Chapter-III

Biological Studies of ethyl 2 (1-amino cyclobutyl)-5-(benzoyloxy)-6-hydroxyl pyrimidine-4-carboxylate sulfonamide, carboxamide and 2-amino pyrimidine derivatives
3.0. Ethyl 2-(1-aminocyclobutyl)-5-(benzoyloxy)-6-hydroxy pyrimidine-4-carboxylate sulfonamide and carboxamide derivatives and 2-amino pyrimidine derivatives as anticancer agents

3.0.1. Introduction to cancer

The first cause of cancer was identified by British surgeon Percivall Pott, who discovered it in 1775. Cancer is a dreaded disease which leads to the death of human population in different parts of the world [1]. Cancer affects people of all ages, even fetuses, but the risk increases with age [2]. Cancer causes about 13% of all human deaths [3]. According to the American Cancer Society, 7.6 million people died from cancer in the world during 2007 [4]. Nearly all cancers are caused by abnormalities in the genetic material of the transformed cells [5]. These abnormalities may be due to the effects of carcinogens, such as chewing tobacco, smoking, radiation, chemicals or infectious agents. Other cancer-promoting genetic abnormalities may be randomly acquired through errors in DNA replication or are inherited and thus present in all cells from birth. The heritability of cancer is usually affected by complex interactions between carcinogens and the host's genome. New aspects of the genetics of cancer pathogenesis, such as DNA methylation, and microRNAs are increasingly recognized as important. Genetic abnormalities found in cancer, typically affect two general classes of genes. Cancer-promoting oncogenes are typically activated in cancer cells, giving these cells new properties such as, hyperactive growth and division protection against programmed cell death. Tumor suppressor genes are then inactivated in cancer cells, resulting in the loss of normal functions in those cells, such as accurate DNA replication, control over the cell cycle, orientation and adhesion within tissues, and interaction with protective cells of the immune system.
Diagnosis usually requires the histologic examination of a tissue biopsy specimen by a pathologist, although the initial indication of malignancy can be symptoms or radiographic imaging abnormalities. Cancer is a diverse class of disease which differ widely in their causes and biology. Normally, the body safeguards against cancer via numerous methods, such as apoptosis, helper molecules (some DNA polymerases), possibly senescence etc.. Cancer is thus a progressive disease and slowly accumulates until a cell begins to act contrary to its function in the animal. Thus cancer often explodes in something akin to a chain reaction caused by a few errors. The reason that cancer is so hard to treat is that, even if there were 10,000,000,000 cancerous cells and one killed all, 10 of these cells can still self-replicate or send error-causing signals to other cells, starting the process over again. This rebellion-like scenario is an undesirable survival of the fittest, where the driving forces of evolution itself work against the body’s design and enforcement of order. In fact, once cancer has begun to develop, this same force continues to drive the progression of cancer towards more invasive stages, and is called clonal evolution [6]. Most cancers can be treated and some cured, depending on the specific type, location, and stage. Once diagnosed, cancer is usually treated with a combination of surgery, chemotherapy and radiotherapy. As research develops, treatments are becoming more specific for different types of cancer. There has been significant progress in the development of targeted therapy drugs that act specifically on detectable molecular abnormalities in certain tumors, which minimize the damage to normal cells. The prognosis of cancer patients is mostly influenced by the type of cancer, as well as the stage, or extent of the disease. Cancers are classified by the type of cell that resembles the tumor and, therefore the examples for the general categories are given below:
• **Carcinoma**: Malignant tumors derived from epithelial cells. This group represents the most common cancers, including the forms of breast, prostate, lungs and colon cancer.

• **Sarcoma**: Malignant tumors derived from connective tissue or mesenchymal cells.

• **Lymphoma** and **leukemia**: Malignancies derived from hematopoietic cells

• **Germ cell tumor**: Tumors derived from totipotent cells. In adults most often found in the testicle and ovary, in fetuses, babies, and young children most often found on the body midline, particularly at the tip of the tailbone.

• **Blastic tumor or blastoma**: A tumor (usually malignant) which resembles an immature or embryonic tissue. Many of these tumors are most common in children.

3.0.2. **Leukemia**

Leukemia means "white blood" in Greek. It occurs when there is an excess of abnormal white blood cells in the blood. It is a cancer of blood, namely the bone marrow (the soft inner part of the bone) and the lymph system. In leukemia, abnormal and immature white blood cells are produced in the bone marrow and lymph system. The immature white blood cells are called leukocytes. In some individuals, leukocytes are so numerous that the blood actually has a whitish tinge. When abnormal and immature white blood cells are produced, production of normal cells decreases and the ability to fight infection decreases. The ability to fight infection decreases because the leukemic cells accumulate and lessen the production of oxygen-carrying red blood cells, blood-clotting cells (platelets), and normal leukocytes. If left untreated, the
surplus leukemic cells overwhelm the bone marrow, enter the bloodstream, and will eventually invade other parts of the body, such as the lymph nodes, spleen, liver, brain, and spinal cord. The differentation of leukemic cells is as shown in figure.

**Differentiation of Leukemic cells**

Leukemia is clinically and pathologically subdivided into a variety of large groups. The first division is between its *acute* and *chronic* forms:

- **Acute leukemia** is characterized by the rapid increase of immature blood cells. This crowding makes the bone marrow unable to produce healthy blood cells. Immediate treatment is required in acute leukemia due to the rapid progression and accumulation of the malignant cells, which then spill over into the
bloodstream and spread to other organs of the body. Acute forms of leukemia are the most common forms of leukemia in children.

- **Chronic leukemia** is distinguished by the excessive build up of relatively mature, but still abnormal, white blood cells. Typically taking months or years to progress, the cells are produced at a much higher rate than normal cells, resulting in many abnormal white blood cells in the blood. Whereas acute leukemia must be treated immediately, chronic forms are sometimes monitored for some time before treatment to ensure maximum effectiveness of therapy. Chronic leukemia mostly occurs in older people, but can theoretically occur in any age group. Additionally, the diseases are subdivided according to which kind of blood cell is affected. This divides leukemia into lymphoblastic or lymphocytic leukemia and myeloid or myelogenous leukemia:

- In lymphoblastic or lymphocytic leukemia, the cancerous change takes place in a marrow cell that normally goes to form lymphocytes, which are infection-fighting immune system cells. Most lymphocytic leukemia involve a specific subtype of lymphocyte, the B cell.

- In myeloid or myelogenous leukemia, the cancerous change takes place in a marrow cell that normally goes to form red blood cells, some other types of white cells and platelets. Combining these two classifications provides a total of four main categories:

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Acute</th>
<th>Chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytic leukemia</td>
<td>Acute lymphoblastic leukemia (ALL)</td>
<td>Chronic lymphocytic leukemia (CLL)</td>
</tr>
<tr>
<td>(or &quot;lymphoblastic&quot;)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myelogenous leukemia</td>
<td>Acute myelogenous leukemia (AML)</td>
<td>Chronic myelogenous leukemia (CML)</td>
</tr>
<tr>
<td>(also &quot;myeloid&quot; or &quot;nonlymphocytic&quot;)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.0.3. Chronic myelogenous leukemia

Leukemia and lymphoma are cancers arising from hematopoetic cell lineage (hence: hematological cancers). Leukemia originates from haematopoetic stem cells or cells at different stages of myeloid or erythroid differentiation which spread throughout the body. Hematological cancers can be classified by their derivation from erythroid, myeloid or lymphoid cells at specific stages of development as determined by their morphology and by protein markers. Chronic myelogenous leukemia (CML) is a disease of hematopoetic stem cell characterized by hyper proliferation of often immature cells of the myeloid, megakaryocytic and erythroid lineages. Its diagnostic chromosomal change is the ‘Philadelphia chromosome’ resulting from a translocation between chromosomes 9 and 22 that creates the BCR-Abl fusion gene. The BCR-Abl protein activates several signaling pathways that promote cell proliferation, block apoptosis and decrease cell adhesion, thereby blocking maturation and causing release of immature cells into the blood. After several years, CML turns into blast crisis, a rapidly lethal disease resembling acute leukemia. CML is treated by chemotherapy, interferon therapy, and allogenic stem cell transplantation. An important component of the current therapy is a specific inhibitor of the Abl kinase, imatinib, which represents one of the great successes in molecular cancer research.

Chronic myelogenous leukemia cells
3.0.4. Cancer therapy

In principle, a range of different therapies are available for the treatment of human cancers. Surgery, irradiation, chemotherapy or combination of any of these can be used to treat cancer and type of therapy depends strongly on the classification of the cancer and different types. Surgery and radiation are treatment choices for localized cancers, in contrast, leukemia, lymphomas and metastatic or locally advanced carcinomas and soft tissue cancers require drug chemotherapy and delete is in some cases supplemented by radiotherapy or surgery for primary cancers or metastases. Conversely, surgery can be followed by chemotherapy or irradiation to attack residual local tumor or metastases. This is called ‘adjuvant’ treatment. Accordingly, chemotherapy applied before surgery to eliminate the tumor mass and facilitate its complete resection is often called ‘neo-adjuvant’ treatment.

3.0.5. Chemotherapy

The standard chemotherapy regimens for a cancer are usually designated as ‘first-line’, if it fails, ‘second-line’ of therapy can be attempted. The efficacies of chemotherapy and radiotherapy are extremely dependent on the tumor type. Some cancers, e.g. testicular cancers and certain lymphomas are highly sensitive, whereas renal cell carcinomas appear to be less sensitive than many cancerous tissues. In cancer chemotherapy a wide range of different drugs are employed. Some drugs are targeted at the cancer itself, but other treatments are employed to stabilize specific body functions in the patient or for pain relief.

The most important component in the treatment of many cancers is cytotoxic drug therapy, often simply called chemotherapy. In this kind of therapy, chemical compounds are employed that block DNA synthesis, transcription and/or mitosis in
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the cancer cells, often driving them into apoptosis. A different type of anti-cancer
drugs summarized as ‘biological agents’ bind to receptor molecules in the cancer cells
that are not directly involved in DNA replication or mitosis, but regulate them.
Examples for this type of drugs are hormones and antihormones used in the treatment
of breast cancer and prostate cancer. Since the advent of recombinant DNA
biotechnology, cytokines and growth factors can be produced at reasonable cost and
can be used in cancer therapy.

3.0.6. Molecular Limitation – Cancer therapy

However today, surgery, radiotherapy, and chemotherapy, i.e. ‘scalpel, ray,
and pill’, remain the standard tumor therapies. The cancer drugs that are most widely
used today were basically developed in the 1950’s to 1970’s. With current therapies,
between 30% and 40% of all cancer patients die of their disease. On the other hand,
cure rates are still abysmal for some cancers that spread early and do not respond well
to surgery. Mortalities exceed 95% for pancreatic carcinoma, and 85% for lungs
cancer in the early stages and some acute leukemia in adults. The rates for other
common cancers fall between these extremes. As a rule, carcinomas can be cured by
chemotherapy or by radiotherapy as long as they are confined to the organ where they
originate, Basal cell carcinoma and squamous carcinoma of the skin, e.g., are almost
always detected before they metastasize and can be cured by local surgery,
radiotherapy or drug application. A few formerly generally lethal tumors like
testicular cancer, Wilms tumors and certain hematological cancer respond excellently
to current therapies. In contrast, cure rates for metastasized carcinomas have
generally remained dismal, although present day drug and radiation therapies often
alleviate symptoms and prolonged survival. Regarding this state of things, two conclusions are evident.

1. Cancer prevention is superior to cancer treatment. Therefore, prevention ought to be consigned a high priority. At the least, if cancers cannot be prevented completely, they ought to be detected at an early stage, while cures are still feasible.

2. Better therapies are required, most urgently for advanced stage and/or metastatic carcinomas.

Therefore, cancer therapy is the area, in which expectations are highest for the applications resulting from insights into the molecular biology of cancers. Indeed, novel drugs have been developed based on such insights. Moreover, the understanding of established therapies like cytotoxic chemotherapy and radiotherapy has also been deepened. Finally current status of exploiting cancer is flaming the cancer researcher to set up the current molecular evolution in cancer therapy. This could be achieved by screening a novel anti cancer drug using the previously molecular screened, the older anticancer compounds have been chemically modified or replaced by related substances to increase their \textit{in vivo} efficacy and diminish their toxicity, to increase their solubility etc. These developments are beginning to have a significant impact on cancer therapy.

Cancer has proven to be a difficult disease to treat, and few effective drugs are available. Identification of novel, efficient, selective, and less toxic anticancer agents remains an important and challenging goal in medicinal chemistry. The past two decades have seen a dramatic change in cancer treatment paradigms. A need for effective anticancer therapeutic agents, as well as a well defined pharmacokinetic
property of the drug is felt. Hence, understanding of the molecular mechanisms involved in cancer lead to novel ways in the development of new anticancer compounds. Induction of apoptosis is another indicator for drug activity in cancer cells [7]. Recently many chemotherapeutic compounds have been shown to have antiproliferative effects by inhibiting cell cycle at certain checkpoints. Similarly, cell cycle-mediated apoptosis is also gaining importance because certain compounds are believed to function via this pathway [8]. Furthermore, apoptosis provides a number of clues with respect to effective anticancer therapy, and many chemotherapeutic agents reportedly exert their antitumor effects by inducing apoptosis in cancer cells [9]. Considerable attention has been devoted to the sequence of events with respect to apoptotic cell death and its role in mediation of the lethal effects of the diverse antineoplastic agents. These studies have become a focus of interest in cancer chemotherapy to shed light on the mechanism of action of candidate drugs.

In discovering small anti-cancer agents, a notable role is played by heterocyclic structures, and among these, a growing attention focuses on the synthesis and study of the biological properties of compounds containing various combinations of pyrimidine moieties [10]. An important application of small molecule libraries is the preparation of a directed or focused combinatorial library for assay against a specific biological target. 4-pyrimidines substituted in the 2 position were proven to be biologically very potent and selective [11-17]. Pyrimidine derivatives comprise a diverse and interesting group of drugs [18]. Earlier, a comprehensive review concerning pyrimidines has been published by Brown [19]. A wide spectrum of pharmacological activities has been reported for these compounds. Pyrimidine derivatives also possess wide range of pharmacological action on central nerves system, especially on anti-HIV agents and cyclooxygenase (COX) inhibitors and
ability to inhibit the bacterial enzyme MurB. Based on this background, we decided to explore the pyrimidine pharmacophore as a biomimetic replacement for the phosphate group. It is expected that this would result in highly potent and selective anticancer agents. The design, synthesis and biological study of new anticancer compounds to obtain compounds with enhanced activity are an ongoing research project in our group. We have recently reported the synthesis of novel derivatives of diazaspiro bicyclo hydantoin and induction of apoptosis leukemia cells [20, 21]. We have also developed a series of pyrimidine derivatives and investigated their anticancer properties against Ehrlich ascites tumor cells [22-24]. In this study, we investigated the apoptosis induced by the newly synthesized pyrimidine analogues against human leukemia cells.

3.1. Materials and methods

Human cell lines, K562 and CEM were purchased from National Center for Cell Science, Pune, India. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL of Penicillin, and 100 μg of streptomycin/ml and incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Leukemia (K562 and CEM) cells growing in log phase were treated with 10, 50 and 100 μM concentrations of 2,4,5,6-substituted pyrimidine derivatives IX(a-i) and VIII(a-i). Since the compounds were dissolved in DMSO, it was used as negative control. We employed trypan blue dye 10 exclusion and MTT assay to assess the cytotoxicity. In addition, we have performed cell cycle analysis and DNA fragmentation assay to determine the apoptosis. Each experiment was repeated for a minimum of two times.
3.1.1. Cytotoxicity assay

MTT [3-(4,5-dimethylthiazol-2-yl-2,5-tetrazolium bromide)] assay was used for the measurement of the cytotoxicity of synthesized compounds IX(a-i) and VIII(a-i) as described previously [25]. In brief, exponentially growing K562 or CEM cells (1×10^4 cells/well) were plated in duplicates and incubated with 10, 50 and 100 μM of IX(a-i) and VIII(a-i). Cells were harvested after 48 and 72 h of treatment and incubated with MTT (0.5 mg/ml) at 37 °C. The water soluble tetrazolium salt, [3-(4,5 dimethylthiazol-2-yl-2,5-tetrazolium bromide)] was metabolized to the water insoluble formazan by intact mitochondrial dehydrogenases. The formazan was then solubilized by adding detergent. The viability of the cells was estimated on the basis of formazan formed, which was detected spectrophotometrically by optical density at 570 nm. The mean absorbance of culture medium was used as the blank and was subtracted. IC$_{50}$ values (concentration of compound causing 50% inhibition of cell growth) were estimated after 72 h of compound treatment. The absorbance of vehicle cells was taken as 100% viability and the values of treated cells were calculated as a percentage of control and presented as histograms (Fig. 1 and Fig. 2).

3.1.2. Cell cycle analysis

Cellular DNA content was measured by flow cytometry. Approximately 7.5×10^4 cells/ml were cultured and treated with 10, 50 and 100 μM concentrations of IXe or VIIIf (Fig. 3). Cells were harvested after 48 h of treatment, washed, fixed in 70% ethanol and incubated with RNase a (Sigma-Aldrich, USA). Propidium iodide (PI, 50 μg/ml, Sigma-Aldrich, USA) was added half an hour before acquiring the flow cytometric reading (FACScan, BD Biosciences, USA). A minimum of 10,000 cells were acquired per sample and histograms were analyzed by using WinMDI 2.8 software.
3.1.3. DNA fragmentation assay

To investigate the effect of IXe and VIIIf with respect to induction of oligonucleosomal DNA Fragmentation, DNA fragmentation assay was performed. Briefly, K562 cells were cultured in presence IXe at 10, 50 and 100 μM or VIIIf at 10, 50 and 100 μM for 72 h. Cells were harvested and genomic DNA was extracted using standard protocol. DNA was resuspended in 100 μl of TE buffer. The DNA samples were run on 1% agarose gel and visualized by ethidium bromide staining and photographed (Fig. 4).

3.2. Results and discussion

Recent report has shown that 2,4,5-trisubstituted pyrimidines lead the compounds with potential antitumor activity. They suggest electron donating groups at the 2-position of the pyrimidine will determine the activity. Inspired by this, we derivatized pyrimidine ring by substituting electron withdrawing and electron releasing groups along with cyclobutyl carboxamide IX(a-i) and sulfonamide VIII(a-i) moiety at position 2 of the pyrimidine ring. In addition, we also introduced hydroxyl group at position 6, ethyl carboxylate at position 4 and phenyl carboxylate at position 5 of the pyrimidine. To investigate the cytotoxic effects of 2,4,5,6-tetrasubstituted pyrimidines on the growth of leukemia cells, a dose response study was conducted using MTT assay (Fig. 1 and 2). Results showed that, the cytotoxicity induced by the derivatives IX(a-i) and VIII(a-i) was in a concentration and time dependent manner. Interestingly, the DMSO control corresponding to the highest concentrations of compounds tested did not show any significant toxic effect and it was taken as 100%. The relative percentage was calculated for the treated compounds (Fig.1 and 2). Based on these results IC<sub>50</sub> values were calculated for 72 h and
tabulated in Table 1. Compounds IXc, IXd, IXe, IXf, IXg and IXi in carboxamide series and compounds VIIIb, VIIIc, VIIIId, VIIIIf and VIIIi in sulphonamide series showed good cytotoxicity. As can be seen from Table 1, the electronic property and position of the group on the phenyl ring attached to the carboxamide/sulphonamide moiety determines the activity of these compounds. Among carboxamide derivatives IX(a–i), we found that compounds with halogen, nitro and tert-butyl as substituents on phenyl ring are more cytotoxic compared to unsubstituted (IXh) and methoxy substituted (IXb) derivatives. Compound IXg with chloro at para position on the phenyl ring of carboxamide showed good activity with an IC<sub>50</sub> of 14 µM against K562 cells. Among halogen substitution containing sulfonamide derivatives, compound VIIIc with chloro groups at two meta positions showed good activity (IC<sub>50</sub>: 15µM) compared to a chloro at para position (VIIIId, IC<sub>50</sub>: 28 µM) for K562 cells. Similarly, in nitro group containing sulfonamide derivatives, activity varies with the position. Compound VIIIb with nitro group at meta position exhibited more activity (IC<sub>50</sub>: 26 µM) relative to compound VIIIe with an ortho nitro group (IC<sub>50</sub>: 36 µM) and compound VIIIa with a para nitro (IC<sub>50</sub>: 43 µM) against K562 cells. Besides electron withdrawing, electron releasing groups also played a role in enhancing the activity. Compound VIIIIf with tert-butyl group at para position exhibited 13 more activity than VIIIi containing methyl group at the same position. This could be due to the presence of three electron releasing groups (methyl groups) in VIIIIf and only one electron releasing group (methyl group) in VIIIi. More importantly, alkyl group (methyl) directly attached to the sulfur atom of the sulfonamide also showed modest activity. Interestingly, compound VIIIg containing only phenyl ring lost the activity. This suggest that, electron releasing or electron withdrawing group on the phenyl ring, or the groups directly attached to the sulfonamide moiety contributed to the
enhancement of cytotoxicity of these compounds. Our previous study on the cytotoxic effect of thiazolidinone derivatives suggested that, the electron donating groups on the C-terminal of the phenyl group at 4th position resulted in increasing the activity [25]. Inspired by these results, in this series, we have chosen two molecules IXe and VIIIf containing tert-butyl group at 4th position from carboxamide and sulfonamide derivatives respectively for further studies. Firstly, to evaluate the effect of IXe and VIIIf on cell cycle progression we have carried out flow cytometry analysis. Results showed that both IXe and VIIIf did not induce any apoptosis upto 50 µM. At 100 µM we have seen a remarkable accumulation of subG1 cells followed by the decline of G1, S and G2/M phase cells (Fig. 3). Therefore, our studies further confirm that growth inhibition could be due to apoptosis. The formation of distinct DNA fragments of oligonucleosomal size is a biochemical hallmark of apoptosis in many cells. Hence we were interested to see whether IXe or VIIIf could induce DNA damage. To test this, K562 cells treated with 10, 50 and 100 µM of IXe or VIIIf for 72 hr were harvested and used for extraction of chromosomal DNA. The extracted DNA was run on agarose gel. The observed smear could be the result of DNA breakage at multiple positions across the chromosomal DNA (Fig. 4). The DNA damage induced by compound IXe was in a concentration dependent manner and it was maximum at 100 µM. Compound VIIIf induces limited strand breaks in all the three concentrations tested. Interestingly, we did not see any strand breaks in DMSO control. These results indicate that IXe is able to induce significant amounts of DNA strand breaks including double-strand breaks (DSBs).
Table 1  Structure and IC$_{50}$ values of the synthesized compounds IX(a-i) and VIII(a-i)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>IC$_{50}$ (μM)</th>
<th>K562</th>
<th>CEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IXa</td>
<td><img src="image" alt="Structure IXa" /></td>
<td>45.2 ± 6.8</td>
<td>54.2 ± 7.2</td>
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<tr>
<td>IXb</td>
<td><img src="image" alt="Structure IXb" /></td>
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<tr>
<td>IXc</td>
<td><img src="image" alt="Structure IXc" /></td>
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<tr>
<td>IXd</td>
<td><img src="image" alt="Structure IXd" /></td>
<td>40.0 ± 5.2</td>
<td>34.7 ± 3.0</td>
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<tr>
<td>IXe</td>
<td><img src="image" alt="Structure IXe" /></td>
<td>36.1 ± 3.4</td>
<td>24.3 ± 2.3</td>
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<tr>
<td>IXf</td>
<td><img src="image" alt="Structure IXf" /></td>
<td>50.2 ± 7.1</td>
<td>22.1 ± 2.1</td>
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</tr>
<tr>
<td>IXg</td>
<td><img src="image" alt="Structure IXg" /></td>
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<tr>
<td>IXh</td>
<td><img src="image" alt="Structure IXh" /></td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>IXi</td>
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<td>48.6 ± 7.2</td>
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<tr>
<td>VIIIa</td>
<td><img src="image" alt="Structure VIIIa" /></td>
<td>43.1 ± 6.5</td>
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<tr>
<td>VIIIb</td>
<td><img src="image" alt="Structure VIIIb" /></td>
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<td>45.1 ± 6.8</td>
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</tr>
<tr>
<td>VIIIc</td>
<td><img src="image" alt="Structure VIIIc" /></td>
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<td>25.3 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>VIIIId</td>
<td><img src="image" alt="Structure VIIIId" /></td>
<td>28.4 ± 4.1</td>
<td>30.3 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>VIIIe</td>
<td><img src="image" alt="Structure VIIIe" /></td>
<td>36.5 ± 4.9</td>
<td>42.1 ± 6.5</td>
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<tr>
<td>VIIIIf</td>
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<td>VIIIg</td>
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<tr>
<td>VIIIh</td>
<td><img src="image" alt="Structure VIIIh" /></td>
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<tr>
<td>VIIIi</td>
<td><img src="image" alt="Structure VIIIi" /></td>
<td>24.1 ± 3.1</td>
<td>20.0 ± 3.4</td>
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</table>
Fig. 1 Cytotoxicity as measured by MTT assay. K562 cells treated with 10, 50 and 100 µM of compounds IX(a-i) and VIII(a-i) for 48 and 72 h were harvested and used for the assay. DMSO treated cells were used as vehicle control. Data are representative of the mean of two separate experiments done in duplicate.
Fig. 2

Cytotoxicity as measured by MTT assay. CEM cells treated with 10, 50 and 100 µM of compounds IX(a-i) and VIII(a-i) for 48 and 72 h were harvested and used for the assay. DMSO treated cells were used as vehicle control. Data are representative of the mean of two separate experiments done in duplicate.
Fig. 3 Cell cycle analysis of K562 cells treated with IXe or VIIIIf. K562 cells (0.75X10⁵ cells/ml) were incubated at 37 °C with IXe or VIIIIf (10, 50 and 100 μM). Following 48 h of incubation, cells were fixed and stained with propidium iodide and subjected to FACS analysis. Panel A and B show histograms comparing the effect of IXe and VIIIIf at specific cell cycle stages. In both the Panel A and B, the first histogram represents DMSO treated cells. Panel C and D show the quantification of cells in different stages of cell cycle followed by treatment with IXe and VIIIIf respectively.
Fig. 4 Detection of IXe or VIII{f} induced DNA damage in K562 cells. The chromosomal DNA was extracted from K562 cells following treatment with different concentrations of IXe (a) and VIII{f} (b). The purified DNA was then resolved on a 1% agarose gel at 30 V for 6 h. In both panels, Lane 1: DMSO, Lane 2-4: K562 cells treated with 10, 50 and 100 µM, respectively. “M” is Marker.

3.3. Conclusion

In summary, a series of 2,4,5,6-substituted pyrimidine derivatives were synthesized and evaluated for antiproliferative activity against human leukemia cells. From the current investigation, structure-activity relationships of those compounds suggest that both electron donating and electron withdrawing groups on the phenyl ring attached to the sulfonamide group will determine the anticancer activity. Compounds with halogen substitution at different positions on the phenyl ring of the
aryl carboxamide and sulphonamide showed good cytotoxicity. From DNA fragment analysis, it is confirmed that tert-butyl group containing carboxamide derivative IXe is able to induce significant amounts of DNA strand breaks including double-strand breaks compared to that of the sulphonamide derivative VIIIff. The detailed investigation on the structure-activity relationship should consider the substitution pattern on phenyl ring as a means to lead the discovery for more potent cytotoxic compounds.
3.4. Ethyl 2-(1-aminocyclobutyl)-5-(benzoyloxy)-6-hydroxy pyrimidine-4-carboxylate sulfonamide and carboxamide derivatives and 2-amino pyrimidine derivatives as antioxidant agents

3.4.1. Introduction:

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols [26]. Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which is the cause of rancidity [27]. Antioxidant activity could be measured simply by placing the fat in a closed container with oxygen and measuring the rate of oxygen consumption. However, it was the identification of vitamins A, C, and E as antioxidants that revolutionized the field and led to the realization of the importance of antioxidants in the biochemistry of living organisms [28,29]. The possible mechanisms of action of antioxidants were first explored when it was recognized that a substance with anti-oxidative activity is likely to be one that readily oxidized by itself [30]. Research into how vitamin E prevents the process of lipid peroxidation led to the identification of antioxidants as reducing agents that prevent oxidative reactions, often by scavenging reactive oxygen species before they can damage cells [31].
The structure of the antioxidant vitamin ascorbic acid (vitamin C).

Although oxidation reactions are crucial for life, they can also be damaging. Plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells. As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. However, it is unknown whether oxidative stress is the cause or the consequence of disease. Antioxidants are also widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer and coronary heart disease. Although initial studies suggested that antioxidant supplements might promote health, later, large clinical trials did not detect any benefit and suggested instead that excess supplementation may be harmful [32]. In addition to these uses of natural antioxidants in medicine, these compounds have many industrial uses, such as preservatives in food and cosmetics and preventing the degradation of rubber and gasoline. Oxidative stress is thought to contribute to the development of a wide range of diseases including Alzheimer’s disease [33,34], Parkinson’s disease [35], the pathologies caused by diabetes, [36,37] rheumatoid arthritis, [38] and neurodegeneration in neuron diseases [39]. In many of these cases,
it is unclear if oxidants trigger the disease, or if they are produced as a secondary consequence of the disease or from general tissue damage [40]. One case in which this link is particularly well-understood is the role of oxidative stress in cardiovascular disease. Here, low density lipoprotein (LDL) oxidation appears to trigger the process of atherogenesis, which results in atherosclerosis, and finally cardiovascular disease [41,42]. A low calorie diet extends median and maximum lifespan in many animals. This effect may involve a reduction in oxidative stress [43], while there is some evidence to support the role of oxidative stress in aging model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* [44,45], the evidence in mammals is less clear [46-48]. Indeed, a 2009 review of experiments in mice concluded that almost all manipulations of antioxidant systems had no effect on aging [49]. Diets high in fruit and vegetables, which are high in antioxidants, promote health and reduce the effects of aging. One reason for this might be the fact that consuming antioxidant molecules such as polyphenols and vitamin E will produce changes in other parts of metabolism, so it may be other effects that are the real reason and these compounds are important in human nutrition [50,51].

![Antioxidant neutralizing a free radical](image)

Antioxidant neutralizing a free radical
3.4.2. The oxidative challenge in biology

A paradox in metabolism is that while the vast majority of complex life on earth requires oxygen for its existence, oxygen is a highly reactive molecule that damages living organisms by producing reactive oxygen species [52]. Consequently, organisms contain a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids [53]. In general, antioxidant systems either prevent these reactive species from being formed, or remove them before they can damage vital components of the cell [54]. However, since reactive oxygen species have useful functions in cells, such as redox signaling, the function of antioxidant systems is not to remove oxidants entirely, but instead to keep them at an optimum level [55]. The reactive oxygen species produced in cells include hydrogen peroxide (H$_2$O$_2$), hypochlorous acid (HOCl), and free radicals such as the hydroxyl radical (·OH) and the superoxide anion (O$_2^-$) [56]. The hydroxyl radical is particularly unstable and will react rapidly and non-specifically with most biological molecules. This species is produced from hydrogen peroxide in metal-catalyzed redox reactions such as the Fenton reaction [57]. These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or proteins [58]. Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms [59,60], while damage to proteins causes enzyme inhibition, denaturation and protein degradation [61]. The use of oxygen as part of the process for generating metabolic energy produces reactive oxygen species [62]. In this process, the superoxide anion is produced as a by-product of several steps in the electron transport chain [63]. Particularly important is the reduction of coenzyme Q in complex III, since a highly
reactive free radical is formed as an intermediate ($Q^-$). This unstable intermediate can lead to electron "leakage", when electrons jump directly to oxygen and form the superoxide anion, instead of moving through the normal series of well-controlled reactions of the electron transport chain [64]. Peroxide is also produced from the oxidation of reduced flavoproteins, such as complex I [65]. Although these enzymes can produce oxidants, the relative importance of the electron transfer chain to other processes that generate peroxide is unclear [66,67]. In plants, algae, and cyanobacteria, reactive oxygen species are also produced during photosynthesis [68], particularly under the conditions of high light intensity [69]. This effect is partly offset by the involvement of carotenoids in photoinhibition, which involves these antioxidants reacting with over-reduced forms of the photosynthetic reaction centres to prevent the production of reactive oxygen species [70,71]. Oxidation and UV degradation are also frequently linked, mainly because UV radiation creates free radicals by bond breakage. The free radicals then react with oxygen to produce peroxy radicals which cause yet further damage, often in a chain reaction. Other polymers susceptible to oxidation include polypropylene and polyethylene. The former is more sensitive owing to the presence of secondary carbon atoms present in every repeat unit. Attack occurs at this point because the free radical formed is more stable than one formed on a primary carbon atom. Oxidation of polyethylene tends to occur at weak links in the chain, such as branch points in low density polyethylene.

Antioxidant properties of pyrimidine derivatives have also been reported by several authors [72,73]. Wijtmas et al have reported the synthesis and chain breaking antioxidant activity of novel 6-substituted-2,4-dimethyl-3-pyridinol derivative. They have clearly explained the importance of hydroxyl group and of free radical autooxidation and inhibition of the same by phenolic compounds [74]. In search of
novel pyrimidine derivatives possessing antioxidant activity, we have synthesized and characterized a series of new ethyl 2-(1-aminocyclobutyl)-5-(benzoyloxy)-6-hydroxypyrimidine-4-carboxylate derivatives and evaluated their antioxidant activity.

3.5. Materials and methods

3.5.1. DPPH assay

Free radical scavenging activity of the newly synthesised pyrimidine derivatives VIII(a-i) was studied by the diphenyl picryl hydrazyl (DPPH) assay method [75]. Drug stock solution (1 mM) was diluted to different concentrations in the range of 0.1 µM to 100 µM in methanol. DPPH methanol solution (1 ml, 0.3 mmol) was added to 2.5 ml of drug solutions of different concentrations and allowed to react at room temperature. After 30 min the absorbent values were measured at 518 nm and converted into the percentage antioxidant activity. Methanol was used as the solvent and ascorbic acid as the standard. The inhibitory concentration (IC\textsubscript{50}) value, representing the concentration required to exhibit 50% antioxidant activity, was extrapolated from the graph plotted with percentage inhibition on the y axis and concentration on the x axis (Fig. 5). Results are presented in Table 2. All experiments were carried out in triplicate and at least on two separate occasions. The L-Ascorbic acid was used as positive controls. The inhibition ratio (percent) of the tested compounds was expressed as percentage of DPPH\textsuperscript{•} elimination calculated according to the following equation:

\[
\% \text{ Inhibition} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of tested product}}{\text{Absorbance of control}} \right) \times 100
\]
Fig. 5. Free-radical scavenging activity of three most potent ethyl 2-(1-aminocyclobutyl)-5-(benzoyloxy)-6-hydroxypyrimidine-4-carboxylate derivatives VIII(a-i), viz VIIIe, VIIg and VIIIh, measured using the DPPH assay and compared with Ascorbic acid which is used as standard.

3.5.2. Assay of hydroxyl radical-induced DNA strand scission

This assay was done according to the method of Keum et al. (2000) with minor modifications. The reaction mixture (30 µl) contained 10 mM Tris-HCl 1 mM EDTA buffer (pH 8.0), φX174 RF1 DNA (0.3 µg), and H₂O₂ (0.04 M). Various concentrations of the synthesized molecules dissolved in DMSO (final concentrations of the molecules in each assay were 1 µM, 5 µM, 10 µM, 25 µM, 50 µM, & 100 µM, respectively) were added prior to H₂O₂ addition. Hydroxyl radicals were generated by irradiation of the reaction mixtures at a distance of 5 cm with a 12 W UV lamp (Spectroline, Spectronics Co.). After incubation at room temperature for 20 min, the reaction was terminated by the addition of a loading buffer (0.25% bromophenol blue tracking dye and 40% sucrose), and the mixtures were then analyzed by 0.8%
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Statistical analysis

All results are expressed as mean ± SD. The significance of difference was calculated by Student’s t test and values <0.05 were considered to be significant.

3.6. Results and discussion

The change in color from violet to yellow and subsequent fall in absorbance of the stable radical DPPH• was measured at 518 nm for different concentrations of newly synthesized ethyl 2-(1-aminocyclobutyl)-5-(benzoyloxy)-6-hydroxypyrimidine-4-carboxylate derivatives VIII(a-i) scheme 1, and the results were plotted (Fig. 5). The IC50 values (the concentration required to inhibit radical formation by 50%), were determined from the curves plotted and tabulated (Table 2). Since IC50 is a measure of inhibitory concentration, a lower IC50 value would reflect greater antioxidant activity of the sample. Hence compound VIIIe displayed highest antioxidant activity with lowest IC50 (0.15 µM) among all the tested derivatives, when compared with the IC50 value of standard antioxidant Ascarbic acid (0.08 µM). Compound VIIIa, VIIIb and VIIIc derivatives showed no antioxidant activity. Compound VIIIg (IC50= 0.7 µM) and compound VIIIh (IC50= 1.2 µM) also showed moderate DPPH• radical-scavenging activity.

Furthermore, to illustrate the protective effect of synthesized molecules on DNA strand scission, in this study, the φX174 RF1 DNA cleavage by hydroxyl radicals generated by UV photolysis of H2O2 was measured by agarose gel electrophoresis. In accordance with their potency to inhibit OH• radical in DPPH assay, VIIIe from all the pyrimidine derivatives VIII(a-i) elicited almost anticipated
level of potency in inhibiting \( \text{OH}^\cdot \) radical induced DNA strand scission in vitro. Fig. (6) shows the effect of different concentration of compound \( \text{VIII}e \) on the DNA strand cleavage. Treatment of super coiled DNA with UV plus \( \text{H}_2\text{O}_2 \) led to conversion of the DNA to open circular form (lane 2). However, the compound \( \text{VIII}e \) exhibits a dose-dependent protection of DNA under oxidative stress (lanes 4-8). When the DNA was incubated with 50 \( \mu\text{M} \) of compound \( \text{VIII}e \), the prevention of DNA strand scission was clearly observed (lane 7). Moreover, nearly complete protection by compound \( \text{VIII}e \) was found at a dose of 100 \( \mu\text{M} \) (lane 8).

It is noteworthy that compounds \( \text{VIII}g \) and \( \text{VIII}h \) also showed significant activity to protect DNA from the strand breaking activity of \( \text{OH}^\cdot \) radicals, but at very high dose (1 mM to 20 mM, respectively). Furthermore, a very important observation is that all the molecules had no effect in plasmid conformation when they were tested alone at the highest concentration. Based on the above findings, it would be interesting and worthy to further investigate the potential effectiveness of this type of molecules in prevention and probably in treatment of diseases caused by the overproduction of radicals.
Table 2: IC$_{50}$ values of ethyl 2-(1-aminocyclobutyl)-5-(benzoyloxy)-6-hydroxy pyrimidine-4-carboxylate derivatives VIII(a-i) for their antioxidant activity in DPPH radical scavenging assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIIIa</td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td>NIL</td>
</tr>
<tr>
<td>VIIIb</td>
<td><img src="image2" alt="Chemical Structure" /></td>
<td>NIL</td>
</tr>
<tr>
<td>VIIIc</td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>NIL</td>
</tr>
<tr>
<td>VIIIId</td>
<td><img src="image4" alt="Chemical Structure" /></td>
<td>10.0±0.80</td>
</tr>
<tr>
<td>VIIIe</td>
<td><img src="image5" alt="Chemical Structure" /></td>
<td>0.15±0.02</td>
</tr>
<tr>
<td>VIIIIf</td>
<td><img src="image6" alt="Chemical Structure" /></td>
<td>2.0±0.30</td>
</tr>
<tr>
<td>VIIIg</td>
<td><img src="image7" alt="Chemical Structure" /></td>
<td>0.70±0.02</td>
</tr>
<tr>
<td>VIIIh</td>
<td><img src="image8" alt="Chemical Structure" /></td>
<td>1.2±0.30</td>
</tr>
<tr>
<td>VIIIi</td>
<td><img src="image9" alt="Chemical Structure" /></td>
<td>5.0±0.60</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>-</td>
<td>0.080±0.001</td>
</tr>
</tbody>
</table>
**Fig. 6.** Protection effect of ethyl 2-(1-(2-nitrobenzenesulfonylamino) cyclobutyl)-5-(benzoyloxy)-6-hydroxy pyrimidine-4-carboxylate (VIIIe) on DNA strand scission induced by H$_2$O$_2$ and UV. ϕX174 RFI super coiled DNA was exposed to UV plus H$_2$O$_2$ (lane 2) or plus H$_2$O$_2$ in the presence of final concentrations of 1 µM (lane 3), 5 µM (lane 4), 10 µM (lane 5), 25 µM (lane 6), 50 µM (lane 7), and 100 µM (lane 8) of compound VIIIe. Lane 1 represents native ϕX174 super coiled DNA without any treatment. Arrows indicate distinct forms of bacteriophage DNA: OC (open circular); SC (super coiled).

### 3.7. Conclusion

In conclusion, a series of 2,4,5,6-substituted pyrimidine derivatives were synthesized and evaluated for antioxidant activity. From the current investigation, structure-activity relationships of those compounds suggest both electron donating and electron withdrawing groups on the phenyl ring attached to the sulfonamide group will determine the antioxidant activity. In particular compounds VIIIg and VIIIh showed significant activity to protect DNA from the strand breaking activity of OH$^-$ radicals. Further detailed investigation on the structure-activity relationship should consider the substitution pattern on phenyl ring as a means to lead the discovery for more potent cytotoxic compounds.
3.8. Xanthine oxidase inhibition studies of a novel class of 2-aminopyrimidine

3.8.1 Introduction

Xanthine oxidase (XO) is a homodimer of catalytically independent subunits with an approximate molecular mass of 150 kD each [76]. XO, a form of xanthine oxidoreductase that generates reactive oxygen species [77], is an enzyme that catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. This enzyme plays an important role in the metabolism of purines in some species, including humans [78, 79]. Any defect in purine metabolism results in an increase in the level of uric acid. This eventually leads to the deposition of sodium hydrogen urate monohydrate crystals in joints. The disease associated with this phenomenon is known as gout [80]. Historically, it was known as "The Disease of Kings" [81] or "Rich man's disease" [82]. Moreover, increase in the level of XO also causes renal stone formation and ischemic myocardium and reactive oxygen species (ROS) induced diseases [83]. In humans, inhibition of xanthine oxidase reduces the production of uric acid, and several medications that inhibit xanthine oxidase are indicated for treatment of hyperuricemia and related medical conditions including gout [84]. Xanthine oxidase inhibitors are being investigated for management of reperfusion injury. Xanthine oxidase inhibitors are of two kinds: purine analogues and others. Purine analogues include allopurinol, oxypurinol and tisopurine [85]. Others include febuxostat [86] and inositol. Allopurinol is a main suicide substrate of XO, which upon oxidation yields the product, alloxanthine or oxypurinol (pyrazolo[3,4-d]pyrimidine-2,6-one).
Allopurinol inactivates the enzyme by coordinating irreversibly to the reduced form of XO, thus inhibiting formation of uric acid [87]. Allopurinol has been used widely for the clinical control of uric acid production in gout and Hyperuricemia [88-90]. Although xanthine oxidase/xanthine dehydrogenase inhibitory activity has recently been discovered in some newly synthesized compounds and previously known compounds [91-97], no clinically effective XO inhibitors for the treatment of hyperuricemia have been developed since allopurinol was introduced for clinical use in 1963 [98].
The active site of XO is composed of a molybdopterin unit with the molybdenum atom also coordinated by terminal oxygen (oxo), sulfur atoms and a terminal hydroxide [99]. In the reaction with xanthine to form uric acid, an oxygen atom is transferred from molybdenum to xanthine. The reformation of the active molybdenum center occurs by the addition of water. Like other known molybdenum containing oxidoreductases, the oxygen atom introduced to the substrate by XO originates from water rather than from dioxygen (O2). Although the inhibitor binds very tightly to the enzyme, the inhibition is time dependent [100, 101]. Furthermore, it is necessary to maintain an effective concentration of the inhibitor in the organ because the inhibitor dissociates from the enzyme by spontaneous oxidation of molybdenum Mo (IV) to Mo (VI), with a half-life of 300 min at 25°C, with concomitant recovery of enzyme activity [100]. Apart from allopurinol, several pteridines [102], thiazoles [103], phenyl pyrazoles [104], aryl triazoles [105], and flavonoids [106, 107] are also reported to be inhibitors of xanthine oxidase. In the present study, we have systematically synthesized 2-amino pyrimidine derivatives XII(a-j) and investigated their potency to inhibit the xanthine oxidase.
3.9 Materials and methods

3.9.1. XO activity assay

The enzyme activity was monitored spectrophotometrically by measuring uric acid formation at 293 nm with saturated concentration of xanthine (20 µM) as the substrate (if not mentioned otherwise for a specific experiment) in 1 ml of 50 mM phosphate buffer, pH 7.5, at 25°C [108]. In all cases the reaction was started by the addition of 12 nM of XO.

3.9.2. XO inhibition

Inhibition of XO-catalyzed xanthine to uric acid formation reaction by various 2-amino pyrimidine derivatives XII(a-j) based inhibitors was measured by observing the decrease in the uric acid formation at 293 nm. The assay mixture contained both the substrate and the inhibitor in order to have equal competition of the substrate and inhibitor for the enzyme active site.

3.9.3. Unit definition

One unit of xanthine oxidase is defined as the amount of the enzyme that produces 1 nmol of uric acid in 1 ml of assay volume per min at 25°C or it is monitored as the amount of the enzyme that is responsible for a change in absorbance of 0.011 at 293 nm in 1 ml assay solution in 1 min at 25°C. Concentration of the enzyme was calculated using the molar extinction coefficient of XO (450 nm = 36 mM⁻¹ ·cm⁻¹) [109]. The pH and the concentration of XO were 7.5 and 2.25 units/ml, respectively, for all the assays.

3.10. Results and discussion

We have synthesized 2-aminopyrimidine derivatives XII(a-j) by coupling with different heterocyclic/aromatic substituted acids. The formation of product was
confirmed by $^1$H NMR spectral data which shows the absence of a -NH$_2$ peak which corresponds to the primary amine and presence of an amide -NH peak around $\delta$ value 9-10. The formation of the products was also confirmed by IR and LCMS data. The synthesized molecules were evaluated for their xanthine oxidase inhibition activity by measuring the formation of uric acid and calculated the IC$_{50}$ values (Table 1). A few of the synthesized molecules showed good activity. Compound XIIe showed significant activity with an IC$_{50}$ value of 1.21 $\mu$M. Compounds XIIb, XIIf and XIIj showed good activities with IC$_{50}$ values of 1.65 $\mu$M, 2.30 $\mu$M and 4.30 $\mu$M respectively. Compounds XIIc, XIIg, XIIh and XIII showed moderate activities with IC$_{50}$ values 20.06 $\mu$M, 18.54 $\mu$M, 15.72 $\mu$M and 10.01 $\mu$M respectively. Compounds XIIa and XIId were almost inactive towards xanthine oxidation inhibition. The compound XIIe bearing a free aldehyde group showed most significant activity with an IC$_{50}$ value of 1.21 $\mu$M. The potent activity of this compound may be due to the presence of this aldehyde group which can undergo further oxidation. The standard drug allopurinol that showed the activity with an IC$_{50}$ value of 0.82 $\mu$M is comparable to that of molecule XIIe which is as active as the standard drug allopurinol. Compound XIIb, showed good activity towards XO inhibition with an IC$_{50}$ value of 1.65 $\mu$M is having a fluorine atom as substituent on the phenyl ring and compound XIIf containing a free hydroxyl group on the phenyl ring showed XO inhibition activity with an IC$_{50}$ value of 2.30 $\mu$M. Compound XIIa and XIId showed almost nil activity towards xanthine oxidation inhibition. The poor activity of these may be attributed to the presence of methyl and ethyl groups on the phenyl ring in compound XIIa and oxo and thiaxo groups in compound XIId which are not susceptible for oxidation.
Table 3: IC\textsubscript{50} values of allopurinol and 2-amino pyrimidine derivatives XII(a-j)-based inhibitors of xanthine oxidase (all the IC\textsubscript{50} values were calculated from the percentage inhibition of the xanthine oxidase-mediated conversion of xanthine (10µM) to uric acid).

![Diagram of compound XII(a-j)]

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structure</th>
<th>IC\textsubscript{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XIIa</td>
<td><img src="image" alt="Structure XIIa" /></td>
<td>Nil</td>
</tr>
<tr>
<td>XIIb</td>
<td><img src="image" alt="Structure XIIb" /></td>
<td>1.65±0.01</td>
</tr>
<tr>
<td>XIIc</td>
<td><img src="image" alt="Structure XIIc" /></td>
<td>20.06±2.51</td>
</tr>
<tr>
<td>XIIId</td>
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<td>Nil</td>
</tr>
<tr>
<td>XIIe</td>
<td><img src="image" alt="Structure XIIe" /></td>
<td>1.21±0.05</td>
</tr>
<tr>
<td>XIIf</td>
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<td>2.30±0.03</td>
</tr>
<tr>
<td>XIIg</td>
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<td>18.54±1.66</td>
</tr>
<tr>
<td>XIIh</td>
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<td>15.72±0.13</td>
</tr>
<tr>
<td>XIIi</td>
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</tr>
<tr>
<td>XIIj</td>
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</tr>
<tr>
<td>Allopurinol</td>
<td>-</td>
<td>0.82±0.03</td>
</tr>
</tbody>
</table>
3.11. Conclusion

In conclusion, we have synthesised and characterized a series of 2-amino substituted pyrimidine derivatives XII(a-j) as a new class of potential xanthine oxidase inhibitors. A few of the pyrimidine derivatives showed potent activities comparable to that of allopurinol. In particular, 5-formyl-2-methoxy-N-(pyrimidin-2-yl)benzamide XIIe showed significant inhibitory activity.
3.12. Reference:

2. Dieter Kabelitz, Daniela Wesch, Wei He, Cancer Research., 2007, 67, 05.
82. Gout *The Free Medical Dictionary*