et al., 2006; Subhashini et al., 2005). Celecoxib exerts its anti-carcinogenic effects in both COX-2-dependent and –independent mechanisms. In general, the anticarcinogenic mechanisms of celecoxib involve blocking cell cycle progression and angiogenesis and inducing apoptosis.

Inhibition of Cell Cycle Progression

Molecular mechanisms involved in cell cycle arrest induced by celecoxib treatment are at least partly understood. Celecoxib can inhibit protein kinase B (PKB/Akt) or its upstream kinase phosphoinositide-dependent kinase 1 (PDK-1) (Lin et al., 2004; Basu et al., 2004; Arico et al., 2002; Zhu et al., 2004; Kulp et al., 2004). In cell free assays, PDK-1 appears to be a direct target of celecoxib. It, however, is only a weak PDK-1 inhibitor; the concentration required for 50% inhibition (IC_{50}) is 48 μM. Celecoxib, by means of its 4-methylphenyl moiety, inhibits PDK-1 by competing with ATP at the ATP binding site of PDK-1 (Zhu et al., 2004). Although many studies (Arico et al., 2002; Zhang et al., 2004) have reported that PKB is inhibited by celecoxib, whether celecoxib binds directly to PKB or acts by means of another celecoxib target, such as PDK-1, is unclear. However, 4-[5- (2,5-dimethylphenyl)-3(trifl uoromethyl)-1 *H* -pyrazol-1-yl] benzene sulfonamide, which is a celecoxib analogue that lacks COX-2 inhibitory activity, also inhibits PDK-1 and PKB activity. Inhibition of PKB by celecoxib prevents the cell proliferation–promoting effects of PKB and could be one mechanism by which celecoxib induces a cell cycle block.

Induction of Apoptosis

Many studies have shown that celecoxib exerts its anticarcinogenic effect in various cancer cell lines by inducing apoptosis (Chen et al., 2007; Fantappie et al.,

2007; Fukada et al., 2007; Haupt et al., 2006; Zhang et al., 2004; Hsu et al., 2000). Apoptosis, or programmed cell death, can be induced by the extrinsic pathway through activation of death receptors or by the intrinsic pathway by means of the release of cytochrome *c* from the mitochondria (Roy et al., 2007; Zhang et al., 2006; Subhashini et al., 2005; Li et al., 2002; Green and Evan, 2002). Both pathways require the activation of various caspases, which cleave various proteins and activate DNases, leading to DNA fragmentation (Subhashini et al., 2005; Cryns and Yuan, 2002). Evidence that the intrinsic apoptotic pathway appears to be activated by celecoxib (Jendrossek et al., 2003) includes the observations that expression of the antiapoptotic proteins Bcl-2, Bcl-xL, Mcl-1 and survivin decreases after treatment of cancer cells with celecoxib, whereas expression of the proapoptotic protein Bad increases (Fukada et al., 2007; Tsujii et al., 1997; Nam et al., 2004; Wun et al., 2004; Dandekar et al., 2005), and rapid release of cytochrome *c* from mitochondria and the activation of Apaf-1 and caspases 3, 8, and 9 are observed (Zhang et al., 2006; Narayanan et al., 2003; Jendrossek et al., 2003; Catalano et al., 2004; Kern et al., 2002). Evidence that the extrinsic apoptotic pathway is activated after celecoxib treatment includes the increased expression of the death receptor DR5 in celecoxib treated human non – small-cell lung carcinoma cells and activation of the

FAS – FADD pathway in celecoxib-treated cervical carcinoma cells (Kim et al., 2004; Qiu et al., 2004; Liu et al., 2004).

One target for selective COX-2 inhibitors is PDK-1 or downstream substrate PKB/AKT (Pang et al., 2007; Basu et al., 2004; Arico et al., 2002; Zhu et al., 2004; Kulp et al., 2004; Hsu et al., 2000). PKB induces antiapoptotic effects by phosphorylating and then inactivating the proapoptotic protein BAD (i.e., the Bcl-2 or Bcl-X antagonist), by phosphorylating procaspase 9 to prevent its cleavage to active caspase 9, or by phosphorylating the apoptosis signal regulating kinase 1, which inhibits the stress-activated protein kinase pathway and other kinases (Handa et al., 2004; Datta et al., 1999). Inhibition of PKB by celecoxib reduces all of these activities and promotes apoptosis.

1.7 Purpose of the study

Chronic myelogenous leukemia (CML) is a clonal neoplastic disorder of hematopoietic stem cells that accounts for 15 to 20% of newly diagnosed cases of adult leukemia. The causative molecular event in CML is the genetic transposition of ABL and BCR sequences to form a BCR-ABL fusion gene, leading to the expression of a constitutively active, chimeric BCR-ABL protein-tyrosine kinase. Approximately 95% of CML patients have this rearrangement which is identifiable in most cases as Philadelphia (Ph) translocation, t(9;22). Expression of the BCR-ABL gene is

sufficient to cause chronic phase CML, while disease progression to acute phase or blast crisis phase is thought to depend on additional genetic changes.

Selective inhibition of the BCR-ABL tyrosine kinase by imatinib is a promising new therapeutic strategy in patients with CML. Despite high rates of hematologic and cytogenetic responses, primary refractory disease and secondary resistance have been observed in a proportion of patients on imatinib monotherapy. Clinical studies have demonstrated durable responses in chronic phase patients whereas most responding patients in blast crisis relapse despite continued therapy. Mechanisms of imatinib resistance identified from *in vitro* studies include several-fold increase in the amount of BCR-ABL protein, amplification of the *BCR-ABL* gene, point mutations in the ABL kinase domain and over expression of the multidrug resistance P-glycoprotein.

Development of new therapeutic strategies to overcome imatinib mesylate resistance in accelerated CML has been the focus of many recent investigations. In the literature, at least 3 distinct approaches have been reported. First, recent efforts have led to the identification of several novel ABL inhibitors capable of inhibiting some or all of mutant ABL kinases, which include PD180970, BMS-354825 and AP23464. In addition, the BCR-ABL chaperone heat shock protein 90 inhibitors

geldanamycin and 17-allyaminogelanamycin have also been shown to inhibit the growth of imatinib-resistant hematopoietic cells found in patients with T315I and E255K mutation. Second, cotreatment of imatinib-resistant cells with antileukemic agents such as As_2O_3, decitabine, the farnesyl transferase inhibitor SCH66336, and the histone deacetylase inhibitors suberoylanilide hydroxamic acid (SAHA) and butyrate could enhance the antiproliferative activity of imatinib mesylate. Third, the combination of different target-directed therapeutic agents such as the proteasome inhibitor bortezomib in conjunction with the cyclin-dependent kinase inhibitor flavopiridol or with SAHA has also been shown to effectively induce apoptosis in imatinib-resistant cells. Combined treatment with these agents and imatinib is beneficial in cell lines that have residual sensitivity to imatinib monotherapy, with synergistic growth inhibition achieved only at doses of imatinib that overcome resistance. In some imatinib-resistant cell lines, combination treatments that use low doses of imatinib lead to antagonism. Apoptosis studies suggest that this can be explained in part by the reduced proapoptotic activity of imatinib in resistant cell lines. This provides a rational approach for dose-adjusted administration of imatinib when combined with other agents.

Cyclooxygenases convert arachidonic acid to prostaglandin H_2 in a two-step reaction. Prostaglandin H_2 is further converted by specific prostaglandin synthases

to prostaglandin E_2, prostaglandin $F_{2\alpha}$, or prostaglandin D_2. Two isoforms of cyclooxygenases, COX-1 and COX-2 have been reported. Traditional NSAIDs are nonselective inhibitors of both COX-1 and COX-2. Although COX-1 is constitutively expressed in many tissues, COX-2 is detected negligibly in most tissues but can generally be induced by cytokines and stress in various tissues. Increased COX-2 expression appears to be involved in the development of cancer by promoting cell division, inhibiting apoptosis, altering cell adhesion and enhancing metastasis, and stimulating neovascularization. The inhibition of COX-1 activity by traditional NSAIDs blocks the cytoprotective activities of COX-1 in gastric mucosal lining leading to unwanted side effects. To circumvent the side effects associated with COX-1 inhibition, selective COX-2 inhibitors, such as celecoxib and rofecoxib, were developed. Clinical studies, indeed, found that the selective COX-2 inhibitors had fewer gastrointestinal side effects than traditional NSAIDs but had anti-inflammatory activities that were similar to those of traditional NSAIDs.

Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, is the only non-steroidal anti-inflammatory drug so far which has been approved by the FDA for adjuvant treatment of patients with familial adenomatous polyposis. Recently, COX-2 inhibitors have also gained attention, either alone or in combination with other chemotherapeutic agents and/or radiation therapy, in the treatment of cancer.

Celecoxib exerted antitumor effects in a wide variety of cancers. It also showed synergistic antitumor effects when combined with gemcitaine or 5-fluorouracil in patients with advanced pancreatic cancer, and it enhanced the response to paclitaxel and carboplatin in early-stage non–small cell lung cancer. Celecoxib also exerts antileukemic effects in K562 cells by cell cycle arrest, caspase-3 activation and down regulation of COX-2 expression. These effects of celecoxib were shown to be synergistic with hydroxyurea or imatinib.

Drug resistance is one of the major factors explaining chemotherapy failure in patients with cancer. Cancer cells possess intrinsic drug resistance to several compounds or they can rapidly acquire it upon chemotherapy. Among the several types of drug resistance, one of the most studied concerns is the over expression of the membrane-associated "ATP binding cassette" (ABC) family transporters. Among mammalian ABC transporters, P-glycoprotein (P-gp) family and multidrug resistance associated protein (MRP) families have a major role in drug transport. P-glycoprotein family consists of two classes: Class I (MDR1 in humans, MDR1a and MDR1b in rodents) and class II (MDR 2 or 3 in humans and MDR 2 in rodents).

Over expression of MDR1, a membrane P-glycoprotein (P-gp) of 170 kDa expels drugs and xenobiotics in an energy dependent manner, thus reducing their intracellular accumulation and thereby causing drug resistance. To date, efforts to

combat the over expression of MDR1 in the clinic have involved the use of functional modulators or reversal agents that block the MDR1 mediated efflux of anti cancer drugs. Among many different regulators of MDR1 transcription, Reactive Oxygen Species (ROS) and Cyclooxygenase-2 (COX-2) were shown to be important. A direct link between COX-2 and MDR-1 expression has been shown confirming the involvement of COX-2 in the regulation of expression of MDR-1.

In the light of the above scenario, the present study is undertaken to understand the role of COX-2 and MDR-1 in the development of resistance to imatinib in K562 cell line and to analyze the effects of celecoxib alone and in combination with imatinib in inducing apoptosis in imatinib-resistant cells.

Objectives

The specific objectives of the present study are:

- To develop and characterize Imatinib-resistant K562 (IR-K562) cells.
- To analyze the role of COX-2 and MDR-1 in the development of IR-K562 cells using Celecoxib, a COX-2 specific inhibitor.
- To elucidate the molecular mechanisms involved in celecoxib and imatinib-induced anti-proliferative effects individually and in combination in K562 and IR-K562 cells.

2.1 Materials

The human chronic myeloid leukemia cell line, K562, was obtained from National Centre for Cell Science (NCCS), Pune, India.

Phosphate buffered saline (PBS), RPMI 1640, Fetal Bovine Serum (FBS), Penicillin, Gentamycin and Streptomycin were purchased from GIBCO, Ltd. (BRL Life Technologies, Inc., Grand Island, NY). MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], Proteinase K, RNase A, Propidium iodide, Phenylmethylsulfonyl fluoride (PMSF), Leupeptin, Aprotinin, Pepstatin A, Trypsin, Tween-20, Triton X-100, Ponceau S, Igepal CA-630, Sodium orthovanadate, Sodium Bicarbonate, EDTA and Calcium chloride were purchased from Sigma Chemical Company (St.Louis, USA). Low fat milk powder was purchased from E-Merck. Nitrocellulose membranes and the enhanced chemiluminescence (ECL) kit were purchased from Amersham Life Science (Amersham, Bucks, UK). X-ray film and development solutions were from Kodak. Mouse monoclonal antibodies against cytochrome *c* and Bax were purchased from Santa Cruz, CA, USA. Polyclonal antibodies of Bcl-2 and PARP were purchased from R&D systems Inc, USA. FITC conjugated anti rabbit IgG was purchased from Molecular Probes Europe (Leiden, The Netherlands).

Acrylamide, N, N'-Methylene-bis-acrlylamide, Sodium Dodecyl Sulfate (SDS), Ammonium persulfate, β-Mercaptoethanol and Bromophenol blue were purchased from Bio-Rad Laboratories (Richmond, USA).

Imatinib was kindly provided by Natco Pharma Ltd., Hyderabad, India. It is the beta form of imatinib approved and marketed in India for the treatment of CML. Its potency was comparable to Imatinib (alpha form) from Novartis (the discoverer of Imatinib/Gleevec®/Glivec®). Celecoxib was a generous gift from Unichem Laboratories Ltd., Mumbai, India.

2.2 Cell culture and treatment

The human chronic myeloid leukemia K562 cells were grown in suspension in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. The cultured cells were passed twice each week, seeding at a density of about $2x10^5$ cells/ml. For treatment exponentially growing K562 cells were collected and resuspended in fresh culture medium. Stock solutions of Imatinib (10 mM) and Celecoxib (10 mM) in DMSO were prepared and stored at –20 °C. The final concentration of DMSO in all

the cultures was 0.1%. Cell viability was determined by the trypan blue dye exclusion method.

2.3 Development of Imatinib-resistant K562 (IR-K562) cells

K562 cells were treated with increasing concentrations of Imatinib incrementing at the concentration of 0.1 μM starting from 0.001 μM. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Live, resistant cells were selected by Ficoll Histopaq density gradient and were passed twice each week, seeding at a density of about $2x10^5$ cells/ml. At each concentration of imatinib increased, cells were grown for one week. Once the cells have attained 1 μM resistance to imatinib, they were maintained in the RPMI medium containing 1 μM imatinib. 24 h before the experiment, the cells were removed from imatinib-containing medium and maintained in imatinib-free medium.

2.4 Cell proliferation assay

Cell proliferation was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) staining as described by Mosmann (1983). The MTT assay is based on the reduction of the tetrazolium salt, MTT, by viable cells.

The dehydrogenases using NADH or NADPH as coenzyme can convert the yellow form of the MTT salt to insoluble, purple formazan crystals (Liu *et al.*, 1997). Formazan solution is read spectrophotometrically after the crystals are dissolved by organic solvent (DMSO). K562 and IR-K562 cells ($5x10^3$ cells/well) were incubated in 96-well plates in the presence or absence of imatinib (1, 10, 100 nM, 1, 10, 100 μM) and/or Celecoxib (1, 10, 100 μM) for 24, 48, 72 h in a final volume of 100 μl. At the end of the treatment, 20 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for an additional 4 hours at 37 °C. The purple-blue MTT formazan precipitate was dissolved in 100 μl of DMSO. The activity of the mitochondria, reflecting cellular growth and viability, was evaluated by measuring the optical density at 570 nm on a multi-well plate reader. Each concentration was tested in three different experiments run in four replicates. Means and standard deviations were calculated and reported as the percentage of growth vs control. The viable cells were counted by the trypan blue exclusion assay with a hemocytometer.

2.5 Morphological differentiation

K562 and IR-K562 cells were incubated with Imatinib (100 nM and 10 μM) or Celecoxib (40μM and 10μM) and in their combination. The appearance of

morphological differentiation was assessed after 24 h. The cells were viewed on a phase contrast inverted microscope and photographed with Nikon F-601 AF Camera.

2.6 Scanning Electron Microscopy

After treatment cells were collected, washed with PBS and concentrated to $1x10^5$ cells/ ml, one drop of such suspension was placed onto a plastic cover slip previously coated with 1% poly-L-lysine (Sigma, St.Louis, MO, USA) in water. The cover slip was then allowed to stand in a small Petri dish at room temperature for 15-30 min to facilitate the cells to adhere to the cover slip. Fixative (15% glutaraldehyde in 0.01 mol/L phosphate buffer, pH 7.4) was added to the Petri dish containing cover slip and the cells were fixed for 1 h at 40 °C. The cover slip was then taken through graded alcohols for dehydration and dried by the critical-point technique (EMS850 critical point drier Electron Microscopy Sciences). After trimming, mounting, and coating with gold-platinum (JFC 1600 JEOL Co auto fine sputter coater), the specimens were observed with SEM (JSM-5600, JEOL Co).

2.7 Transmission Electron microscopy

Electron microscopy was performed to confirm that the ultrastructural features of apoptosis were present in cells exposed to imatinib and celecoxib. In the present study, K562 cells treated with Imatinib 100 nM, Celecoxib 40 μM and combination of Imatinib (100 nM) and Celecoxib (40 μM) for 24 h were fixed in 2.5% glutaraldehyde (pH 7.3) buffered with 0.1 M sodium cacodylate overnight at 4 ^{o}C and then washed with 0.1 M sodium cacodylate buffer for 15 min before post- fixation with 1% osmium tetroxides buffered with 0.1 M sodium cacodylate for 1 h on ice. After another wash with 0.1 M sodium cacodylate buffer for 15 min, cells were dehydrated with increasing concentrations of alcohol (30%, 50%, 70%, 80%, 90%, and 100%; three times at each concentration) for 10 min each. Next, cells were infiltrated with propylene oxide for 15 min, followed by 1:1 propylene oxide: epoxy resin for 2 h, and finally 100% epoxy resin for 2 h. Cells were embedded with fresh epoxy resin into molds and placed in a 60 °C oven for 2 h. Semi thin sections were examined to confirm proper cross-sectional orientation before ultrathin sectioning. Ultrathin sections (Leica ultracut CUT) were stained with uranyl acetate and lead citrate and were examined under TEM (H7500 Hitachi Co).

2.8 DNA fragmentation assay

K562 and IR-K562 cells were treated with vehicle alone or Imatinib (100 nM and 10 μM respectively) or Celecoxib (40 μM and 10 μM) and combination of Imatinib (100 nM and 10 μM) and Celecoxib (40 μM and 10 μM) for 24 h. DNA laddering was detected by isolating fragmented DNA using the SDS/ Proteinase K/ RNase A extraction method, which allows the isolation of only fragmented DNA without contaminating genomic DNA (Hermann *et al.*, 1994). Five million cells were pelleted, washed in cold PBS and lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2 % Triton X-100 for 20 min at 4 °C. After centrifugation at 14,000 g for 15 min, the supernatant was treated with Proteinase K (0.5 mg/ml) and 1% SDS for 1 h at 50 °C. DNA was extracted twice with buffered phenol and precipitated with 140 mM NaCl and 2 volumes of ethanol at -20 °C overnight. DNA precipitates were washed in 70% ethanol, dissolved in TE, and treated for 1 h at 37 °C with RNase A. Fifteen microliters of DNA was mixed with 3 μl DNA sample buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol), and DNA was resolved in 1 % agarose gel in TBE (44.6 mM Tris, 44.5 mM boric acid and 1 mM EDTA) using 100 bp ladder as DNA standard. DNA fragmentation was visualized upon staining gel with ethidium bromide (0.5 mg/ml) and exposed to UV light. The

presence of apoptosis was indicated by the appearance of a ladder of oligonucleosomal DNA fragments that are approximately 180–200 bp multiples.

2.9 Quantification of apoptosis by flow cytometry

One of the major characteristics, which are used to assess apoptosis, is the state and content of nuclear DNA. The later is readily assessed by flow cytometric quantitation of red fluorescence from fixed propidium iodide-stained, RNase-treated cells. Apoptotic activity is heralded by sub-G0-G1 events on DNA histograms, as described by Nicoletti *et al.* (1991). The advantage of flow cytometry is that it permits measurements on a large number of cells within a short time period. Flow cytometry has been used to analyze numerous features of the apoptotic process (Gorczyca, 1999; Vermes *et al.*, 2000; Lecoeur et al., 2002). Apoptosis starts with cell shrinkage expressed by changes in light - scatter signals. The FSC-SSC pattern obtained by flow cytometry was used to detect the changes in size and granularity induced by the apoptotic process. The FS-SSC histogram was used to divide the cells into separate populations, differing in size and granulation. Gates were set on the populations and the staining of the cells in the gate, was measured as mean fluorescence intensity (MFI), peak position of fluorescence intensity or relative number of positive cells (%), depending on the agent.

To quantitate apoptosis, a flow cytometric analysis using propidium iodide was performed as described previously. Cells which were less intensively stained than G1 cells (sub- G1 cells) in flow cytometric histograms were considered apoptotic cells. DNA analysis of the aforesaid samples by flow cytometry was performed to evaluate the percentage of apoptotic cells. As is generally accepted, apoptotic cells can be recognized by their diminished stainability with DNA specific fluorochromes, which could be attributed to DNA degradation and its subsequent leakage from the cell. The method for DNA labeling was as described previously (Reddy et al., 2003) with minor modifications. Briefly, K562 and IR-K562 cells were treated with Imatinib (100 nM and 10 µM respectively) or Celecoxib (40 µM and 10 µM respectively) and combination of Imatinib and Celecoxib for 24 h. After treatment, cells were prepared as single cell suspension in 200 µl PBS, fixed with 2 ml of ice-cold 70 % ethanol, and maintained at 4 °C overnight. The cells were harvested by centrifugation at 500x g for 10 min, resuspended in 500 µl PBS supplemented with 0.1 % Triton X-100 and RNase A (100 µg/ml), incubated at 37 °C for 30 min, and stained with 50 µg/ml propidium iodide (PI) in the dark at 4 °C for 30 min. The red fluorescence of individual cells was measured with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). A minimum of 10,000 events was collected

per sample. The relative DNA content per cell was obtained by measuring the fluorescence of PI that bound stoichiometrically to DNA.

2.10 Measurement of ROS

ROS production in control and treated K562 and IR-K562 cells was measured using the dye 2, 7-dichloro dihydro fluorescein diacetate (DCFH-DA). DCFHDA, a non-fluorescent cell-permeable compound becomes the fluorescent compound, 2, 7-dichlorofluorescein (DCF), upon oxidation by ROS. Cells seeded at a density of 2 x 10^6 in 60 mm culture dishes were first pre-incubated with imatinib (100 nM or 10 µM), celecoxib (40 µM or 10 µM) or both imatinib and celecoxib for 2 h. Cells were harvested after 10 min incubation with DCFH-DA (10 µM) and washed with PBS. ROS measurement was carried out on FACS Calibur flow cytometer. Data were collected using the data acquisition program CELL Quest (BectonDickinson, San Jose, CA). DCF was measured with the following excitation and emission wavelengths: lexc = 488 nm, lem = 525 nm. 10,000 cells were analyzed per sample.

2.11 Flow cytometric analysis of mitochondrial membrane potential

K562 and IR-K562 cells were treated with imatinib (100 nM or 10 µM), celecoxib (40 µM or 10 µM) or both imatinib and celecoxib for 24 h. The cells were harvested and changes in the mitochondrial membrane potentials were measured by the uptake of cation Rhodamine 123 into mitochondria (Seuduto and Grotyohann, 1999). Untreated control cells were used to determine the normal uptake of this cation, and the percentage of treated cells with low membrane potentials were then calculated. Briefly, the cells were centrifuged at 800x g for 10 min and resuspended in 1 ml of Rhodamine 123 (10 µg/ml) for 30 min at room temperature, and washed once in PBS. The cells were resuspended in PBS. The samples (10000 events) were analyzed for fluorescence (FL-1 detector, filter 430/30 nm band pass) using a FACscan (Becton Dickson, San Jose, CA).

2.12 Preparation of whole cell extracts and immunoblot analysis

The protocol was based on Sambrook et al. (1989). To prepare the whole cell extract, cells were washed with PBS and suspended in a lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1%NP 40, 0.5% deoxy cholic acid, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml leupeptin,

20 μg/ml aprotinin). After 30 min of shaking at 4 °C, the mixtures were centrifuged (10,000x g) for 10 min, and the supernatants were collected as the whole-cell extracts. The protein content was determined according to the Bradford method (Bradford, 1976). An equal amount of total cell lysate was resolved on 8-12 % SDS-PAGE gels along with protein molecular weight standards, and then transferred onto Nitrocellulose membranes. The membranes were blocked with 5% w/v nonfat dry milk and then incubated with the primary antibodies (COX-2, Cytochrome *c*, PARP, Bcl-2, Bax, Akt, pAkt, pTyr, MDR1, cytochrome P450, PKC, NF-κB (RelA)) in 10 ml of antibody-diluted buffer (1X Tris-buffered Saline and 0.05% Tween 20 with 5% milk) with gentle shaking at 4 °C for 8-12 h and then incubated with peroxidase conjugated secondary antibodies. Signals were detected using an ECL Western blotting detection kit.

2.13 Detection of cytochrome c release

Release of cytochrome *c* from mitochondria to cytosol was measured by Western blot as previously described (Chandra et al., 1998) with some modifications. Cells were washed twice with ice cold PBS and sonicated (3 x 5 sec on ice) in buffer containing 20 mM HEPES (pH 7.2), 10 mM KCl, 1.5 mM EDTA, 1 mM EGTA, 250

mM sucrose and protease inhibitors. The homogenates were centrifuged at 750x g for 5 min, and the supernatant was then centrifuged at 10,000X g for another 5 min. The supernatant was subjected to further ultracentrifugation at 100,000x g for 60 min. The resulting supernatant represented the cytosolic fraction. Following the quantification, proteins were separated on polyacrylamide gel, transferred onto a nitrocellulose membrane and probed with antibody against cytochrome *c* followed by incubation with a secondary antibody conjugated with horseradish peroxidase. Detection was performed using the ECL kit.

2.14 Detection of NF-κB translocation by Western blot analysis

K562 and IR-K562 cells at a density of 5 x 10^6 were seeded in 90 mm culture dishes. Cells were incubated with Imatinib (100 nM and 10 μM) or Celecoxib (40 μM and 10 μM) and combination of Imatinib and Celecoxib for 24 h. Cells were harvested and then used for nuclear protein extraction. The cells were washed with PBS and 200 μl of ice cold lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 1% NP-40, 1 mM PMSF) was added, and incubated for 5 min on ice with 3-4 vortexings of 10 sec each. The nuclei were then harvested by centrifugation at 16,000 rpm for 1 min. The nuclear pellet was resuspended in 40 μl of nuclear protein extraction buffer (420 mM NaCl, 10 mM HEPES, 10 mM MgCl2, 1 mM EDTA, 0.1

mM DTT and 25% glycerol) and incubated on ice for 30 min with intermittent vortexing of 10 sec each. The sample was then centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was collected and nuclear protein was estimated by Bradford method (Bradford, 1976). NF-κB translocation into nucleus was studied by Western blot analysis using Rel A (p65) antibody.

2.15 RT-PCR analysis

RT-PCR analysis was carried out for BCR-ABL, COX-2 and MDR-1 as described by Weisberg and Griffin, 2000, Hanif *et al*., 1996 and Marroni *et al*., 2003 respectively. Total RNA was isolated from K562 and IR-K562 cells treated with Imatinib (100 nM and 10 µM respectively), Celecoxib (40 µM and 10 µM respectively) and in combination of Imatinib and Celecoxib using TRIzol reagent. Reverse transcription of 1 µg of total RNA isolated was achieved by mixing the RNA with 10 µl of 2x PCR master mix, 1 µl of deoxynucleotides, 1 µl of oligo dT, 0.25 µl RNAase inhibitor (10 U/µl), and 0.5 µl of Reverse Transcriptase (200 U/µl) in a 20 µl volume. This was followed by incubation of the mixture at 42 °C for 60 min, and then for 5 min at 95 °C.

Table 1: Primer sequences and conditions used for the RT-PCR analysis

Primers (5' → 3')	A (°C)	D (°C)	E (°C)	No. of Cycles	Size (bp)
B2A2					
FP:CATTCCGCTGACCATCAATA	60	94	72	30	158
RP:GGCTTCACTCAGACCCTGAGG					
COX-2					
FP:TCAAATGAGATTGTGGGAAAATTGGT	54	94	72	35	303
RP: AGATCATCTTTGTCTGAGTATTTT					
MDR-1					
FP: TGATTACCATCAGGCTCGCCAA					
RP: TAGCGATTTTCTTAGTACTTT	54	94	72	30	305
ABL Kinase					
FP: GCGCAACAAGCCCACTGTCTATGG					
RP: GGACTGCACCCGAGAGCCTGGC	68	94	72	30	579
GAPDH					
FP: GAAGGTGAAGGTCGGAGTC					
RP: GAAGATGGTGATGGGATTC	54	94	72	30	352

A = Annealing Temperature, D = Denaturation Temperature, E = Extension Temperature

Four microliters of the RT product was taken and PCR was carried out in PTC 200 thermal cycler (MJ Research**)**. The primer sequences and the annealing temperatures used are given in Table 1.The PCR product was visualized on 1 % agarose gels under UV light. The GAPDH primers served as control.

2.16 PCR-based RFLP

The PCR amplicon generated by ABL specific kinase primers were digested with the restriction enzymes – PstI and SmaI (from MWG Biotech, India) as follows: The digestion reaction mixture consisted of 10 µl of amplicon, 2.5 µl of appropriate 10 X reaction buffer, 1 µl of restriction enzyme and 11.5 µl of sterile distilled water. The mixture was incubated at 37 °C (for PstI) or 30 °C (for SmaI) for 2h. 10 µl of the restricted samples were mixed with 6 X gel loading dye and were loaded on 2 % ethidium bromide (0.5 µg/ml) stained Agarose gel and electrophoresed at constant voltage (30 V) for 1.5 h in tris-borate-EDTA buffer. The bands were visualized & documented under UV light in a gel documentation system (BioRad, USA).

2.17 SSCP analysis

The amplicons generated using ABL specific primers were mixed with equal volumes of 2X gel loading dye (95% formamide, 10 mM NaOH, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). The samples were denatured at 94 °C for 10 min and immediately kept on ice till loading on the gel. A composite gel consisting of 1% agarose and 6% polyacrylamide was prepared by mixing the ingredients as follows (Orita et al., 1989):

Total volume – 50 ml

1. 29:1 Acrylamide:bis acrylamide solution – 10 ml
2. Glycerol – 8 ml
3. 10 X TBE – 5 ml
4. 10% ammonium per sulphate – 175 µl
5. 2% agarose solution (maintained at ~ 60-65 °C) – 25 ml
6. TEMED – 12 µl
7. Distilled water to make up volume to 50 ml.

After preparation of the solution, it was immediately poured into the gel plates and allowed for polymerization for about an hour. The samples were loaded on the gel and electrophoresed at 50 V for 16-18 h at 4 °C. the gel was then subjected to silver staining as per the procedure described by Peng et al., (1995).

Silver staining

The gel was fixed in a solution containing 12 % acetic acid, 50% methanol and 0.02% formaldehyde for about 2 h under gentle agitation. The gel was then washed in 50% ethanol for 20 min twice. Subsequently, the gel was pretreated with freshly prepared 0.02% sodium thiosulphate for 1 min, rinsed in distilled water thrice and impregnated with 0.2% silver nitrate and 0.03% formaldehyde

for 20-30 min and rinsed in distilled water thrice. The amplicons were then visualized in a solution containing 6% sodium carbonate and 0.02% formaldehyde for 3-5 min and transferring the gel into a solution containing 50% methanol and 16% acetic acid to stop the reaction. The gel was then dried and documented.

2.18 Gel extraction of the PCR products

The PCR products amplified using ABL specific primers were resolved in 1% agarose-TBE gel and the amplified bands were cut and extracted using Gel purification kit (Qiagen, Germany) as per manufacturer's instructions.

2.19 Sequencing of ABL kinase domain

The gel extracted PCR amplicons generated using ABL specific primers were subjected to sequencing directly in both the directions from MWG Biotech, India. The sequences obtained were then compared with the already existing sequence for any point mutations in database using "BLASTn" available at the website http://www.ncbi.nlm.nih.gov/BLAST.

2.20 PGE_2 estimation

K562 and IR-K562 cells at a density of 5 x 10^6 were seeded in 90 mm culture dishes. They were incubated with Imatinib (100 nM and 10 μM) or Celecoxib (40 μM and 10 μM) and combination of Imatinib (100 nM and 10 μM) and Celecoxib (40 μM and 10 μM) for 24 h. At the end of the treatment period, culture medium was collected to determine the amount of PGE_2 secreted by these cells and stored at –80 °C. The quantitative analysis of PGE_2 released into the medium was assessed using PGE_2 immunoassay kit as per manufacturer's instructions (Cayman, USA).

2.21 Effect of PGE_2 on the sensitivity of K562 cells to imatinib

To determine the effect of PGE_2 on the sensitivity of K562 cells towards imatinib, cells were grown in the presence of PGE_2 (3 and 6 μg/ml) for 3 days continuously and then MTT assay was carried out with different concentrations (100 nM, 1 μM, 10 μM) of imatinib as described above.

2.22 *COX-2 gene knockdown using siRNA*

siRNA synthesis

Double strand siRNA oligos were synthesized as described by Donzé & Picard (2002). Briefly, desalted DNA oligonucleotides were ordered from Sigma (India). For each transcription reaction, 1 nM of each oligonucleotide was annealed in 50 µl of TE buffer (10 mM Tris–HCl pH 8.0, and 1 mM EDTA) by heating at 95 ℃; after 2 min, the heating block was switched off and allowed to cool down slowly to obtain dsDNA. Transcription was performed in 50 µl of transcription mix: 1X T7 transcription buffer (40 mM Tris–HCl pH 7.9, 6 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl and 2 mM spermidine) 1 mM NTPs, 0.1 U yeast pyrophosphatase (Sigma), 40 U RnaseOUT (Life Technologies) and 100 U T7 RNA polymerase (Invitrogen) containing 200 pM of the dsDNA as template. After incubation at 37 ℃ for 2 h, 1 U RNase-free DNase (Genetix) was added at 37 ℃ for 15 min. Sense and antisense 21-nt RNAs generated in separate reactions were annealed by mixing both crude transcription reactions, heating at 95 ℃ for 5 min followed by 1 h at 37 ℃ to obtain T7 RNA polymerase synthesized small interfering double-stranded RNA (T7 siRNA).

The mixture (100 µl) was then adjusted to 0.2 M sodium acetate pH 5.2, and precipitated with 2.5 volumes ethanol. After centrifugation, the pellet was washed once with 70% ethanol, dried, and resuspended in 50 µl of water.

siRNA transfection and cell proliferation assay

IR-K562 cells ($1x10^6$) were transfected with COX-2 siRNA by using Lipofectamine-2000 (Invitrogen). The analysis was performed after 48 h. After confirming the transfection efficiency by RT-PCR analysis using COX-2 specific primers, cell proliferation assay was carried out with imatinib (100nM, 1 µM, 10 µM).

2.23 Statistical analysis

The results were expressed as mean ± SEM of data obtained from three independent experiments. Statistical analysis of differences was carried out by analysis of variance (ANOVA). The level of significance was set at $P< 0.05$.

3.1 100-fold resistance to imatinib was developed

Imatinib-resistant K562 (IR-K562) cells were developed by growing K562 cells in medium containing increasing concentrations of imatinib. IR-K562 cells were treated with imatinib (0.1 µM – 100 µM) for 24, 48 and 72 h and the IC_{50} of imatinib was determined by MTT assay. IR-K562 cells showed a dose dependent decrease

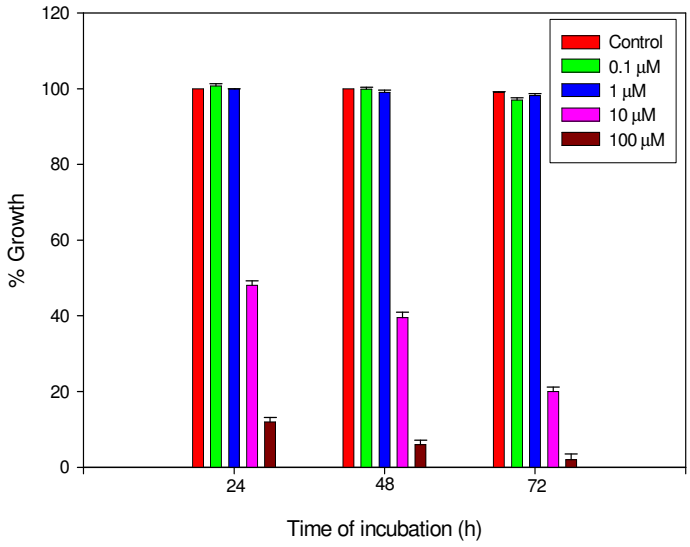


Fig. 11: Effect of Imatinib on the cell proliferation of IR-K562 cells (MTT Assay).

IR-K562 cells were treated with 0.1 µM, 1 µM, 10 µM and 100 µM of imatinib for 24, 48 and 72 h. The percent viable cells were calculated in comparison to untreated cells. The number of cells in the control was taken as 100%.

in proliferation above 1 μM concentration of imatinib with no effect at 0.1 μM and 1 μM concentrations suggesting the acquirement of resistance. 50% inhibition in the cell proliferation was observed at 10 μM concentration of imatinib after 24 h incubation (Fig. 11). Fold resistance developed to imatinib was found to be 100.

3.2 BCR-ABL independent development of resistance to imatinib in IR-K562 cells

Western blot analysis and RT-PCR analysis for BCR-ABL showed no difference in the expression pattern of BCR-ABL in K562 and IR-K562 cells at protein or mRNA level (Fig. 12) thus eliminating the over expression of BCR-ABL as a mechanism of development of resistance to imatinib.

3.3 Screening for ABL kinase domain mutations

Imatinib resistance, in general, is mainly caused due to the point mutations in the ABL kinase domain, which inhibits binding of imatinib to the domain. To check whether this mechanism is involved in the imatinib resistance of K562 cells, three methods have been employed – PCR-based RFLP, SSCP and direct sequencing

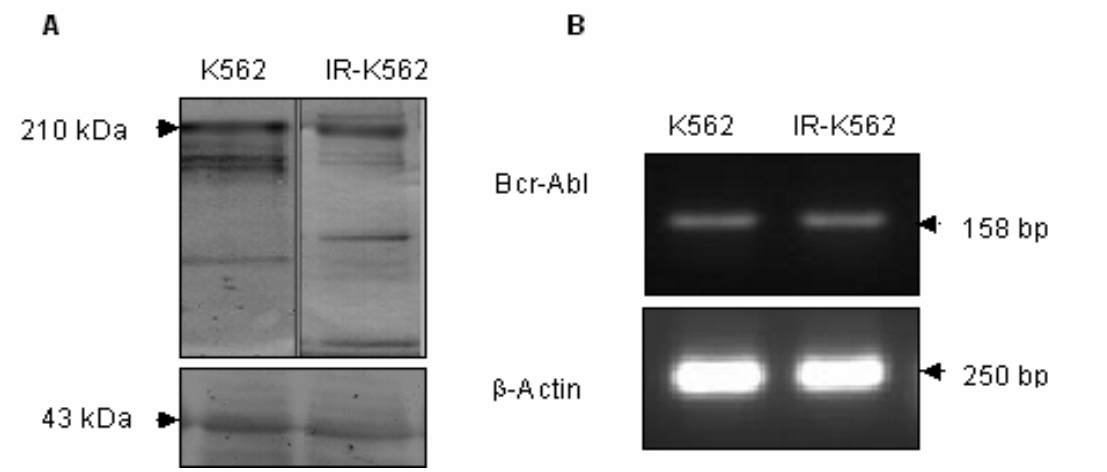


Fig. 12: BCR-ABL kinase activity and expression in K562 and IR-K562 cells.

A. Western blot analysis of BCR-ABL protein expression in K562 and IR-K562 cells.

B. RT-PCR analysis of BCR-ABL mRNA expression. β-Actin was used as control in both the analyses.

of the kinase domain. All the three methods showed no mutation in the kinase domain (Fig. 13-15).

3.3.1 PCR Based RFLP

The PCR amplicons from ABL kinase specific primers were subjected to PCR based RFLP with two different restriction enzymes as mentioned in materials and methods. The results did not show any difference in the movement of the restricted

fragments suggestion that there might be no mutation in the sequence sites which these enzymes recognize and cut (Fig. 13).

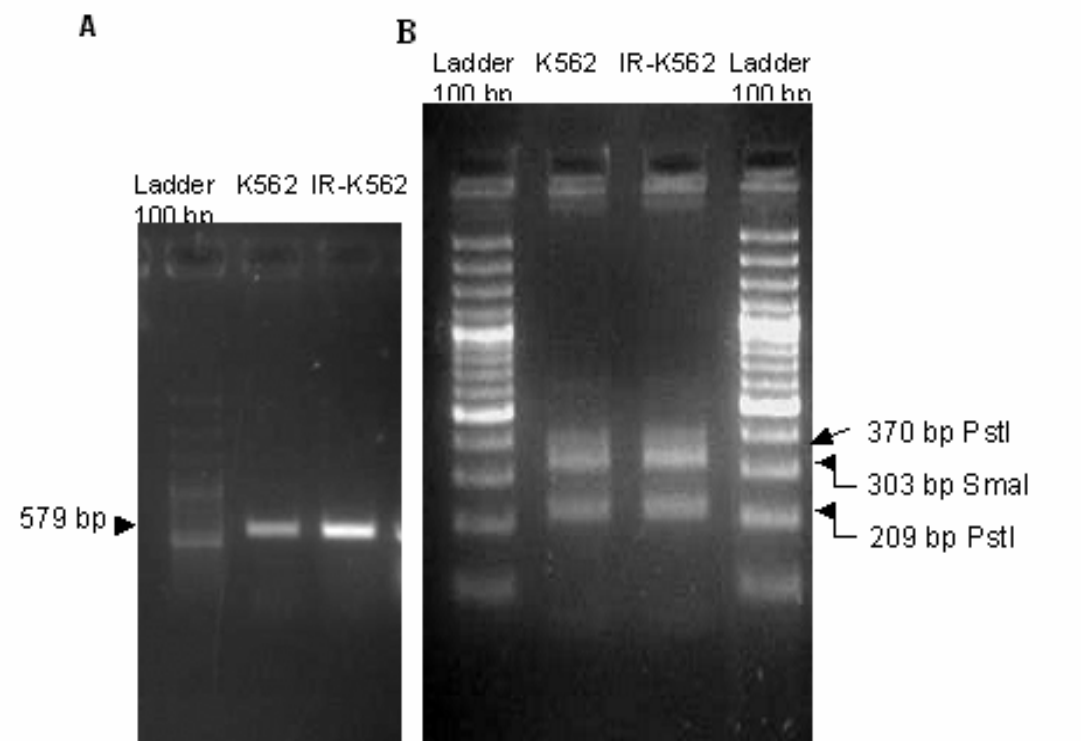


Fig. 13: PCR based RFLP.

A. *The PCR amplicons generated from ABL kinase specific primers (579 bp).*

B. Restriction digested PCR amplicons generated from ABL kinase specific primers using PstI and SmaI enzymes and the digested samples were loaded on 2% ethidium bromide-agarose gels and visualized and photographed.

3.3.2 SSCP analysis

The PCR amplicons generated using ABL kinase specific primers were subjected to SCCP analysis through a silver stained composite gel electrophoretic

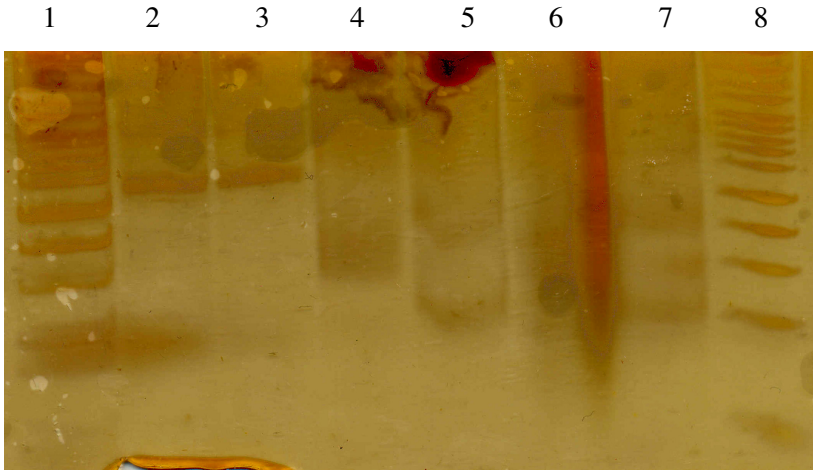
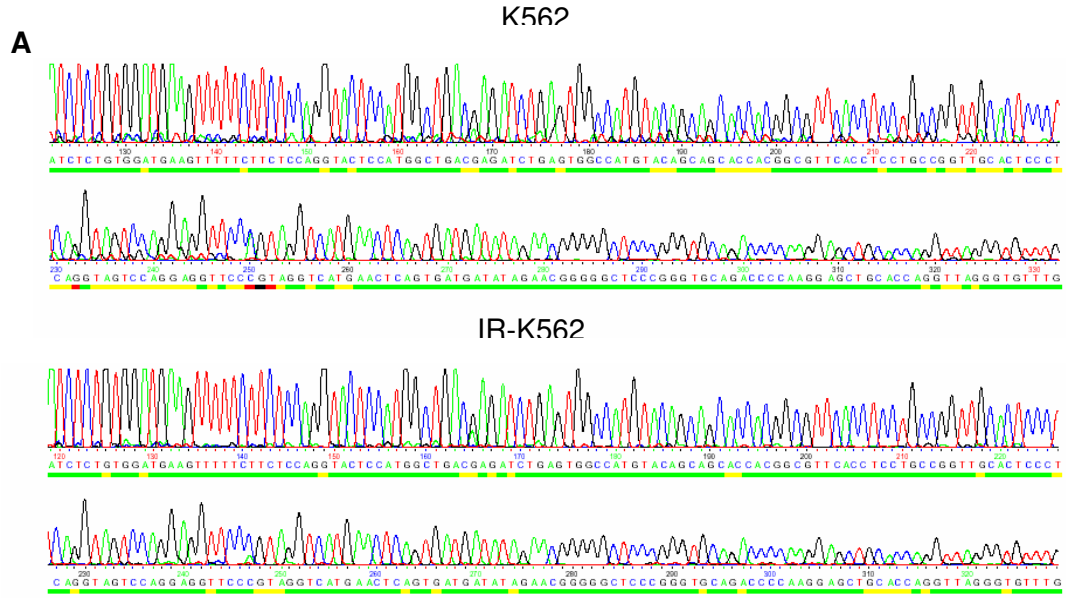


Fig. 14: SSCP analysis.

The PCR amplicons of the ABL kinase domain from K562 and IR-K562 cells were subjected to SSCP analysis (Lanes 2 & 3). The restricted digested products of the PCR amplicons using PstI and SmaI were also subjected to SSCP analysis. Lanes 4 & 5 represent ABL kinase amplicon from K562 cells digested with PstI ans SmaI respectively. Lanes 6 & 7 represent ABL kinase amplicon from IR-K562 cells digested with PstI and SmaI respectively. Lanes 1 & 8 are 100 bp marker.

analysis. It was observed that both K562 and IR-K562 cells showed a single band at 579 bp and even the restriction digested samples also showed same pattern in both the cell types (Fig. 14).

A



B

GGACACCATGGAGGTGGAAGAGTTCTTGAAAGAAGCTGCAGTCATGAAAGAGATCAAA
GGACACCATGGAGGTGGAAGAGTTCTTGAAAGAAGCTGCAGTCATGAAAGAGATCAAA
CACCCTAACCTGGTGCAGCTCCTTGGGGTCTGCACCCGGGAGCCCCCGTTCTATATCA
CACCCTAACCTGGTGCAGCTCCTTGGGGTCTGCACCCGGGAGCCCCCGTTCTATATCA
TCACTGAGTTCATGACCTACGGGAACCTCCTGGACTACCTGAGGGAGTGCAACCGGCA
TCACTGAGTTCATGACCTACGGGAACCTCCTGGACTACCTGAGGGAGTGCAACCGGCA
GGAGGTGAACGCCGTGGTGCTGCTGTACATGGCCACTCAGATCTCGTCAGCCATGGAG
GGAGGTGAACGCCGTGGTGCTGCTGTACATGGCCACTCAGATCTCGTCAGCCATGGAG
TACCTGGAGAAGAAAAACTTCATCCACAGAGATCTTGCTGCCCGAAACTGCCTGGTAGG
TACCTGGAGAAGAAAAACTTCATCCACAGAGATCTTGCTGCCCGAAACTGCCTGGTAGG
GGAGAACCACTTGGTGAAGGTAGCTGATTTTGGCCTGAGCA
GGAGAACCACTTGGTGAAGGTAGCTGATTTTGGCCTGAGCA

Fig. 15: Sequence analysis of the ABL kinase domain amplified using kinase specific primers.

A.The ABL kinase domain from K562 and IR-K562 cells was amplified using specific primers through RT-PCR and the PCR amplicons were directly sequenced. A. Electropherogram showing the peaks after direct sequencing.

B. Comparison of the sequences obtained from K562 (red) and IR-K562 (blue) cells.

3.4 MDR1 over expression in IR-K562 cells

MDR1 is involved in drug resistance in many cancers and to elucidate its role in development of imatinib resistance in K562 cells, Western blot and RT-PCR

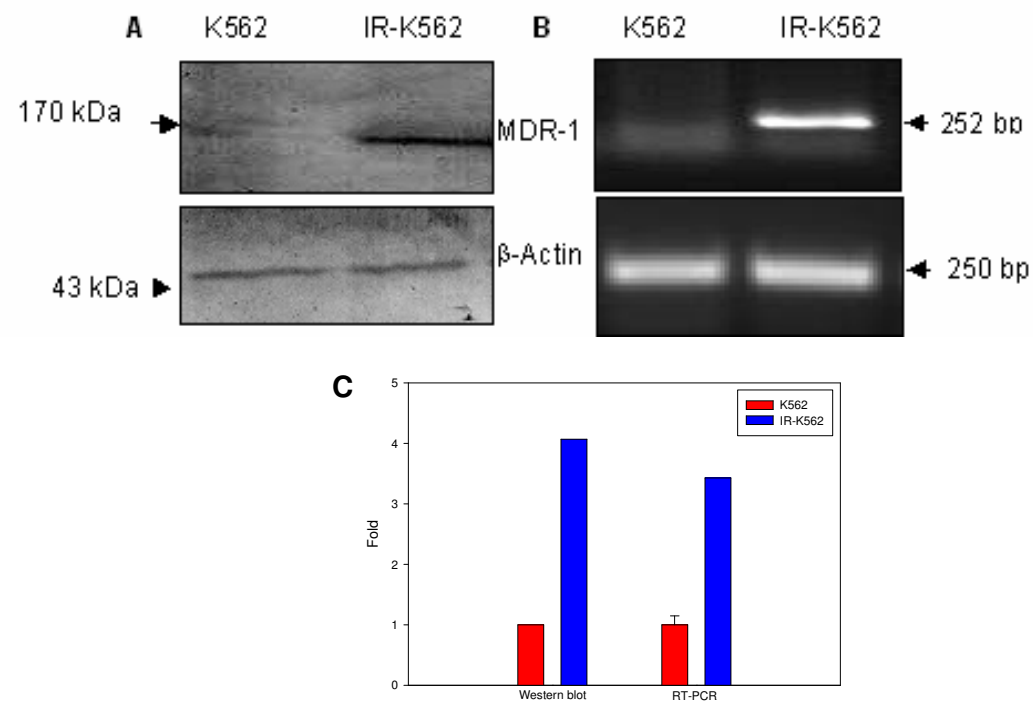


Fig. 16: Expression of MDR1 in K562 and IR-K562 cells.

A. Western blot analysis of MDR1 protein expression in K562 and IR-K562 cells.
B. RT-PCR analysis of MDR1 mRNA expression. β-Actin was used as control in both the analyses.
C. Bar graph showing the fold increase in MDR1 expression in both Western blot and RT-PCR analysis.

analyses were carried out. These studies reveal that MDR1 is over expressed in IR-K562 cells both at mRNA and protein level. MDR1 expression is undetectable in K562 cells (Fig. 16).

3.5 Generation of ROS in IR-K562 cells

To determine the mechanism involved in the development of resistance to imatinib, ROS levels were measured. Compared to K562 cells there is an increase in the generation of ROS in IR-K562 cells (Fig. 17).

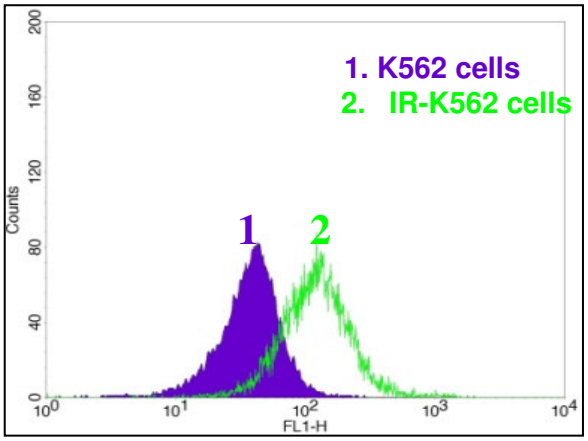


Fig. 17: Measurement of ROS by FACS analysis in K562 and IR-K562 cells.

3.6 Activation of NF-κB

To determine the downstream signaling pathways activated by ROS, translocation of NF-κB into nucleus was studied by Western blot analysis using p60

(Rel A component of NF-κB) antibodies. The result clearly demonstrated an increased NF-κB translocation (2.2 fold increase) from cytosol to nucleus in IR-K562 cells (Fig 18). This result suggests that ROS is generated in IR-K562 cells, which results in the activation of NF-κB and thus gene transcription leading to resistance.

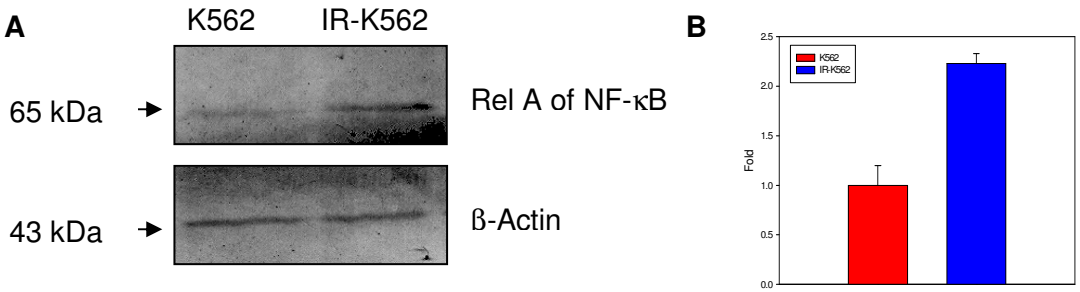


Fig. 18: Western blot analysis of NF-κB.

A. Western blot analysis of NF-κB Rel A translocation into nucleus in K562 and IR-K562 cells. B. Bar graph showing fold increase.

3.7 Activation of Cytochrome P450

To check whether the development of resistance to imatinib is due to the xenobiotic cytochrome P450 pathway, the key isozyme known for the drug detoxification, Western blot analysis was carried. These studies showed an increased expression of Cytochrome P450, 1A1 in IR-K562 cells (Fig. 19).

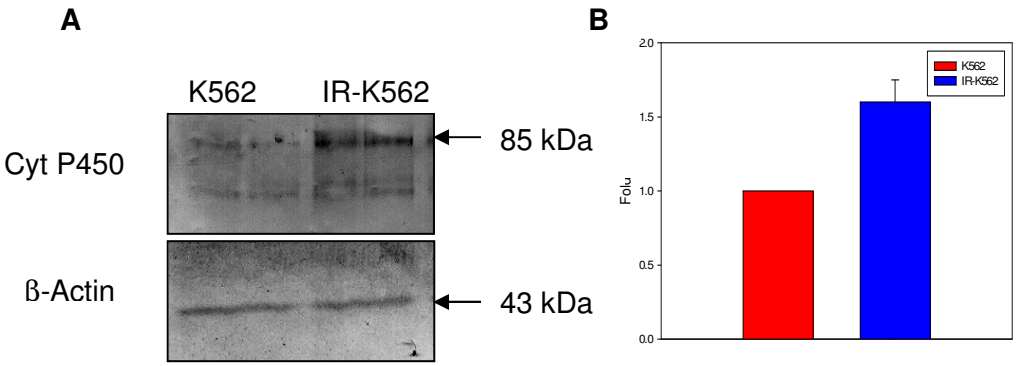


Fig. 19: Western blot analysis of Cytochrome P450, 1A1 isozyme.

A. Western blot analysis of Cytochrome P450 1A1 expression in K562 and IR-K562 cells.
B. Bar graph showing fold increase.

3.8 PGE_2 release and COX-2 over expression

The release of PGE_2 into the culture medium was measured by employing ELISA kit as described in the materials and methods. There is a significant increase in the PGE_2 levels in IR-K562 cells compared to K562 cells (Fig. 20) and so the expression of COX-2 was studied by Western blot and RT-PCR analysis. The results showed an overexpression of COX-2 in IR-K562 cells with undetectable COX-2 in K562 cells (Fig. 20).

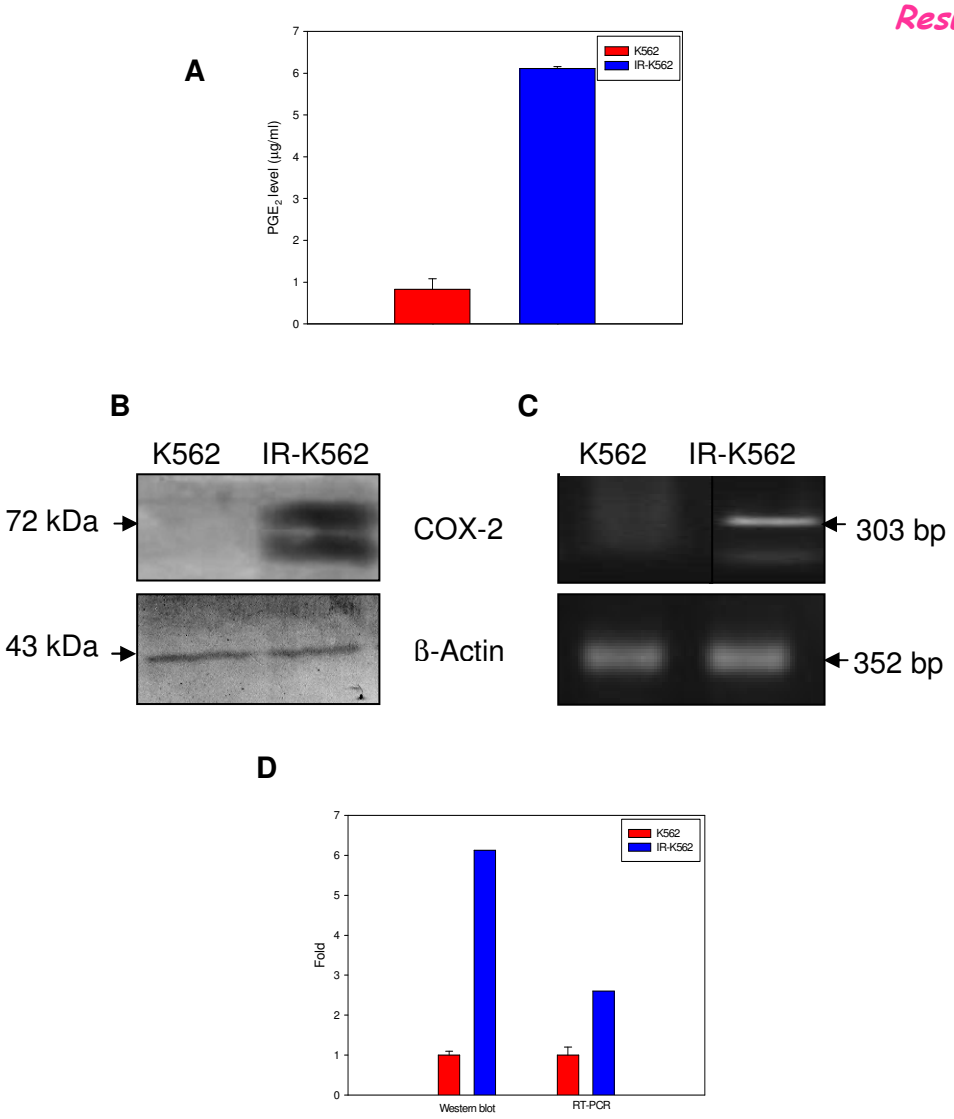


Fig. 20: PGE_2 release and expression of COX-2.

A. PGE_2 levels.
B. Western blot analysis of COX-2 expression in K562 and IR-K562 cells.
C. RT-PCR analysis of COX-2.
D. Bar graph showing fold increase in both Western blot and RT-PCR analysis.

3.9 *COX-2* gene knockdown using siRNA and cell proliferation assay

To confirm that COX-2 over expression is responsible for the imatinib resistance in K562 cells, COX-2 gene was knocked down using siRNA and then sensitivity of these cells to imatinib was checked by MTT assay. Approximately 80% of IR-K562 cells showed COX-2 gene knocked down as demonstrated by RT-PCR analysis (Fig. 21A).

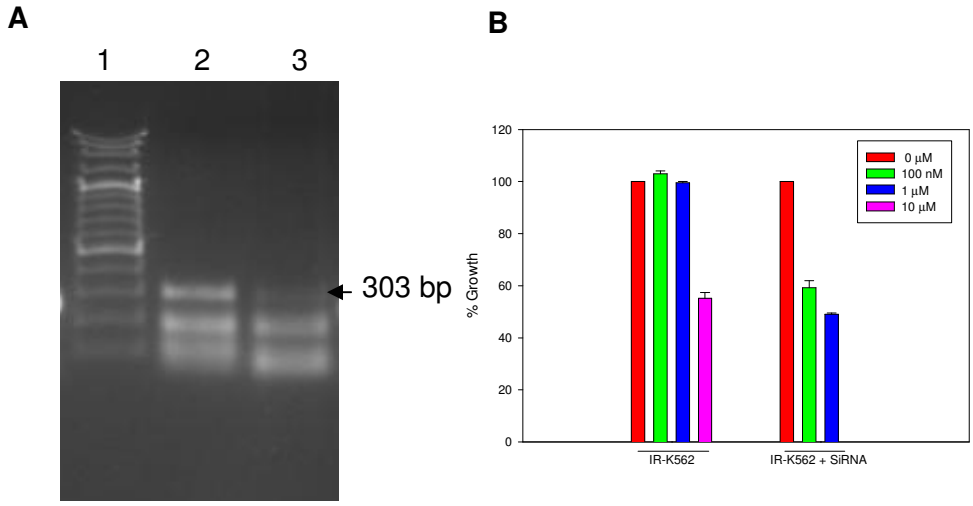


Fig. 21: COX-2 gene knockdown using siRNA.

A. Agarose gel showing COX-2 expression after RT-PCR analysis in IR-K562 cells (Lane 2) and IR-K562 transfected with siRNA (Lane 3); Lane1 is 100 bp ladder.
B. Graph showing effect of Imatinib on the cell proliferation of IR-K562 cells and IR-K562 cells transfected with COX-2 siRNA (MTT Assay).

Cell proliferation assay showed 40% inhibition in the growth of IR-K562 cells transfected with siRNA at 100 nM concentration of imatinib (IC_{50} of imatinib in K562 cells) (Fig. 21B) indicating that COX-2 overexpression is responsible for the development of resistance to imatinib in K562 cells.

3.10 Effects of Imatinib and Celecoxib on the growth of K562 and IR-K562 cells

Cells were cultured in 10% FBS containing medium with or without Imatinib (1 nM-10 μM) and/or Celecoxib (10, 20, 40, 80 μM) for 24, 48, 72 h and cell proliferation was evaluated by the MTT assay. Under these experimental conditions a dose dependent decrease in K562 cell proliferation was observed until 72 h after Imatinib (Fig.22A) and Celecoxib (Fig.22B) treatment with the IC_{50} value being at 100 nM and 40 μM (at 24 h) where the percent inhibition was 49% and 51% respectively. Since the 50% inhibition was observed in cells exposed to 100 nM imatinib and 40 μM celecoxib for 24 h, further experiments were carried under these conditions in K562 cells.

The effect of Celecoxib and imatinib on the cell proliferation of imatinib-resistant K562 cells (IR-K562) was also determined by MTT assay. For this, 24 h before the assay, IR-K562 cells were maintained in imatinib-free RPMI medium.

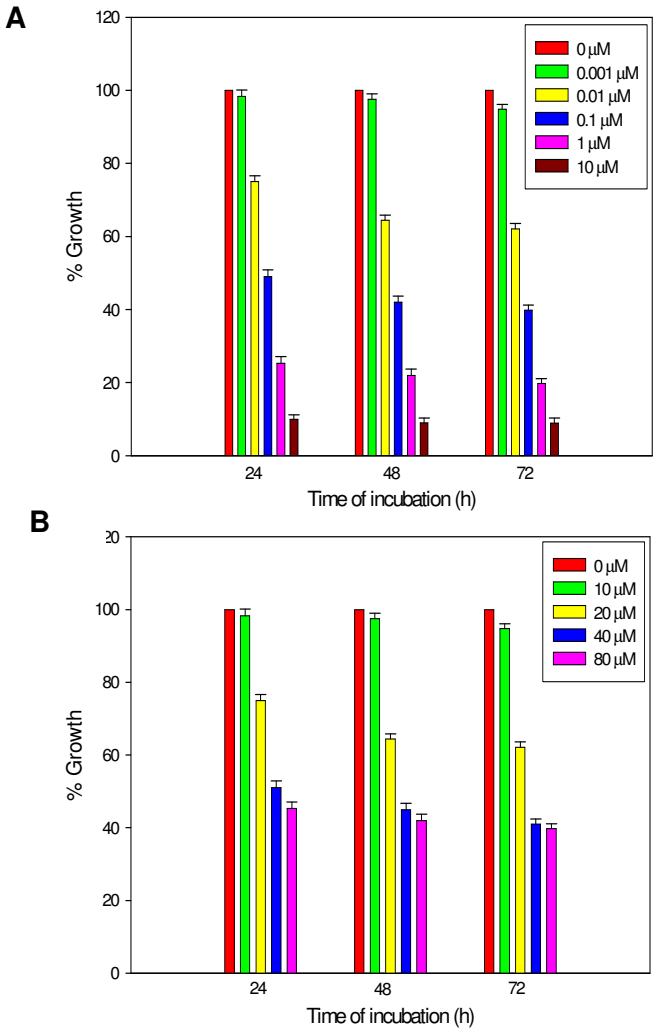


Fig. 22: Effect of Imatinib and Celecoxib on the cell proliferation of K562 cells.

A. K562 cells were treated with 0.001 µM, 0.01 µM, 0.1 µM, 1 µM and 10 µM of imatinib for 24, 48 and 72 h. The percent viable cells were calculated in comparison to untreated cells. The number of cells in the control was taken as 100%.

B. K562 cells were treated with 10 µM, 20 µM, 40 µM and 80 µM of celecoxib for 24, 48 and 72 h. The percent viable cells were calculated in comparison to untreated cells. The number of cells in the control was taken as 100%.

Cells were cultured in 10% FBS containing medium with or without imatinib (1-100 µM) orcelecoxib (1-100 µM) for 24, 48, 72 h and cell proliferation was evaluated. Celecoxib significantly reduced the cell proliferation of IR-K562 with an IC_{50} much

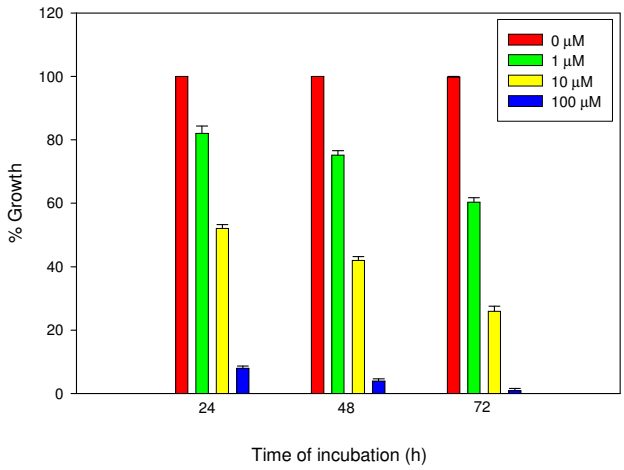


Fig. 23: Effect of Celecoxib on the cell proliferation of IR-K562 cells.

R-K562 cells were treated with 1 µM, 10 µM and 100 µM of celecoxib for 24, 48 and 72 h. The percent viable cells were calculated in comparison to untreated cells. The number of cells in the control was taken as 100%.

lower than that observed for K562 cells (Fig. 23). The IC_{50} value of imatinib and celecoxib for IR-K562 cells is 10 µM for both compared to the IC_{50} values of 100 nM and 40 µM respectively for K562 cells. While the IC_{50} value of imatinib increased by 100 fold (from 100 nM to 10 µM), the IC_{50} value of celecoxib decreased by four folds (from 40 µM to 10 µM) (Table 2).

3.11 Imatinib and celecoxib synergistically inhibited the proliferation of K562 and IR-K562 cells

K562 cells were treated with imatinib (10 nM, 100 nM and 1 µM) and IR-K562 were cells treated with Imatinib (1µM, 10 µM and 100 µM), with or with out celecoxib (1µM, 10 µM and 100 µM) for 24 h and cell proliferation was determined by MTT assay. In the presence of 1 µM celecoxib, the percent inhibition in the growth of both K562 (Fig. 24 A) and IR-K562 (Fig. 24 B) cells was much higher than in the cells grown in its absence at all the concentrations of imatinib studied. However, the percent inhibition in the growth of K562 cells exposed to imatinib in the presence of celecoxib was much lower when compared to that of IR-K562 cells. As a result the IC_{50} of imatinib for K562 cells was 100 nM in the absence of celecoxib and 80 nM in the presence of 1 µM celecoxib. The IC_{50} of imatinib for IR-K562 cells was reduced drastically from 10 µM in the absence of celecoxib to 6 µM in the presence of 1 µM celecoxib (Table 2).

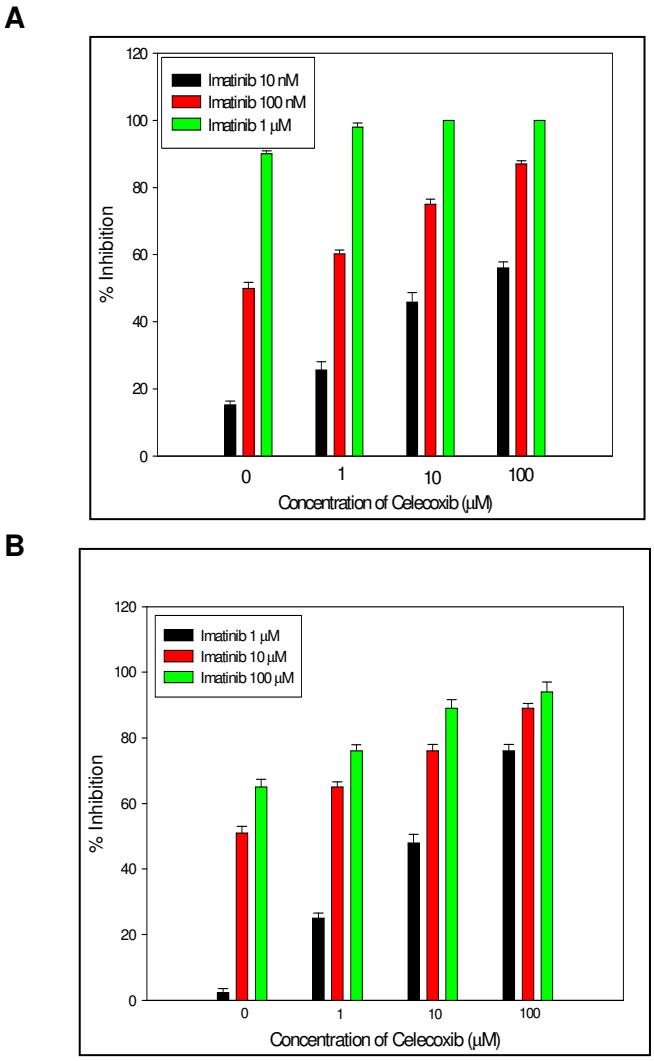


Fig. 24: Synergistic effects of Imatinib and Celecoxib on the cell proliferation of K562 and IR-K562 cells.

K562 cells were treated with imatinib (10 nM, 100 nM and 1 µM) (A) and IR-K562 cells were treated with Imatinib (1µM, 10 µM and 100 µM) (B), with or with out celecoxib (1µM, 10 µM and 100 µM) for 24 h. The percent viable cells were calculated in comparison to untreated cells.

Table 2: IC_{50} values of imatinib and celecoxib in K562 and IR-K562 cells

Drug	K562 cells (IC_{50} μM)	IR-K562 cells (IC_{50} μM)
Imatinib	0.1	10
Celecoxib	40	10
Imatinib + 1 μM Celecoxib	0.08 (Imatinib)	6 (Imatinib)

3.12 Morphological and ultrastructural changes

3.12.1 Phase contrast microscopy

Phase contrast microscopy pictures of K562 and IR-K562 cells treated with imatinib (100 nM or 10 μM) and celecoxib (40 μM or 10 μM) for 24 h were taken to observe the altered morphological features. Cells grown in complete medium in the absence of imatinib or celecoxib were round in shape with characteristic features of lymphoid cells (Fig. 25 A & 25 A).

However, after 24 h of incubation with imatinib (Fig. 25 B & 26 B) and celecoxib (Fig. 25 C & 26 C) showed decrease in cell number with cytoplasmic shrinkage and marked convolution of cellular surfaces. Many cells displayed

protuberances of the plasma membrane that would eventually separate into membrane-bound apoptotic bodies.

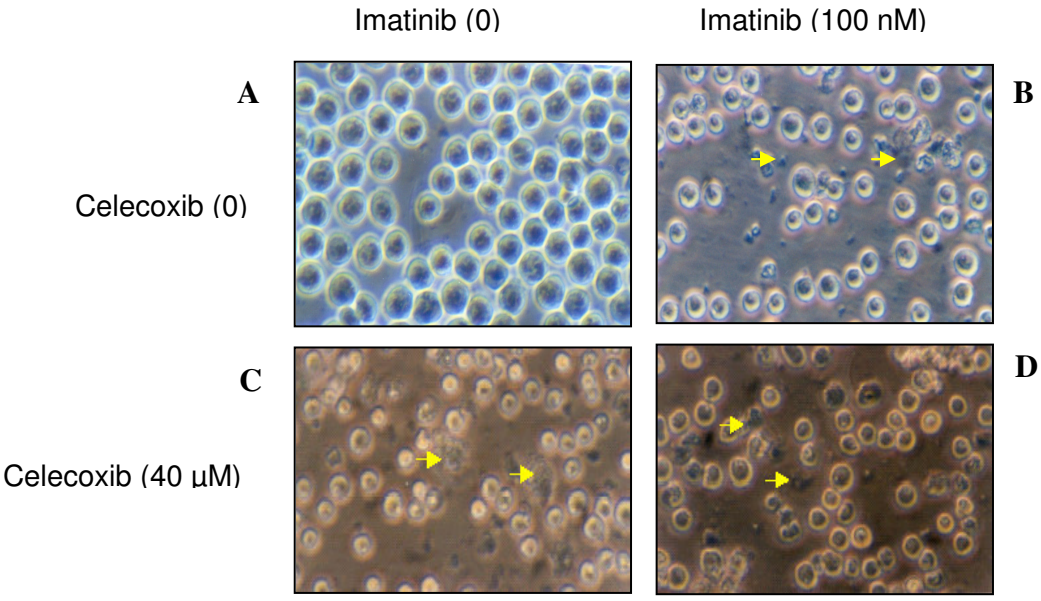


Fig. 25: Phase contrast photomicrographs showing the effect of Imatinib and Celecoxib in K562 cells.

Phase contrast pictomicrographs showing the effect of imatinib and celecoxib on K562 cells. K562 cells were treated with imatinib (100 nM) and celecoxib (40 µM) for 24 h and cells were photographed under phase contrast microscopy (Magnification 400X). Arrows indicate a typical apoptotic cell with apoptotic bodies.

3.12.2 Ultrastructural changes - SEM & TEM

In the light of changes observed under phase contrast microscope, further studies were undertaken for detailed analysis of morphological and ultrastructural

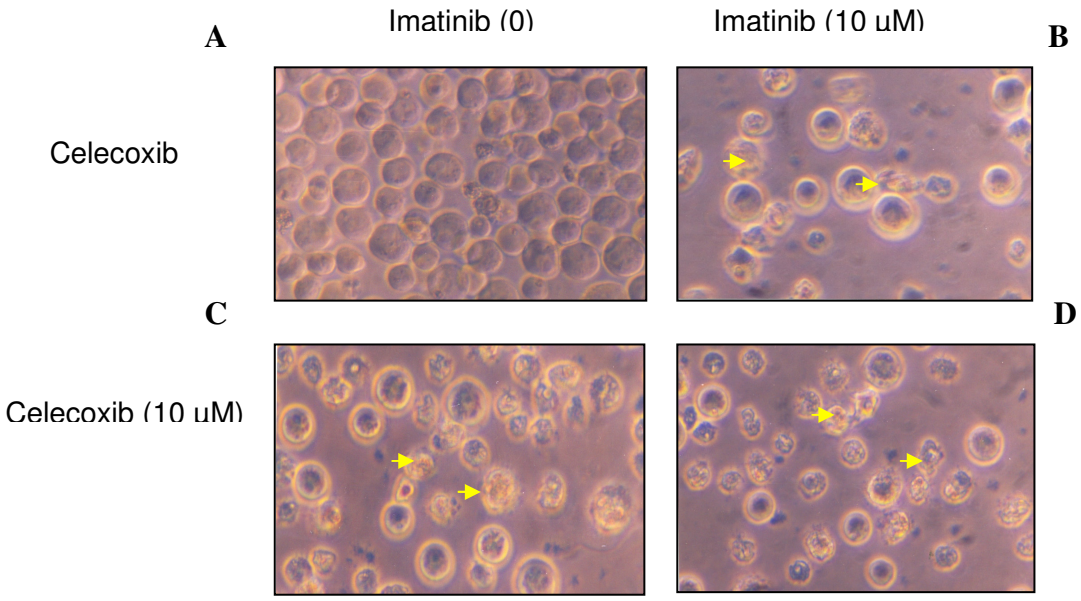


Fig. 26: Phase contrast photomicrographs showing the effect of Imatinib and Celecoxib in IR-K562 cells.

Phase contrast pictomicrographs showing the effect of imatinib and celecoxib on IR-K562 cells. IR-K562 cells were treated with imatinib (10 µM) and celecoxib (10 µM) for 24 h and cells were photographed under phase contrast microscopy (Magnification 400X). Arrows indicate a typical apoptotic cell with apoptotic bodies.

changes on SEM and TEM. To determine whether the antiproliferative effects of imatinib and celecoxib were associated with apoptosis, we examined the ultrastructural changes of K562 cells treated with 100 nM imatinib / 40 µM celecoxib for 24 h. Apoptotic cell death was confirmed by scanning and transmission electron microscopy, which revealed characteristic ultrastructural features of apoptosis. SEM studies of imatinib and celecoxib treated cells revealed the presence of membrane

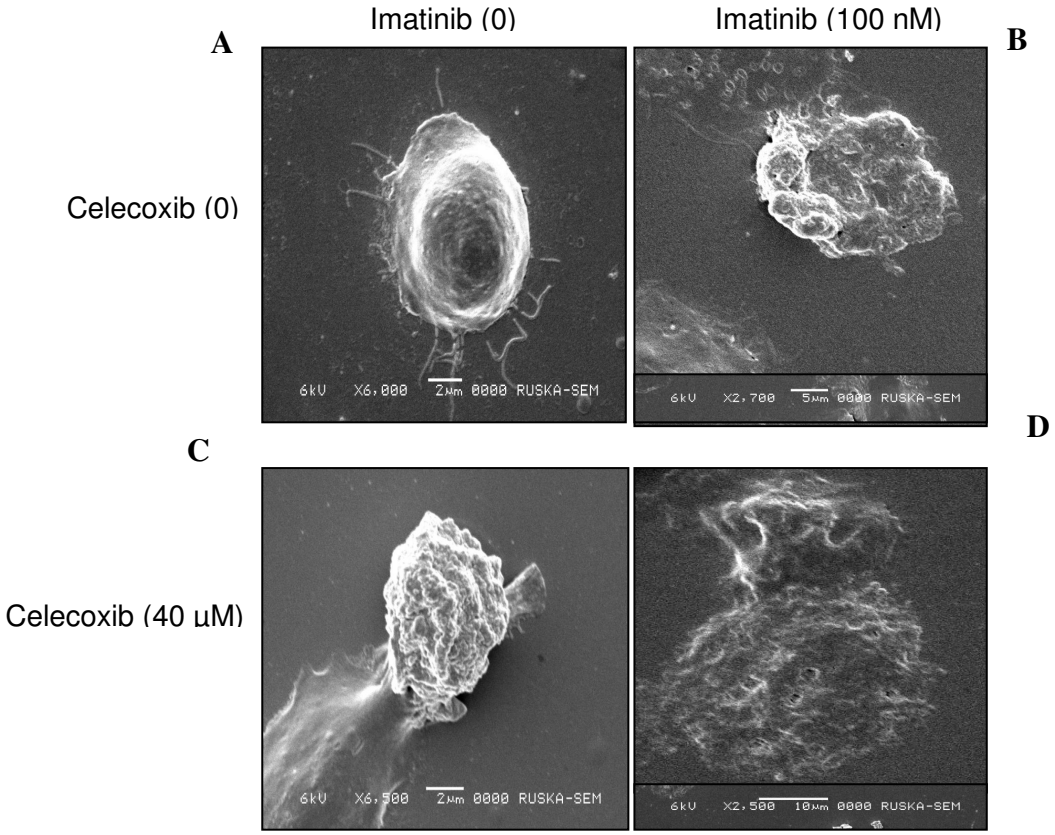


Fig. 27: Scanning electron micrographs showing Imatinib, Celecoxib and Imatinib + Celecoxib treated K562 cells.

Ultra structural morphology, SEM analysis, in K562 cells treated with Imatinib (100 nM) and celecoxib (40 µM) for 24 h. Control cells showing occasional microvilli. Imatinib, Celecoxib and Imatinib and celecoxib treated cells showing clumping and shortening of microvilli, cell shrinkage and membrane blebbing, holes and cytoplasmic extrusions.

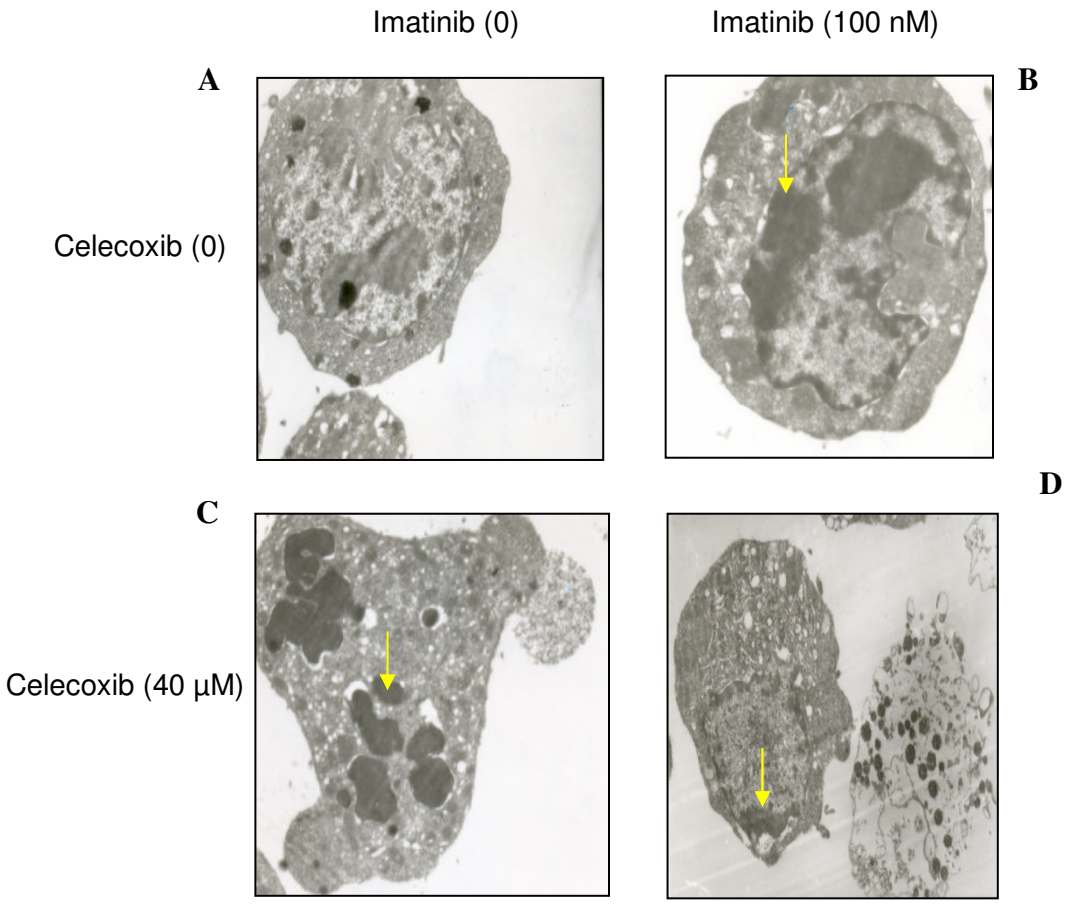


Fig. 28: Transmission electron micrographs of Imatinib, Celecoxib and Imatinib + Celecoxib treated K562 cells.

Ultrastructural changes, TEM analysis, induced by imatinib (100 nM) & celecoixb (40 µM) in K562 cells. Cells were harvested, fixed in 2.5 % glutaraldehyde and analyzed by transmission electron microscopy. Chromatin condensation and nuclear fragmentation is clearly seen in imatinib, celecoxib and imatinib and celecoxib treated cells (arrows). Control cells showed distinguishable diffused interchromatin.

blebbing, which might be due to a deep cytoskeleton rearrangement, causing progressive changes in cell shape, organelle distribution, cell shrinkage and severing

junctions with its neighbors and loss of microvilli (Fig. 27 B, C & D). TEM gives the qualitative bidimensional image of the sectioned samples. The cell volume of the imatinib/celecoxib treated cells was reduced, which indicated shrinkage of cytoplasm, while the plasma membrane remained well defined. The cells showed typical nuclear fragmentation and condensed chromatin with the formation of apoptotic bodies (Fig. 28 B, C & D). However in the control cells, the nuclei are intact with high nucleus/cytoplasm ratio (Fig. 27 A & 28 A).

3.12.3 Imatinib and Celecoxib induced DNA fragmentation in K562 and IR-K562 cells

In addition to morphological evaluation, apoptosis induction by imatinib and celecoxib was ascertained by using an assay developed to measure DNA fragmentation, a biochemical hallmark of apoptosis. During later stages of apoptosis internucleosomal cleavage of cellular DNA by endonucleases to 180 bp or oligomers of 180 bp fragments could be detected by extraction of nuclear DNA and agarose gel electrophoresis. As illustrated in (Fig. 29), agarose gel electrophoresis of DNA extracted from K562 cells and IR-K562 cells treated with imatinib, celecoxib and

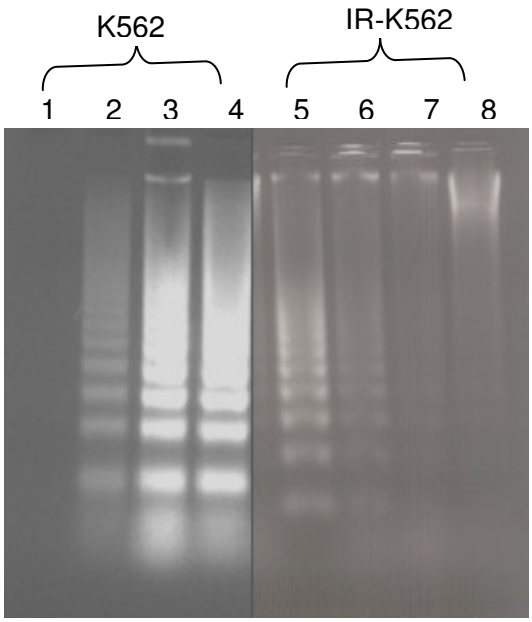


Fig. 29: Agarose gel electrophoresis showing internucleosomal DNA fragmentation induced by Imatinib and Celecoxib in K562 and IR-K562 cells.

Agarose gel electrophoresis of DNA extracted from K562 and IR-K562 cells treated with imatinib and celecoxib for 24 h. After treatment cells were lysed and total cellular DNA was extracted and electrophoresed on a 1% agarose gel containing 0.05 mg/ml ethidium bromide at 5 V/cm. The gels were then photographed under UV illumination.

Lane 1: K562 control cells

Lane 2: K562 cells treated with imatinib 100 nM

Lane 3: K562 cells treated with celecoxib 40 µM

Lane 4: K562 cells treated with imatinib (100 nM) and celecoxib (40 µM)

Lane 5: IR-K562 cells treated with imatinib (10 µM) and celecoxib (10 µM)

Lane 6: IR-K562 cells treated with celecoxib 10 µM

Lane 7: IR-K562 cells treated with imatinib 10 µM

Lane 8: IR-K562 control cells

combination of both at indicated concentrations for 24 h revealed a progressive increase in the non-random fragmentation into a ladder of 180–200 bp (lanes 2-7). Such a pattern corresponds to internucleosomal cleavage, reflecting the endonuclease activity characteristic of apoptosis. Control cells did not show any internucleosomal DNA fragmentation (lanes 1 & 8).

3.13 DNA content assay by fluorescence activated cell sorter (FACS)

The induction of apoptosis in imatinib and celecoxib treated cells was further verified by flow cytometric analysis of DNA content. Loss of DNA is a typical feature of apoptotic cells. Propidium iodide (PI) staining of DNA, which is taken up into the nucleus of apoptotic and necrotic cells, was used to measure the relative numbers of dead cells (Pullen *et al*., 1981). Furthermore, since apoptosis, but not necrosis, involves degradation of DNA, the staining pattern obtained with PI was used to establish whether cell death was due to apoptosis or necrosis. Two different ways of staining with PI are generally observed: PI in the presence of a permeabilising and fixing agent results in the staining of DNA in living, apoptotic and necrotic cells; and PI in a physiological buffer, stain cells that are dead due to apoptosis or necrosis. In the present study, K562 and IR-K562 cells treated with imatinib/ celecoxib/both at indicated concentrations for 24 h were taken for FACS analysis. Figs. 30 and 31

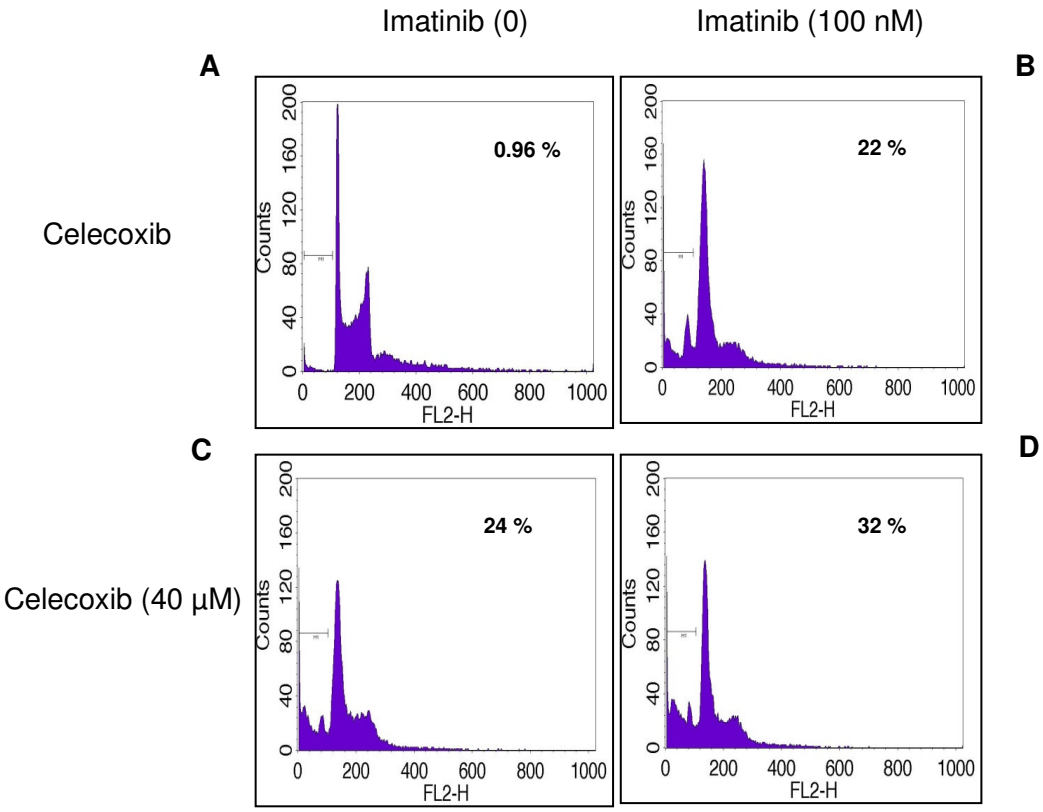


Fig. 30: Quantification of apoptosis by flow cytometric analysis (FACS).

The percentages of apoptotic K562 cells after treatment with imatinib, celecoxib and both for 24 h were determined using propidium iodide staining by flow cytometry.

illustrate the DNA content histograms obtained after PI staining of permeabilized cells. In agreement with DNA fragmentation results, a typical sub-diploid apoptotic peaks (sub G0-G1 phase) were observed in K562 cells treated with 100 nM imatinib (Fig. 30 B), 40 µM celecoxib (Fig. 30 C) and both (Fig. 30 D) for 24 h. Similar

subdiploid peaks were also obtained in IR-K562 cells treated with 10 µM imatinib (Fig. 31 B), 10 µM celecoxib (Fig. 31 C) and both (Fig. 31 D) for 24 h. The FACS analysis of control cells, on the other hand, showed prominent G1, followed by S and G2/M phases (Fig. 30 A & 31 A).

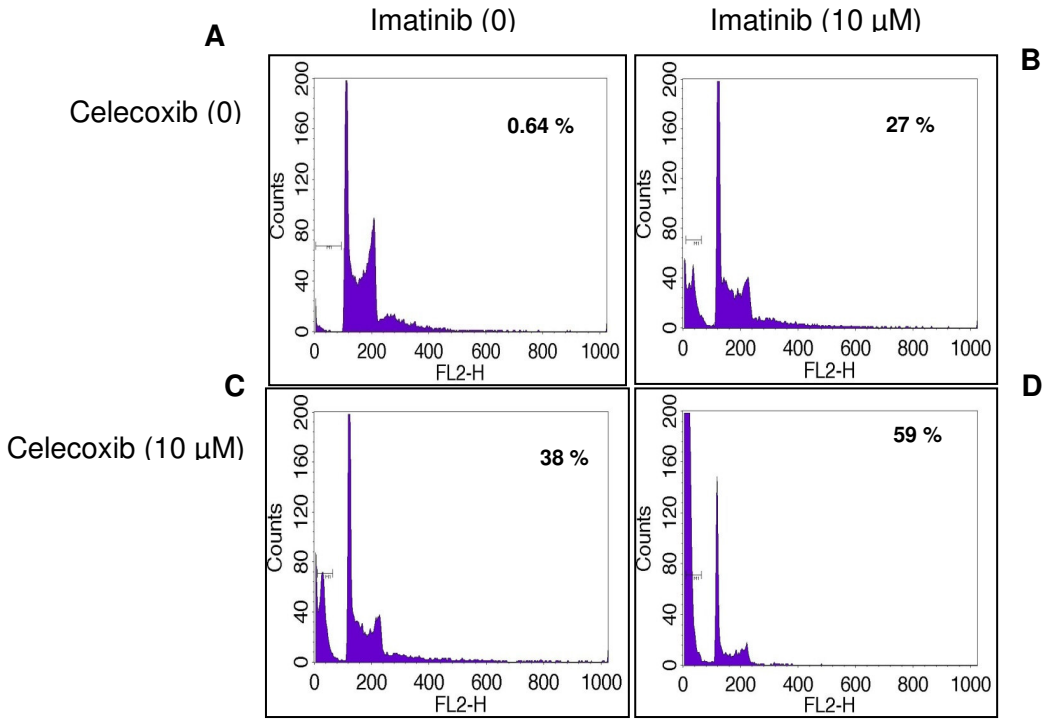


Figure 31: Quantification of apoptosis by flow cytometric analysis (FACS).

The percentages of apoptotic IR-K562 cells after treatment with imatinib, celecoxib and both for 24 h were determined using propidium iodide staining by flow cytometry.

These studies reveal an increase of hypodiploid apoptotic cells in response to imatinib and celecoxib treatment and the decrease of the cells at S and G2 phase of cell cycle. The percent apoptotic cells shown as the sub G0/G1 peak is given in the figure.

3.14 Signal transduction pathways

3.14.1 Imatinib and celecoxib decreased the mitochondrial membrane potential

The decrease in mitochondrial membrane potential ($\Delta\psi m$) is associated with mitochondrial dysfunction (Seuduto and Grotyohann, 1999). In the present study changes in the membrane potential of K562 and IR-K562 cells exposed to imatinib /

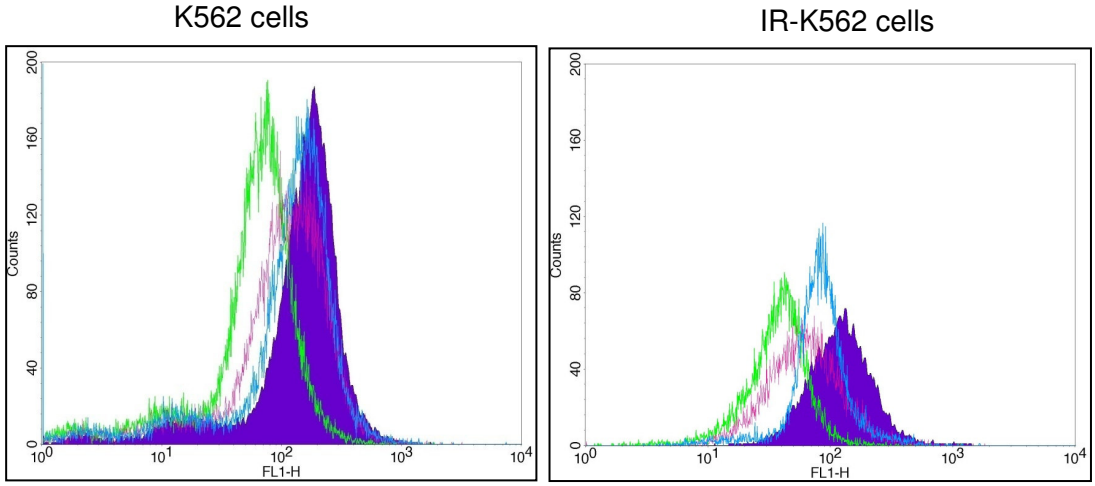


Fig. 32: Measurement of Mitochondrial membrane potential by FACS analysis.

celecoxib /both at indicated concentrations was investigated using FACS for the uptake of rhodamine. The treated cells showed low membrane potentials compared to the untreated control cells (Fig. 32) as indicated by the shift in the fluorescence.

3.14.2 Imatinib and Celecoxib treatment evokes cytochrome c release

One of the major apoptotic pathways is activated by the release of apoptogenic protein, cytochrome *c*, from mitochondria into the cytosol. The release of cytochrome *c*, one of the most important respiratory-chain proteins, from the mitochondria into the cytosol is the hallmark of cells undergoing apoptosis (Liu *et al.*, 1996; Martinou *et al.*, 2000). To specify the molecular basis of apoptosis the release of cytochrome *c* into the cytosol was measured in K562 and IR-K562 cells treated with imatinib and celecoxib, by Western blot analysis employing mouse monoclonal cytochrome *c* antibodies. As shown in Fig. 33, cytochrome *c* was not detectable in the cytoplasm of untreated cells, whereas the levels increased significantly after imatinib and celecoxib treatment. The levels of cytochrome *c* in the cytosol were elevated after cotreatment with imatinib and celecoxib.

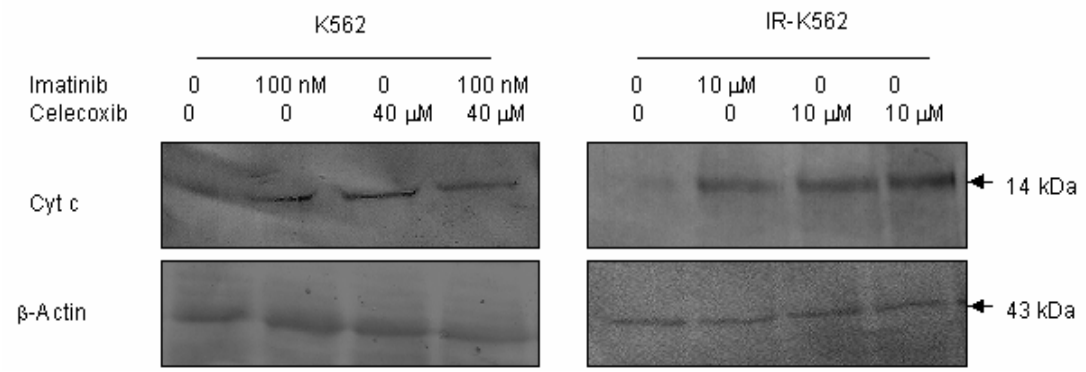


Fig. 33: Western blot analysis showing Imatinib and Celecoxib induced release of cytochrome c into the cytosol in K562 and IR-K562 cells.

K562 and IR-K562 cells were treated with imatinib (100 nM / 10 µM) and celecoxib (40 µM / 10 µM) for 24 h, cytosolic proteins (50 µg) were separated on a 15 % SDS-PAGE, and after electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with mouse monoclonal cytochrome-c antibodies.

3.14.3 PARP cleavage in response to imatinib and celecoxib treatment

PARP, poly (ADP-ribose) polymerase, has been implicated in many cellular processes including apoptosis and DNA repair. PARP is primarily found in the nucleus and is activated by DNA strand breaks. PARP is a 116-kDa protein, which converts nicotinamide adenine dinucleotide (NAD) to nicotinamide and protein-linked ADP-ribose polymers. The DNA repair enzyme, PARP has been recognized as a representative death substrate that is cleaved and inactivated by down-stream

caspases. In response to growth factor withdrawal or on exposure to a variety of chemotherapeutic compounds (Shah *et al.*, 1996), PARP is cleaved to generate 85

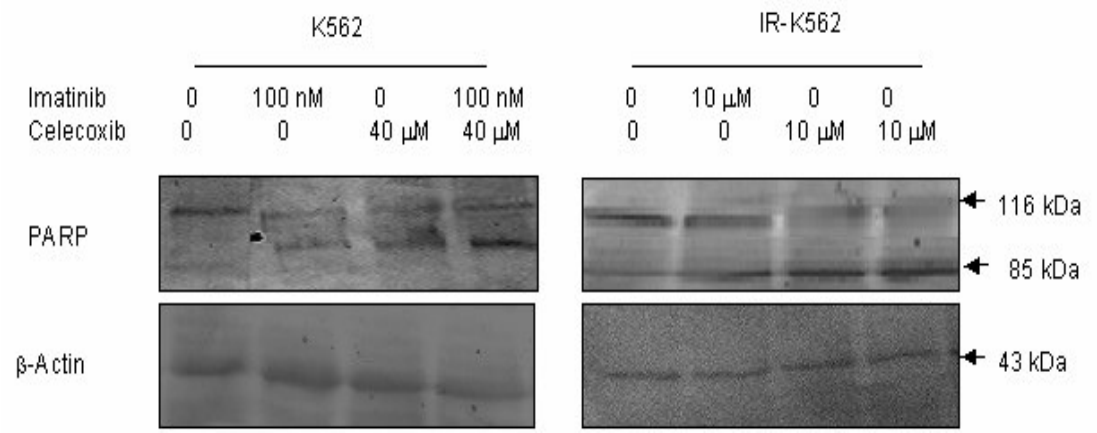


Fig. 34: Western blot analysis showing the cleavage of PARP in cell extracts of Imatinib and Celecoxib treated K562 and IR-K562 cells.

Whole cell lysates from K562 and IR-K562 cells treated with imatinib and celecoxib for 24 h, were fractionated on a 12 % SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and the proteins were probed with anti-PARP antibodies. This antibody recognizes both uncleaved PARP (116 kDa) and the cleaved fragment (85 kDa).

and 23 kDa fragments. To determine whether PARP is cleaved in imatinib and celecoxib induced cell death, K562 and IR-K562 cells were treated with 100 nM & 10 µM imatinib and 40 µM & 10 µM celecoxib for 24 h and PARP cleavage was monitored with PARP antibodies, which specifically recognize the 83 kDa fragment of the cleaved PARP and uncleaved 116 kDa PARP. Fig. 34 illustrates the gradual

increase in the proportion of the 83 kDa cleavage product and decrease in the proportion of 116 kDa uncleaved PARP with the treatment of imatinib and celecoxib. In the control cells, however, no fragment of PARP was observed, except the uncleaved 116 kDa protein. The extent of PARP cleavage in cells treated with imatinib and celecoxib, however, was much higher than the same in imatinib or celecoxib treated cells.

3.14.4 Bcl-2/ Bax ratio modulation

Different proteins of the Bcl-2 family have been implicated in triggering or preventing apoptosis. Bax and Bcl-2 are the proteins associated with the mitochondrial membrane and their ratio is crucial for cell survival. Studies were undertaken to test whether Bcl-2 expression is affected after imatinib/celecoxib treatment in K562 and IR-K562 cells. Changes in the expression of cellular anti-apoptotic proteins, Bcl-2, and of the pro-apoptotic protein Bax, following imatinib and celecoxib treatment for 24 h were examined by Western blotting.

As shown in Fig. 35 untreated cells expressed high levels of Bcl-2 protein where as in cells treated with imatinib and celecoxib the expression of Bcl-2 protein is down regulated. Bax protein levels, however, were not altered on imatinib and celecoxib treatment. As a result of decreased Bcl-2 with no change in Bax, the ratio of Bcl-2/Bax reduced significantly during imatinib and celecoxib treatment.

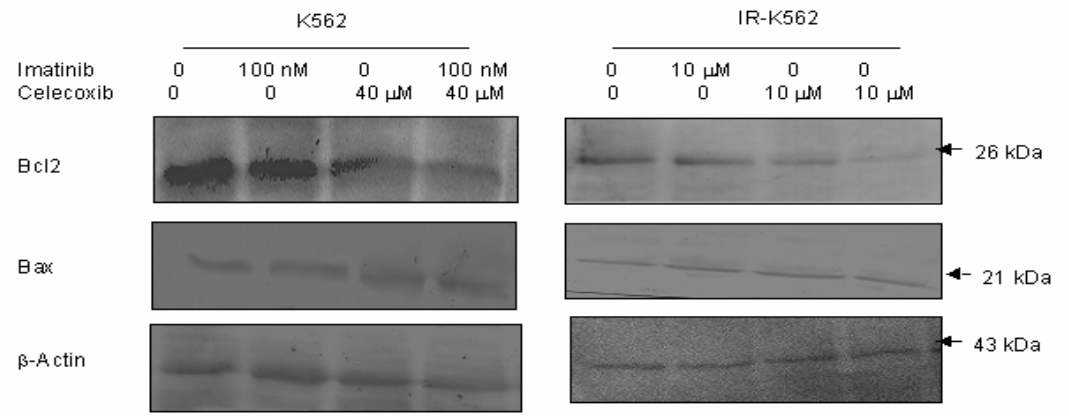


Fig 35: Immunoblot analysis of Bcl-2 and Bax expression in K562 and IR-K562 cells treated with Imatinib and Celecoxib.

Whole cell lysates from K562 and IR-K562 cells treated with imatinib and celecoxib for 24 h, were fractionated on a 15 % SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and the proteins were probed with anti-Bcl2 or Bax antibodies.

3.14.5 Change in the Akt/pAkt expression

Akt, a cell survival protein is activated by various growth and survival factors. The phosphorylated-Akt increases in response to growth factors. Inhibition of p-Akt leads to apoptosis. To examine the possible involvement of p-Akt inhibition by imatinib and celecoxib, Western blot analysis was performed in K562 and IR-K562 cells with specific antibodies. Imatinib and celecoxib cotreated cells showed a marked decrease in p-Akt expression with no change in the expression of Akt (Fig. 36).

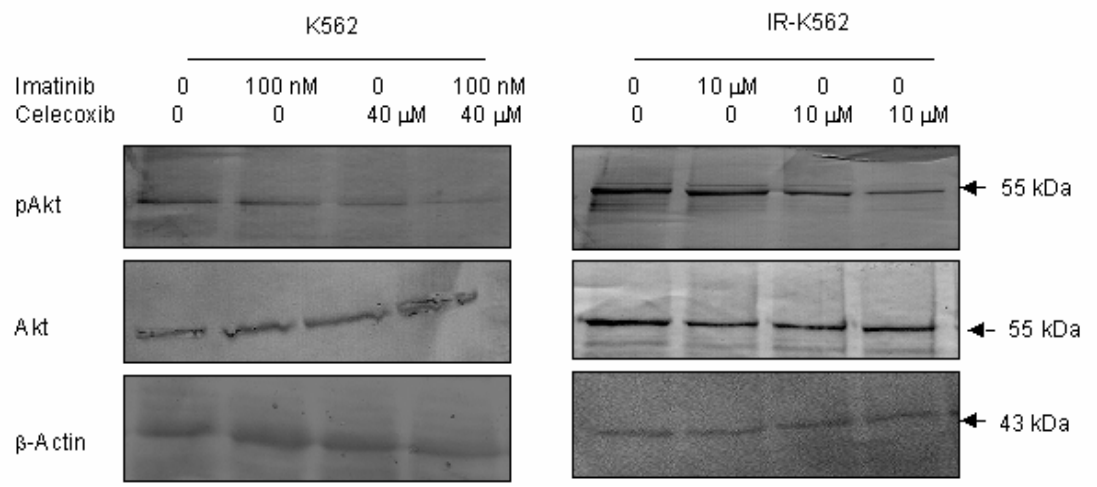


Fig. 36: Immunoblot analysis of Akt/pAkt expression in Imatinib and Celecoxib treated K562 and IR-K562 cells.

Whole cell lysates from K562 and IR-K562 cells treated with imatinib and celecoxib for 24 h, were fractionated on a 12 % SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and the proteins were probed with anti-pAkt or Akt antibodies.

3.14.6 Effect on the BCR-ABL kinase activity and expression

To determine whether the apoptotic effects of celecoxib are mediated through the BCR-ABL tyrosine kinase signaling, Western blot analysis using phospho-tyrosine antibodies and RT-PCR analysis were done. After treatment with imatinib there is a decrease in the kinase activity (Fig. 37) and expression (Fig. 38) of BCR-ABL. However, after celecoxib treatment, in both K562 and IR-K562 cells, there is no change in the activity and expression (Fig. 37 & Fig. 38), indicating that the apoptotic

effects of celecoxib are BCR-ABL independent. This study suggests that antiproliferative effects of celecoxib might be mediated through some other signaling pathway.

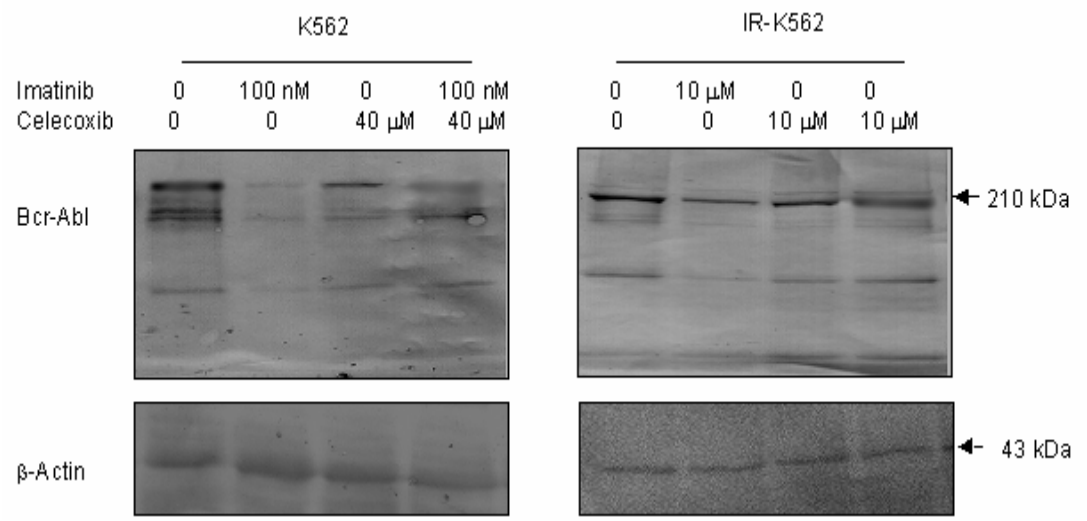


Fig. 37: Western blot analysis of BCR-ABL kinase expression in Imatinib and Celecoxib treated K562 and IR-K562 cells.

Whole cell lysates from K562 and IR-K562 cells treated with imatinib and celecoxib for 24 h, were fractionated on a 7 % SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and the proteins were probed with anti-pTyr antibodies.

3.14.7 Expression of MDR1

MDR1 is responsible for the development of drug resistance and the role of NSAIDs to enhance the cytotoxic effects of doxorubicin and vincristine was reported in T98G human malignant glioma cells (Roller *et al.,* 1999). In view of the above

mentioned points, the effect of celecoxib on MDR1 expression was studied using Western blot analysis and RT-PCR analysis in K562 and IR-K562 cells. These

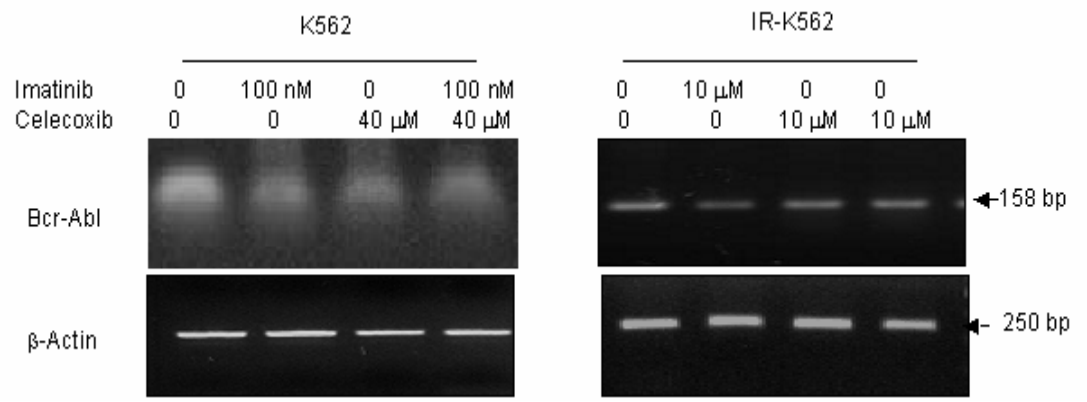


Fig. 38: RT-PCR analysis of BCR-ABL kinase expression in Imatinib and Celecoxib treated K562 and IR-K562 cells.

Total RNA was isolated from K562 and IR-K562 cells treated with imatinib and celecoxib for 24 h and RT-PCR was carried as described in materials.

studies clearly showed that celecoxib down regulates the expression of MDR1 in IR-K562 cells (Fig. 39 & Fig. 40). MDR1 was undetectable in K562 cells in Western blot and RT-PCR analysis.

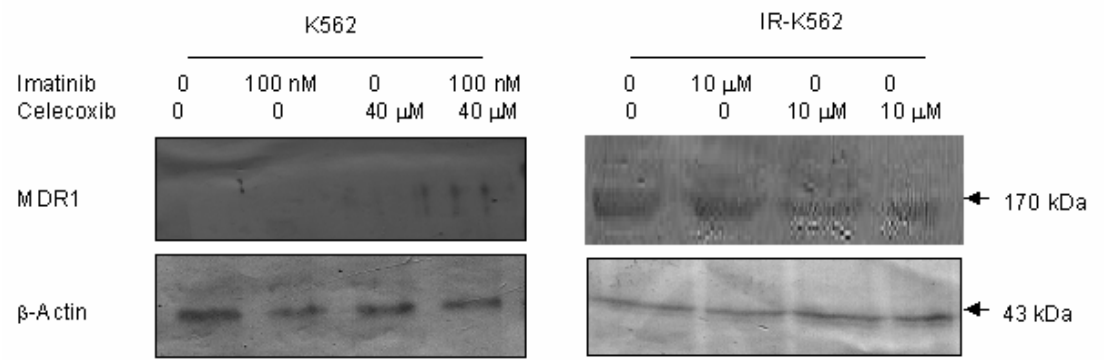


Fig. 39: Immunoblot analysis of MDR1 expression in Imatinib and Celecoxib treated K562 and IR-K562 cells.

Whole cell lysates from K562 and IR-K562 cells treated with imatinib and celecoxib for 24 h, were fractionated on a 7 % SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and the proteins were probed with anti-MDR1 antibodies.

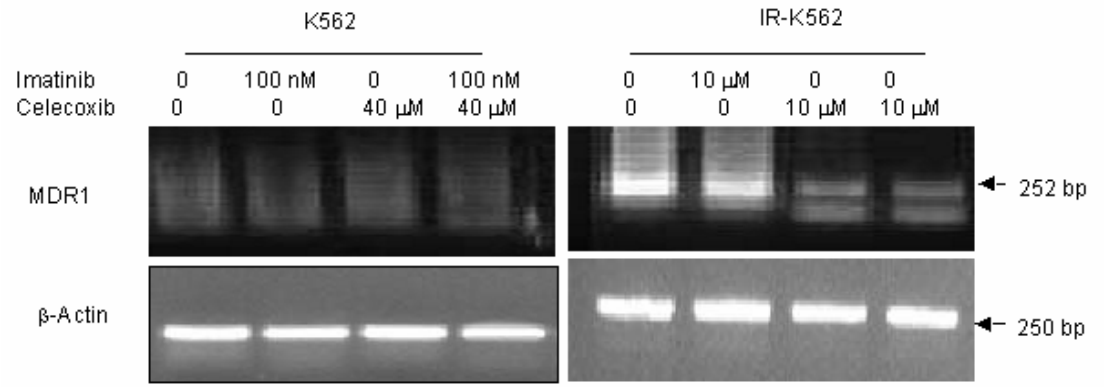


Fig. 40: RT-PCR analysis of MDR1 expression in Imatinib and Celecoxib treated K562 and IR-K562 cells.

Total RNA was isolated from K562 and IR-K562 cells treated with imatinib and celecoxib for 24 h and RT-PCR was carried as described in materials.

3.15 Effect of imatinib and celecoxib on PGE_2 levels

PGE_2 levels were estimated in the culture medium of K562 and IR-K562 cells treated with imatinib and celecoxib. The PGE_2 levels are elevated in IR-K562 cells compared to K562 cells and celecoxib lowered the levels of PGE_2 in both the cell types (Fig. 41).

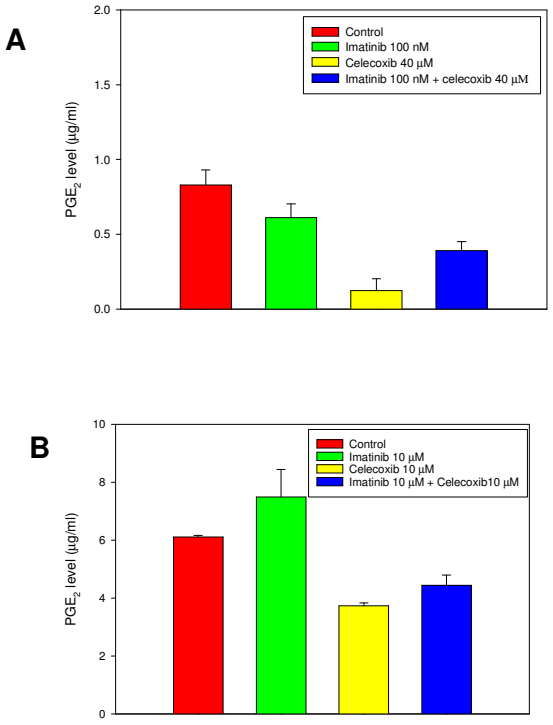


Fig. 41: Effect of imatinib and celecoxib on PGE_2 release in K562 (A) and IRK562 (B) cells.

3.16 Expression of *COX-2*

As COX-2 is over expressed in IR-K562 cells, the effect of celecoxib on COX-2 was studied in K562 and IR-K562 cells. For this, Western blot and RT-PCR analysis were employed and the results indicate inhibition in the expression of COX-2 in IR-K562 cells by celecoxib both at protein and mRNA level (Fig. 42 & Fig. 43). COX-2 was not detected in the untreated control K562 cells.

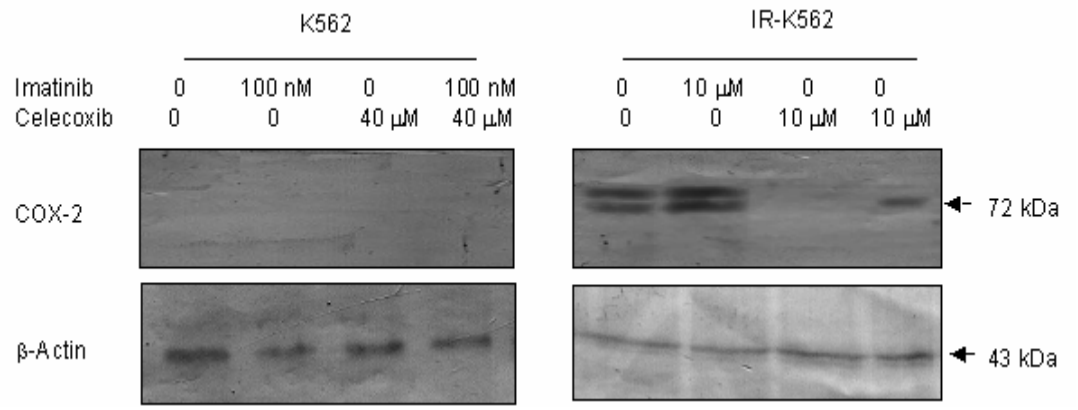


Fig. 42: Immunoblot analysis of COX-2 expression in Imatinib and Celecoxib treated K562 and IR-K562 cells.

Whole cell lysates from K562 and IR-K562 cells treated with imatinib and celecoxib for 24 h, were fractionated on a 12 % SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and the proteins were probed with anti-COX-2 antibodies.

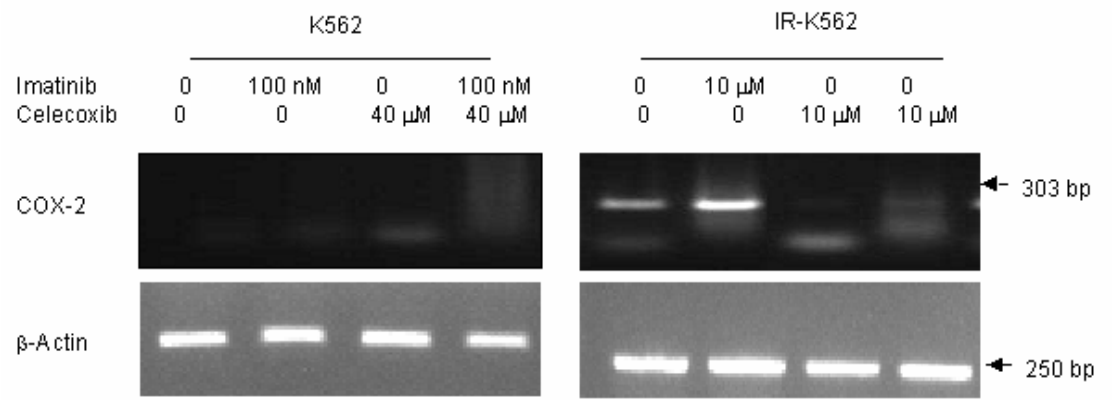


Fig. 43: RT-PCR analysis of COX-2 expression in Imatinib and Celecoxib treated K562 and IR-K562 cells.

Total RNA was isolated from K562 and IR-K562 cells treated with imatinib and celecoxib for 24 h and RT-PCR was carried as described in materials

3.17 Expression of NF-κB and PKC

The downstream effector molecules of BCR-ABL/Akt signaling, NF-κB and PKC, effected by celecoxib are studied by Western blot analysis. The nuclear extracts of imatinib and celecoxib treated K562 and IR-K562 cells were subjected to Western blot analysis using Rel A (p65 component of NF-κB) antibodies. The results indicate a decline in the NF-κB translocation upon treatment with celecoxib (Fig. 44). Similar result was obtained in the Western blot analysis of PKC (Fig. 44).

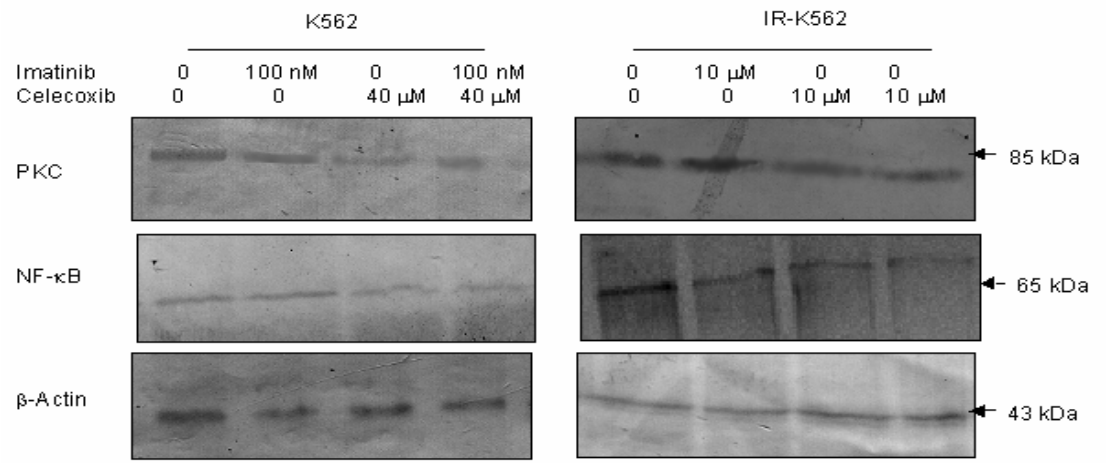


Fig. 44: Immunoblot analysis of PKC and NF-κB expression in Imatinib and Celecoxib treated K562 and IR-K562 cells.

Whole cell lysates from K562 and IR-K562 cells treated with imatinib and celecoxib for 24 h, were fractionated on a 12 % SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and the proteins were probed with anti-PKC and anti-RelA antibodies.

3.18 Effect of PGE_2 on K562 cell proliferation

If elevated COX-2 and PGE_2 levels are responsible for drug resistance in IR-K562 cells, PGE_2 when given externally to K562 cells should also cause drug resistance. To test this hypothesis, K562 cells were exposed continuously for 3 days to 3 µg/ml and 6 µg/ml PGE_2 and then cell proliferation MTT assay in presence of imatinib was carried out. The results indicated that PGE_2 did not effect the proliferation of K562 cells in presence of imatinib (Fig. 45 A). But in presence of

PGE_2 PKC and MDR1 levels are elevated as observed by Western blot analysis. These elevated levels of PKC and MDR1 are reduced by celecoxib treatment (Fig. 45 B). Thus these studies suggest the possible role of COX-2 in the development of resistance to imatinib in K562 cells through the activation of PKC and thus expression of MDR1.

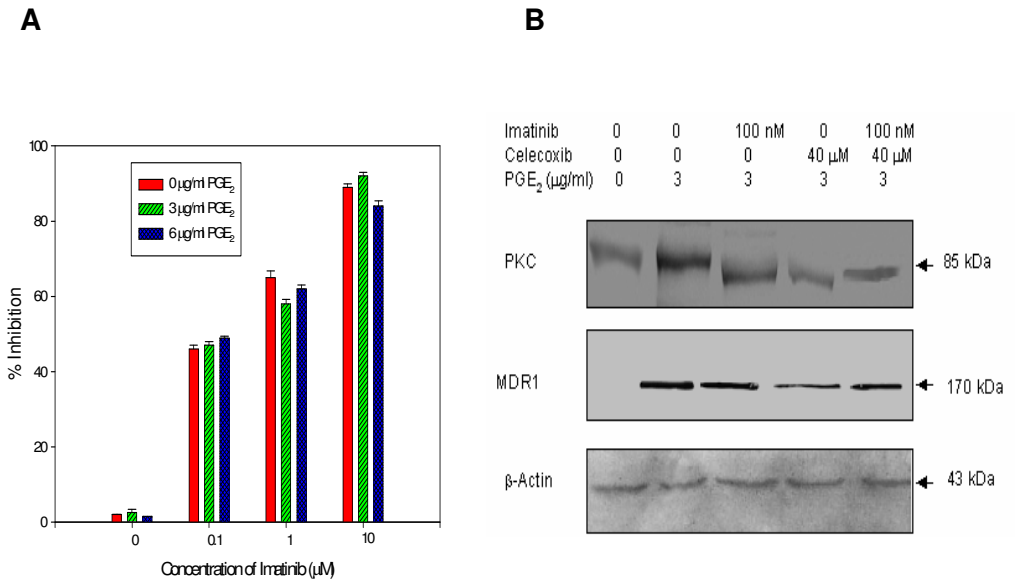


Fig. 45: Effect of PGE_2 on cell proliferation, expression of PKC and MDR1 in K562 cells.

(A) *Effect of PGE_2 on K562 cell proliferation and PKC and MDR1 expression in K562 cells. (B) Bar graphs showing the effect of PGE2 on K562 cell proliferation. (B) Western blot analysis of PGE_2 mediated effect on PKC and MDR1 expression in K562 cells.*

Research in the past 2 decades has established that BCR-ABL is the causal to the pathogenesis of CML, and that constitutive tyrosine kinase activity is central to BCR-ABL's capacity to transform hematopoietic cells *in vitro* and *in vivo* (Daley et al., 1990; Lugo et al., 1990). The activation of multiple signal transduction pathways in BCR-ABL–transformed cells leads to increased proliferation, reduced growth-factor dependence and apoptosis and perturbed interaction with extracellular matrix and stroma.

The development of imatinib mesylate (Gleevec, Novartis Pharmaceuticals, Basel, Switzerland) represented a major success for target-directed cancer chemotherapy and a breakthrough in the management of CML. Imatinib selectively inhibits BCR-ABL by occupying the ABL domain adenosine triphosphate–binding site; it maintains the protein in an inactive conformation, thereby inhibiting its tyrosine kinase activity (Schindler et al., 2000). Despite high rates of hematologic and cytogenetic responses, primary refractory disease and secondary resistance have been observed in a proportion of patients on imatinib monotherapy. Mechanisms of imatinib resistance identified from studies include several-fold increase in the amount of BCR-ABL protein, amplification of the BCR-ABL gene, overexpression of the multidrug resistance P-glycoprotein (MDR1) and most commonly, mutations in the kinase domain of the Abl (Weisberg and Griffin, 2000; le Coutre et al., 2000;

Gorre et al., 2001; Branford et al., 2002). Thus, it is of urgency to develop an alternative strategy to overcome this imatinib mesylate resistance.

Epidemiological studies showed that use of non-steroidal anti-inflammatory drugs (NSAIDs) was associated with a reduced risk of developing several malignant diseases including skin, breast cancer, colon cancer, ovarian cancer etc. (Ulrich et al., 2006; Grau et al., 2006; Meier et al., 2002). Selective COX-2 inhibitors cause fewer serious adverse effects than traditional NSAIDs. Celecoxib, a specific cyclooxygenase-2 (COX-2) inhibitor, has been shown to possess antitumor activity in a variety of cancer cells. At the cellular level, celecoxib inhibits COX-2 and causes cell cycle arrest and induces apoptosis in cancer cells. Antileukemic effects of celecoxib have been observed previously in K562 cells (Subhashini et al., 2005; Zhang et al., 2006). The synergism between celecoxib and imatinib was also reported (Zhang et al., 2006). Tseng et al., (2005) studied the synergistic effects of celecoxib derivative and imatinib in imatinib resistant cells. However, effects of celecoxib in imatinib-resistant cells remained uninvestigated.

Thus this study was planned in an effort to gain insight into the effects of celecoxib, individually and in combination with imatinib, on imatinib-resistant K562 cells and also to understand the biochemical mechanisms underlying COX-2 inhibitor-induced apoptosis.

4.1 Development of imatinib-resistant K562 (IR-K562) cells: Role of COX-2 and MDR1

Imatinib-resistant K562 (IR-K562) cells were developed by continuous exposure of K562 cells to increasing concentrations of imatinib and fold resistance was determined as described in earlier studies (Song et al., 2005). A 100-fold resistance to imatinib was developed. Different mechanisms of resistance were characterized. Weisberg and Griffin (2000) demonstrated that imatinib resistance occurs due to overexpression of Bcr-Abl protein or mRNA. The results from the Western blot and RT-PCR analysis demonstrated that over expression of the Bcr-Abl protein or mRNA was not responsible for the development of resistance in the present study. This result was in agreement with earlier studies (Mahon et al., 2000). The most common mechanism of resistance, mutations in the kinase domain of Abl, was then checked by PCR-based RFLP and sequencing of the Abl kinase domain. The results showed no mutations in the kinase domain suggesting a different mechanism for the resistance development.

MDR1 overexpression, which is another mechanism of development of imatinib resistance, was then studied by Western blot and RT-PCR analysis. The results clearly indicated an over expression of MDR1 at both protein and mRNA level. These results are in concert with other studies where the interaction of imatinib

with Pgp has been studied (Hamada et al., 2003; Mahon et al., 2000). Resistance to doxorubicin in K562 cells has also been attributed to overexpression of MDR1 (Grandjean et al., 2001). The role of MDR1 in protecting cells from apoptosis has been studied in several cellular systems (Johnstone *et al.,* 1999). Few studies have shown that the activation of the cyclooxygenase system might be critical event in the development of MDR1 mediated drug resistance. A close association between MDR and COX-2 has been reported in human hepatocellular carcinoma (Fantappie et al., 2002) and rat renal mesangial cells (Patel et al., 2002). A strong correlation between expression of COX-2 and MDR-1 was also found in tumor specimens derived from breast cancer patients (Ratnasinghe et al., 2001). Furthermore, it has been suggested that COX-2 inhibitors sensitize cells to chemotherapeutic drugs by a functional blockade of p-glycoprotein (Awara et al., 2004). These studies strongly suggest that COX-2 modulates p-glycoprotein expression and is involved in the development of the MDR phenotype (Sorokin, 2004; Kang et al., 2005).

In the light of above, further studies were taken up on the expression of COX-2 in IR-K562 cells. Surprisingly, COX-2 was over expressed both at protein and mRNA level in IR-K562 cells but was undetectable in K562 cells. To further confirm that overexpression of COX-2 is responsible for the development of imatinib resistance in K562 cells, COX-2 gene was knocked down by siRNA and then cell

proliferation assay with imatinib was carried out. The MTT assay clearly showed a 40% inhibition in growth of IR-K562 + siRNA treated cells at 100 nM concentration of imatinib (IC_{50} of imatinib in K562 cells) suggesting that indeed COX-2 overexpression is responsible for imaitnib resistance. These results are also supported from a study reporting that bone marrow COX-2 levels are elevated in CML patients and that increasing levels of COX-2 were significantly associated with shorter survival (Giles et al., 2002). In order to elucidate the pathway though which COX-2 and thereby MDR1 are overexpressed in resistant cells, reactive oxygen species (ROS) levels were measured by FACS. Indeed, ROS levels were greatly increased in IR-K562 cells compared to K562 cells. ROS are produced in the cells as a result of various signaling pathways such as receptor tyrosine kinases (RTKs) which become activated by growth factors –epidermal growth factor, platelet derived growth factor, fibroblast growth factor as well as cytokines (tumor necrosis factor, g-interferon and interleukins), leading to an intracellular tyrosine phosphorylation cascade (Behrend *et al.,* 2003). Reactive oxygen species may also be formed in response to xenobiotic exposure. Redox-sensitive signaling factors regulate multiple processes including proliferation, cell cycle and anti-apoptotic signaling pathways. The modification of gene expression by reactive oxygen species has direct effects on cell proliferation and apoptosis through the activation of transcription factors

including AP-1 and NF-κB pathways. Likewise, reactive oxygen species function as second messengers involved in activation of NF-κB by tumor necrosis factor and cytokines. Alteration in the redox potential of the cells exposed to xenobiotics may affect ROS responsive signaling pathways. ROS activates signal transduction pathways that enhance NF-κB translocation to nucleus (Kabe *et al.,* 2005), which may be critical in regulation of COX-2 and MDR1 expression. Numerous reports indirectly support the notion that intracellular ROS lead to the activation of NF-κB (Kang *et al.,* 2007; Beaudeux *et al.,* 2006; Song *et al.,* 2005). So, we have studied the translocation of NF-κB into the nucleus by Western blot analysis using p65 antibodies. The levels of NF-κB in the nuclear extract of IR-K562 cells have been elevated by two fold compared to K562 cells implying that ROS has activated NF-κB, which positively regulated the expression of MDR1 and COX-2 leading to the development of resistance. COX-2 again induced the expression of MDR1 through PGE_2 activated cAMP-PKC signaling finally leading to a 4-fold increase in the MDR1 expression.

Studies were also done on the cytochrome P450 isozyme, 1A1, by Western blot analysis to see if imatinib is eliminated through the xenobiotic system, which might also be responsible for the development of drug resistance (Rochat, 2005).

Imatinib is metabolized mainly by CYP3A4, CYP2C9, and CYP2D6 (Goldman and Melo, 2001); however other CYPs may also be involved (Marull and Rochat, 2006). In the present study, an increased expression of CYP1A1 was observed, which might eliminate imatinib and thus allowing only a little biologically active imatinib than required for the inhibition of Bcr-Abl.

Thus, the above studies clearly indicate that development of imatinib resistance in K562 cells is primarily due to over expression of MDR1. Since COX-2 is overexpressed in IR-K562 cells, it might also be responsible for the resistance development. Also CYPs that metabolize imatinib and thus allowing very little amount of the drug available for Bcr-Abl inhibition may be responsible for the development of resistance to imatinib. A schematic representation of these results on the mechanism of development of resistance to imatinib in K562 cells is given in Fig. 46.

4.2 Celecoxib shows more potent effects in IR-K562 cells and the effects are synergistic with imatinib

Since COX-2 and MDR1 are overexpressed in IR-K562 cells, it was presumed that use of a COX-2 inhibitor, celecoxib, might reverse the drug resistance in these cells. To test this hypothesis, the inhibitory effects of celecoxib alone or in combination with imatinib were studied. The effects of celecoxib / imatinib / in

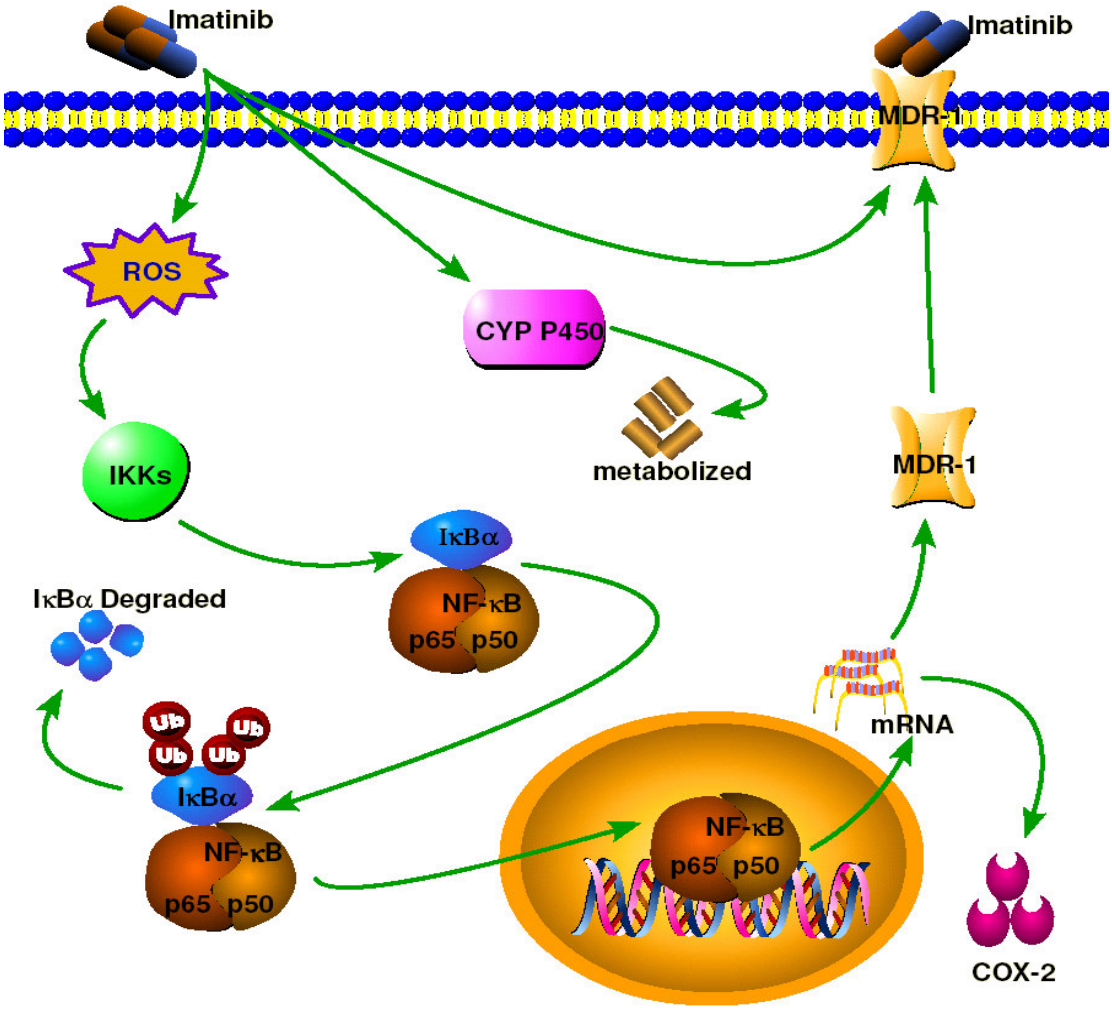


Fig. 46: Schematic representation of the model showing the mechanisms of development of resistance to imatinib in K562 cells.

combination on the viability of K562 and IR-K562 cells were evaluated after 24, 48 and 72 h in culture. These studies have clearly shown the inhibition in the growth of K562 and IR-K562 cells in a dose and time dependent manner. It was interesting to find a 4-fold decrease in the IC_{50} of celecoxib in IR-K562 cells (10 µM) compared to K562 cells (40 µM). This reduction in the IC_{50} itself suggests that the inhibitory effects of celecoxib are COX-2-dependent in IR-K562 cells and COX-2-independent in K562 cells. There is accumulating evidence suggesting that the antineoplastic effect of NSAIDs may not be solely mediated by the inhibition of COX-2 activity and a subsequent decrease of prostaglandin E_2 (PGE_2) synthesis, but by other cellular targets besides COX-2 (Grösch et al., 2006). This assumption is largely based on the observation that significantly higher concentrations of NSAIDs are necessary to inhibit cell growth and to induce apoptosis than those required for the inhibition of prostaglandin production (Tegeder *et al.*, 2001). Furthermore, NSAIDs reduced cell survival not only in COX-2 expressing cells, but also in COX-2 deficient cell lines (Pang *et al.*, 2007; Zhang *et al.*, 1999; Grosch *et al.*, 2001).

Celecoxib also enhanced the sensitivity of cells to imatinib to some extent since in presence of 1 µM celecoxib the IC_{50} of imatinib was reduced to 80 nM from 100 nM in case of K562 cells and 6 µM from 10 µM in IR-K562 cells. The results from the studies on IR-K562 cells suggest that the synergism of imatinib and

celecoxib on proliferation IR-K562 cells is due to the inhibition of COX-2 by celecoxib as observed.

Earlier studies suggest that defects in the process of apoptosis may be closely associated with carcinogenesis and that many cancer cells have defective machinery for self-destruction (Yano *et al.*, 1994). It is suggested that the susceptibility to apoptosis-inducing effects of chemotherapeutic drugs may depend on the intrinsic ability of tumor cells to respond to apoptosis (Yano *et al.*, 1994; Tseng *et al.*, 2002). Apoptosis is a specific mode of cell death recognized by a characteristic pattern of morphological, biochemical, and molecular changes. Imatinib and celecoxib treated cells showed pronounced morphological changes like cell shrinkage, formation of membrane blebs, chromatin condensation and loss of cellular details characteristic of apoptosis as evidenced by phase-contrast and electron microscopic studies. A ladder-like DNA fragmentation pattern, a biochemical marker of apoptosis (cleavage of DNA into nucleosomal size fragments of 180-200 bp) was observed in K562 and IR-K562 cells treated with imatinib or celecoxib or both. The flow cytometer has become the instrument of choice for analysis of cell kinetics and offers a rapid and accurate analysis of a large population of individual cells. Flow cytometric analysis of treated cells showed the increase of hypodiploid apoptotic cells and the decrease of the cells at S and G2 phase of cell

cycle. These results suggest that imatinib and celecoxib induced apoptosis occurs at S and G2 phase of the cell cycle. Similar result induction of apoptosis in multiple myeloma cells and K562 cells (Zhang et al., 2007; Subhashini *et al.,* 2005) by celecoxib was reported.

In mitochondria, cytochrome *c* is required as an electron carrier in oxidative phosphorylation, a process which generates the majority of intracellular ATP (Matsuno-Yagi and Hatefi, 1985). Cytochrome *c* resides in the space between the outer and inner membranes of mitochondria, where it snuggles up to the cytochrome *c* oxidase complex located in the inner membrane. Several apoptosis inducing agents are known to trigger mitochondrial uncoupling leading to the rupture of outer membrane. This in turn causes the release of pro-apoptotic factors such as apoptosis inducing factor (AIF), cytochrome *c* and the apoptosis protease-activating factor (Apaf-1) into the cytosol. In cytoplasm, cytochrome *c* is known to get associated with caspase-9, Apaf-1 and dATP to form the apoptosome complex (Li *et al*., 1997), which inturn activates caspase-9, 3 and 7. Caspase activation leads to the cleavage of cellular substrates and apoptosis. Accumulating scientific evidences indicate the pivotal role of mitochondria in the execution of apoptosis of the cells exposed to various stimuli (Desagher and Martinou, 2000; Green and Reed, 1998). The principal hypothesis proposed for the release of cytochrome *c* from

mitochondria during apoptosis is that of a decrease in the mitochondrial membrane potential, opening of permeability transition pore in the inner membrane causing swelling and rupture of the outer membrane (Szabo and Zoratti, 1991; Bernardi *et al.*, 1992; Von Ashen *et al.*, 2000). The anti-apoptotic protein Bcl-2 acts on mitochondria to stabilize membrane integrity and prevent the opening of the megachannel (Yang *et al.*, 1997; Susin *et al.*, 1998; Tsujimoto and Shimizu, 2000). The present studies showed a decrease in the mitochondrial membrane potential as determined by FACS analysis. To further examine whether cytochrome *c* is released or not into the cytosol in response to imatinib / celecoxib /both treatment Western blot analysis of the cytosolic proteins was done. These studies have shown a significant increase in the release of cytochrome *c* after the treatment. This is in support with the earlier finding that celecoxib induced apoptosis in K562 cells is through cytochrome *c* release into the cytosol (Subhashini *et al.,* 2005).

In the present model, a biochemical evidence is provided for cellular damage in the from of activation of potential substrates for an ICE/CED 3 like proteases during apoptosis called, Poly (ADP) ribose polymerase (PARP). Activation of caspases leads to cell demise (Nicholson and Thornberry, 1997) via cleavage of cellular substrates, such as actin (Mashima *et al.*, 1997), fodrin (Martin *et al.*, 1995), PARP (Lazebnik *et al.*, 1994) and gelsolin (Kothakota *et al.*, 1997). By processes

that are not altogether clear, poly (ADP) ribosylation of variety of proteins facilitates DNA repair. Activation of PARP by DNA damage depletes energy stores and thus may prevent apoptosis. PARP cleavage, on the other hand, seems to be important to preserve the energetic substrates for apoptotic events. In the present study, imatinib / celecoxib / both accelerated the cleavage of PARP leading to the formation of an 85 kDa product. This cleavage of PARP might then preclude the catalytic domains of PARP being recruited to the sites of DNA damage, and presumably disable PARP from coordinating subsequent repair and maintain genome integrity. Also PARP is known to negatively regulate the Ca^{+2} and Mg^{+2} dependent endonucleases (Yoshihara *et al*., 1975; Yoshihara *et al*., 1974; Tanaka et *al*, 1984). Since imatinib and celecoxib are promoting the PARP cleavage in K562 and IR-K562 cells, it may result in activation of Ca^{+2} and Mg^{+2} dependent endonucleases, which would eventually cleave DNA into oligonucleosomal fragments.

Bcl-2 belongs to a growing family of proteins, which can either inhibit (Bcl-2, Bcl-$X_{L,}$ Bcl-2w, Mcl-1, Bfl-1, A1 etc.) or favor (Bax, Bcl-X_S, Bad, Bak, Bik etc.) apoptosis, which in cells reside predominantly in the outer mitochondrial membrane, endoplasmic reticulum, and the outer nuclear envelope (Adams and Cory 1998; Zamzami *et al*., 1996). Bcl-2 is known to protect cells against apoptosis triggered by a wide range of factors. Activated *bcl-2* gene could prevent apoptosis induced by

Bax, another *bcl-2* family gene (Sakamuro *et al.*, 1995). Enhanced expression of Bcl-2 or of its apoptosis-inhibitory homologs is involved in the pathogenesis of numerous human cancers. Overexpression of Bcl-2 is correlated with the progression of prostate carcinoma (McDonnell *et al.*, 1992). Recent studies also show altered expression of Bcl2 as a mechanism of imatinib ressitance (Dai et al., 2004). Ectopic expression of Bcl-2 was shown to impair apoptotic signaling by inactivating c-Jun NH2-terminal kinase, leading to apoptosis (Herrmann *et al.*, 1997). Bcl-2 regulates apoptosis atleast inpart, due to their capacity to act on mitochondria, perhaps as an endogenous inhibitor of the pore forming protein Bax (Antonsson *et al.*, 1997). Bcl-2 proteins regulate the translocation of mitochondrial ions or proteins (cytochrome *c*) into the cytoplasm (Kluck *et al.*, 1997). Bax might function as a death effector molecule that is neutralized by Bcl-2. However, the inhibitory effect of Bcl-2 on apoptosis is determined by the interaction with Bax, a 21 kDa protein with a degree of homology to Bcl-2. Bcl-2 can form heterodimers with Bax and lose its protective effect. When Bcl-2 is present in excess, cells are protected from apoptosis. However, when Bax is in excess and the homodimers of Bax dominate, cells are susceptible to programmed cell death. So, it appears to be the relative ratios of Bcl-2 and Bax that determine the fate of a cell, rather than the absolute concentrations of either (Oltvai *et al.*, 1993). In the present study, imatinib and

celecoxib treated K562 and IR-K562 cells were analyzed for changes in the levels of Bcl-2 protein. The level of Bcl-2 decreased in imatinib and celecoxib treated cells. However, the expression of Bax showed no apparent changes. The net effect resulted in a lowered ratio of Bcl-2/Bax, which might be responsible for imatinib and celecoxib induced apoptosis in K562 and IR-K562 cells. Similar operation was reported in celecoxib-induced apoptosis in chronic myeloid leukemia cells (Zhang *et al.*, 2006).

Expression of the BCR/ABL oncogene up-regulates multiple downstream signaling pathways, including those mediated by phosphatidylinositol 3-kinase (PI3K)/Akt, Ras/mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription (STAT) (Goldman and Melo, 2003; Steelman et al., 2004). Of these pathways, the PI3K/Akt signaling cascade plays a pivotal role in Abl oncogene-mediated proliferation, survival, and transformation (Skorski et al., 1995; Skorski et al., 1997; Neshat et al., 2000; Kharas et al., 2004). Recent evidence indicates that CML cells were susceptible to the growth-inhibitory effects of the PI3K inhibitor LY294002 but not the MAPK inhibitor PD98059 (Kawauchi et al., 2003). In addition, PI3K inhibitors have been shown to synergize with imatinib mesylate in inhibiting CML cell growth (Klejman et al., 2002). The phosphatidylinositol 3'-

kinase/PDK-1/Akt signaling cascade represents a convergence point for a plethora of receptor tyrosine kinase and cytokine-mediated pathways that regulate cell proliferation and offers a framework to account for the ability of many extracellular trophic factors to maintain cell survival (Cantley 2002; Vivanco and Swayers, 2002; Storz and Toker, 2002). Kinetic and molecular modeling data indicate that celecoxib derivatives exert PDK-1 inhibition by competing with ATP for binding, a mechanism shared by many types of kinase inhibitors (Zhu et al., 2004). In order to determine whether celecoxib has any effect on Bcr-Abl kinase, Western blot and RT-PCR analysis were employed. However, any inhibitory effect of celecoxib on BCR/ABL kinase activity or its expression was not observed. These results indicate that celecoxib-induced apoptosis is not mediated though the inhibition of BCR/ABL kinase directly, but may be mediated though a different mechanism.

To test the hypothesis that COX-2 regulates MDR1 in IR-K562 cells, the PGE_2 release and the expression of MDR1 and COX-2, at both mRNA and protein level was analyzed. Treatment with celecoxib resulted in the significant decline in the levels of PGE_2 release, and thus inhibition of COX-2, and MDR1 expression both at mRNA and protein level compared to the untreated controls. Addition of PGE_2 (6 μg/ml), a key product of COX-2, to the K562 cells containing medium induced the

expression of MDR1 at mRNA and protein level compared to the untreated controls. These results clearly demonstrate that the induction of MDR1 in IR-K562 cells is mediated through COX-2 dependent mechanism. Similar observations were reported in rat glomerular mesangial cells, where in the transfection of COX-2 expression vector resulted in increased expression of MDR1 and its expression decreased with NS-398 treatment (Patel *et al.,* 2002). It was also shown that PGE_2 addition to the culture medium of rat primary hepatocytes upregulated MDR1b mRNA expression and MDR1 dependent transporter activity (Ziemann *et al.,* 2002). Furthermore, structurally different cyclooxygenase inhibitors (Indomethacin, Meloxicam, NS-398) mediated inhibition of EGF-induced MDR1 mRNA overexpression, resulting in enhanced intracellular accumulation of MDR1 substrate, rhodamine 123 in rat primary hepatocyte cultures (Ziemann *et al.,* 2002).

To further understand the regulation of MDR1 expression by COX-2, studies were taken up on transcriptional regulators of MDR1. There are evidences indicating that NF-κB is involved in the transcription of MDR1 in colon cancer cells (Bentires-Alj et al., 2003). Elevated levels of NF-κB have been demonstrated in a number of drug resistant cell lines (Zhou *et al.,* 2007; Ahmed *et al.,* 2007; Camp et al., 2004; Wang and Casidy 2003; Arlt and Schafer, 2002). In view of NF-κB's role in transcription of

drug transporters, further studies were taken up on the activation of NF-κB, a positive regulator of MDR1 expression, in the presence and absence of celecoxib in K562 and IR-K562 cells. Treatment with imatinib and celecoxib inhibited the NF-κB activation.

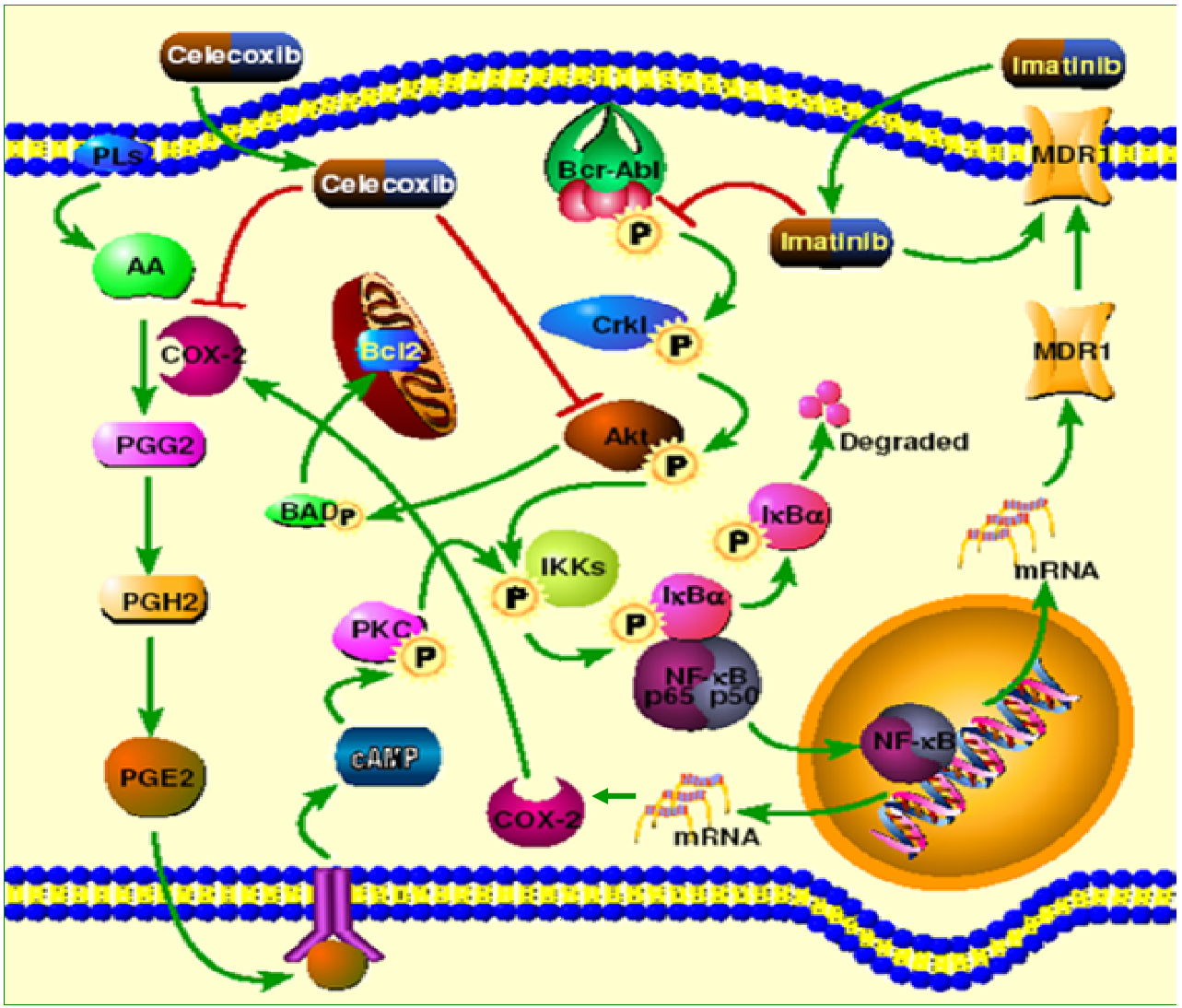
Overexpression of PKC and pAkt are associated with tumorigenesis (Verma et al., 2006; Shukla et al., 2005). Both of them are involved in the activation of NF-κB leading to gene transcription. PKC / NF-κB activation also leads to the COX-2 transcription and thus increase in the PGE_2 levels (Si et al., 2007; Shin et al., 2007). In order to check the upstream signaling leading to NF-κB activation, PKC and Akt/pAkt were studied using Western blot analysis in imatinib and celecoxib treated K562 and IR-K562 cells. Imatinib and celecoxib inhibited PKC activity in both K562 and IR-K562 cells. Both the drugs showed inhibitory effect on pAkt with no effect on the Akt levels. Thus, these results suggest that the antiproliferative signal from imatinib and celecoxib may be transmitted through PKC and Akt/pAkt signaling pathway with NF-κB as the downstream effector molecule.

To check if PKC is involved in COX-2 expression in IR-K562 cells, K562 cells were treated with PGE_2 (6 μg/ml) for 3 days and PKC was analyzed by Western blot analysis. Indeed, PKC levels were increased in K562 cells in presence of PGE_2 than

in untreated controls suggesting its involvement in expression of COX-2. The elevated PKC levels were inhibited upon celecoxib treatment.

In conclusion, this work presents evidence that COX-2 and MDR1 play role in the development of imatinib-resistance in human chronic myeloid leukemia cells and that COX-2 inhibitor, celecoxib, induces apoptosis in imatinib-resistant cells. Imatinib and celecoxib also act in synergy in inducing apoptosis both in K562 and IR-K562. Both imatinib and celecoxib induced ultrastructural changes such as cell shrinkage, formation of membrane blebs, and micronuclei, characteristic of cells undergoing apoptosis. This induction of apoptosis in K562 and IR-K562 cells by imatinib and celecoxib appears to be mediated by cytochrome *c* release, PARP cleavage, Bcl-2 down regulation, inhibition of PKC, pAkt, and NF-κB. Since very high concentrations of celecoxib (40 μM) are required to induce apoptosis in K562 cells, it is suggested that COX-2 independent pathways may be operating in inducing apoptosis. The lowered IC_{50} value of celecoxib (10 μM) in IR-K562 cells compared to that in K562 cells (40 μM) indicate the operation of COX-2 dependent mechanism for the action of celecoxib, more specifically in IR-K562 cells. The overall signal transduction mechanism involved in imatinib and celecoxib induced apoptosis in IR-K562 cells is presented in Fig. 47.

Fig. 47: The overall signal transduction mechanism involved in imatinib and celecoxib induced apoptosis in IR-K562 cells.



Summary

Leukemia is an uncontrolled proliferation of one kind of white blood cell (leukocyte). One of the most common is chronic myelogenous leukemia (also known as chronic myeloid leukemia) or CML. CML arises in a bone marrow stem cell that is the precursor to all the types of blood cells. More than 90% of CML cases are associated with the presence of the Philadelphia chromosome (Ph^+). The Philadelphia chromosome is the result of a reciprocal translocation between 9 and 22 chromosomes that fuses Bcr-encoded sequences to a truncated c-Abl. The fusion protein produced has increased protein tyrosine kinase (TK) activity of Abl that is responsible for the malignancy. The BCR/ABL tyrosine kinase in the cytosol activates various intracellular signaling pathways, those involving Ras, Rap1, B-Raf, Raf-1, Erk, PI-3K, STAT5 and NF-κB, which normally play roles in the regulation of hematopoiesis by hematopoietic cytokines and other extracellular stimuli.

Imatinib mesylate (Gleevec - 2-phenyl amino pyrimidine compound) a specific inhibitor of several TKs, ABL, ABL-related gene product (ARG), c-KIT and PDGF, induces complete hematologic and cytogenetic remissions in most patients with chronic phase CML. Although demonstrating impressive clinical activity against chronic-phase CML, in the accelerated and blast phases of CML the outcome after imatinib therapy is unacceptably poor. Resistant to TK inhibitors was first identified in patients with advanced CML who had a relapse while receiving imatinib. This

resistance was associated with point mutations that rendered ABL kinase resistant to the drug or, less commonly associated with BCR/ABL gene amplification. In addition several other unknown mechanisms may be responsible for the development of resistance against imatinib. These studies emphasize the need to identify novel anti–BCR/ABL therapies to overcome the imatinib resistance in CML patients.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most commonly used drugs in inflammatory diseases, since they are effective in the management of pain, fever, redness, edema arising as a consequence of inflammatory mediator release. Studies have shown that both therapeutic and side effects of NSAIDs are dependent on cyclooxygenase (COX) inhibition. Cyclooxygenases (COX) are the key enzymes that catalyze the conversion of arachidonic acid to prostaglandins and other eicosanoids. COX enzyme had two distinct isoforms with different genetic coding. Although both isoforms had similar amino acid sequence and catalytic activity, they were demonstrated to have different functions. COX-1 is constitutive and cytoprotective, while COX-2 is an inducible enzyme in the inflamed tissues. COX-1 products, prostaglandins, maintain integrity of gastrointestinal system (GIS) by reducing gastric acid secretion, increasing the thickness of mucus layer, stimulating bicarbonate secretion and enhancing mucosal blood flow. Drugs, which inhibit COX-

1 more than COX-2, such as indomethacin, naproxen, ibuprofen, cause more severe damage to the gastric tissues. As a result, studies focused on reduction of the adverse effects of NSAIDs, selective COX-2 inhibitors, such as celecoxib and rofecoxib, have been developed which inhibit COX-2 375 and 800 times more strongly than COX-1, respectively. Epidemiologic and experimental studies have shown that COX-2 inhibitors are effective chemopreventive agents, reducing the risks of many types of tumors, including colon, lung, prostate, and gastric cancers where COX-2 is over expressed. Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, is the only non-steroidal anti-inflammatory drug so far which has been approved by the FDA for adjuvant treatment of patients with familial adenomatous polyposis. Recently, COX-2 inhibitors have also gained attention, either alone or in combination with other chemotherapeutic agents and/or radiation therapy, in the treatment of cancer. Celecoxib exerted antitumor effects in a wide variety of cancers. It also showed synergistic antitumor effects when combined with gemcitaine or 5-fluorouracil in patients with advanced pancreatic cancer, and it enhanced the response to paclitaxel and carboplatin in early-stage non–small cell lung cancer. Celecoxib also exerts antileukemic effects in K562 cells by cell cycle arrest, caspase-3 activation and down regulation of COX-2 expression. These effects of celecoxib were shown to be synergistic with hydroxyurea or imatinib.

Summary

Drug resistance is one of the major factors explaining chemotherapy failure in patients with cancer. Cancer cells possess intrinsic drug resistance to several compounds or they can rapidly acquire it upon chemotherapy. Among the several types of drug resistance, one of the most studied concerns is the over expression of the membrane-associated "ATP binding cassette" (ABC) family transporters. Among mammalian ABC transporters, P-glycoprotein (P-gp) family and multidrug resistance associated protein (MRP) family has a major role in drug transport. P-glycoprotein family consists of two classes: Class I (MDR1 in humans, MDR1a and MDR1b in rodents) and class II (MDR 2 or 3 in humans and MDR 2 in rodents).

Overexpression of MDR1, a membrane P-glycoprotein (P-gp) of 170 kDa expels drugs and xenobiotics in an energy dependent manner, thus reducing their intracellular accumulation and thereby causing drug resistance. To date, efforts to combat the overexpression of MDR1 in the clinic have involved the use of functional modulators or reversal agents that block the MDR1 mediated efflux of anti cancer drugs. Among many different regulators of MDR1 transcription, Reactive Oxygen Species (ROS) and Cyclooxygenase-2 (COX-2) were shown to be important. A direct link between COX-2 and MDR-1 expression has been shown confirming the involvement of COX-2 in the regulation of expression of MDR-1.

In the light of the above scenario, the present study is undertaken to understand the role of COX-2 and MDR-1 in the development of resistance to imatinib in K562 cell line and to analyse the effects of celecoxib alone and in combination with imatinib in inducing apoptosis in imatinib-resistant cells.

COX-2 and MDR1 are over expressed in imatinib-resistant cells

Imatinib-resistant K562 (IR-K562) cells were developed by continuous exposure of K562 cells to increasing concentrations of imatinib. A 100-fold resistance to imatinib was observed in IR-K562 cells. These IR-K562 cells were then employed to understand the mechanism of resistance. The results from the Western blot and RT-PCR analysis demonstrated that over expression of the Bcr-Abl protein or mRNA was not responsible for the development of resistance. No mutations were observed in the kinase domain suggesting a different mechanism for the resistance development. To understand the other possible mechanisms of resistance, MDR-1 and COX-2 were analysed at protein and mRNA levels. The results indicated an over expression of MDR1 at both protein and mRNA level in IR-K562 cells. COX-2 was also over expressed both at protein and mRNA level in IR-K562 cells. These results suggested a possible role of COX-2 and MDR1 in the development of resistance. Knockdown of COX-2 in IR-K562 cells using siRNA demonstrated reversal of resistance to imatinib with 40% inhibition in the growth of cells (IR-K562 +

siRNA) at 100 nM (IC_{50} of imatinib in K562 cells) concentration of imatinib proving that COX-2 over expression is responsible for resistance to imatinib. In addition, cytochrome P450 (CYP1A1) also was over expressed in IR-K562 cells, suggesting the involvement of Phase I drug detoxification systems also in the development of resistance in K562 cells against imatinib. In order to elucidate the pathway through which COX-2 and thereby MDR-1 are over expressed in resistant cells, reactive oxygen species (ROS) levels were measured by FACS. Indeed, ROS levels were greatly increased in IR-K562 cells compared to K562 cells. The levels of NF-κB in the nuclear extract of IR-K562 cells have been elevated by two folds compared to K562 cells, implying that ROS may be responsible for the activation of NF-κB, which positively regulated the expression of MDR1 and COX-2. Further studies indicated that COX-2-induced expression of MDR1 is mediated through PGE_2 and subsequent activation of cAMP-PKC signaling. This was also supported by the results which demonstrated that external addition of PGE_2 to K562 cells increased the expression of MDR1 and PKC suggesting involvement of COX-2 and its products in the development of resistance to imatinib via over expression of MDR1.

Reversal of imatinib resistance in K562 cells by COX-2 inhibitor, Celecoxib

Summary

In the light of a predominant role of COX-2 and MDR1 in the development of imatinib resistance in K562 cells, it was presumed that use of a COX-2 inhibitor, celecoxib, might reverse the drug resistance in these cells. To test this hypothesis, the inhibitory effects of celecoxib alone or in combination with imatinib were studied in imatinib-sensitive and imatinib-resistant K562 cells. These studies have clearly shown the inhibition in the growth of K562 and IR-K562 cells in a dose and time dependent manner. Cell proliferation assay (MTT assay) demonstrated an IC_{50} of 100 nM for imatinib and 40 µM for celecoxib in K562 cells. It was interesting to find a 4-fold decrease in the IC_{50} of celecoxib in IR-K562 cells (10 µM) compared to K562 cells (40 µM). This reduction in the IC_{50} itself suggests that the inhibitory effects of celecoxib are COX-2-dependent in IR-K562 cells and COX-2 independent in K562 cells. Celecoxib at 1 µM concentration enhanced the sensitivity of IR-K562 cells to imatinib by decreasing the IC_{50} of imatinib to 6 µM from 10 µM observed in IR-K562 cells. These results also suggest that the synergism of imatinib and celecoxib on proliferation of IR-K562 cells is due to the inhibition of COX-2 by celecoxib.

Imatinib and celecoxib treated cells (K562 and IR-K562) showed pronounced morphological changes like cell shrinkage, formation of membrane blebs, chromatin condensation and loss of cellular details, characteristic of apoptosis as evidenced by phase-contrast and electron microscopic studies. A ladder-like DNA fragmentation

pattern, a biochemical marker of apoptosis (cleavage of DNA into nucleosomal size fragments of 180-200 bp) was observed in K562 and IR-K562 cells treated with imatinib or celecoxib or both. Flow cytometric analysis of treated cells showed the increase of hypodiploid apoptotic cells and the decrease of the cells at S and G2 phase of cell cycle suggesting the induction of apoptosis. To further examine whether cytochrome *c* is released, or not, into the cytosol in response to imatinib / celecoxib /both treatment, Western blot analysis of the cytosolic proteins was done. These studies have shown a significant increase in the release of cytochrome *c* after the treatment. In the present study, imatinib / celecoxib / both accelerated the cleavage of PARP leading to the formation of 85 kDa product in response to apoptosis. The level of Bcl-2, an anti-apoptotic protein, decreased in imatinib and celecoxib treated cells. However, the expression of Bax, a pro-apoptotic protein, showed no apparent changes. The net effect resulted in a lowered ratio of Bcl-2/Bax, which might be responsible for imatinib and celecoxib induced apoptosis in K562 and IR-K562 cells. In order to determine whether celecoxib has any effect on Bcr-Abl kinase, Western blot and RT-PCR analyses were employed. However, no inhibitory effect of celecoxib on BCR/ABL kinase activity or its expression was not observed. These results indicate that celecoxib-induced apoptosis is not mediated though the inhibition of BCR/ABL kinase directly, but may be mediated though a different

mechanism in both the cell types. Treatment with imatinib and celecoxib inhibited the NF-κB activation. In order to check the upstream signaling leading to NF-κB activation, PKC and Akt/pAkt were studied using Western blot analysis in imatinib and celecoxib treated K562 and IR-K562 cells. Imatinib and celecoxib inhibited PKC activity in K562 and IR-K562 cells. Both the drugs showed inhibitory effect on pAkt with no effect on the Akt levels. Thus, these results suggest that the antiproliferative signal from imatinib and celecoxib may be transmitted through PKC and Akt/pAkt signaling pathway with NF-κB as the downstream effector molecule.

In conclusion, this work presents the evidence that COX-2 and MDR1 play a predominant role in the development of imatinib-resistance in human chronic myeloid leukemia cells and that COX-2 inhibitor, celecoxib, induces apoptosis in imatinib-resistant cells. Imatinib and celecoxib also act in synergy in inducing apoptosis both in K562 and IR-K562. Since over expression of COX-2 is responsible partly for imatinib resistance, celecoxib, a selective COX-2 inhibitor can form a potential candidate for enhancing the sensitivity of imatinib-resistant cells to imatinib and thus ultimately leading to the improved therapy of imatinib-resistant CML. However, further studies are required to test the efficacy of celecoxib on imatinib-resistant CML and other leukemias in animal models.

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1. **Arunasree KM,** Roy KR, Anilkumar K, Aparna A, Reddy GV, Reddanna P (2007). Imatinib-resistant K562 cells are more sensitive to celecoxib, a selective COX-2 inhibitor: Role of COX-2 and MDR-1, *Leukemia Research* (In Press).

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3. Roy KR, **Arunasree KM**, Reddy NP, Dheeraj B, Reddy GV, Reddanna P (2007). Alteration of mitochondrial membrane potential by C-Phycocyanin induces apoptosis in doxorubicin resistant human hepatocellular carcinoma cell line-HepG2. *Biotechnology and Applied Biochemistry* 47:159-167.

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5. Anilkumar K, **Arunasree KM**, Nishantreddy P, Aparna A, Mahipal SVK, Reddy GV, Reddanna P (2007). 15-LOX metabolites 15-(S)-HPETE and 15-(S)-HETE induce apoptosis in acute lymphoblastic leukemia T cells by NADPH Oxidase mediated ROS generation and subsequent activation of extrinsic and intrinsic death pathways. *Archives of Biochemistry and Biophysics* (Submitted).