1. Introduction

1.1. Tumours and cancer

Cancer is generally reckoned by common people as one of the scariest diseases, but it is not a single ailment. It is defined medically as a group of more than 100 life threatening diseases which is caused by out-of-control progressive cellular growth (www.insidecancer.org). It can also occur almost in everybody part where cells grow and divide. In addition cancer can affect any human regardless of colour, caste and creed. If the frequency of cancer is looked up globally, a striking observation emerges. In Australia the skin cancer, in Brazil cervical cancer, in China liver cancer, in Canada leukaemia, in Japan stomach cancer, in United Kingdom lung cancer, in USA colon cancer is most prominent. This trend can be related to heredity, life style, exposure to radiation and exposure to carcinogens.

Tumour is used to indicate an abnormal swelling of body part regardless of its pathogenesis. This uncontrolled proliferation leads to either benign (hyperplasia) or malignant (dysplasia) tumour (Freeman, 1985; Ruddon, 2007). By definition a benign tumour does not grow in uncontrolled fashion and also not invade surrounding tissues and metastasis. This type of growth commonly does not possess any serious threat to health if left untreated. In some cases of serious health hazards where it is space-occupying and forms constantly growing lesions in vital organs such as the brain, or cosmetic reason (superficial skin or visible lump) the tumours are removed by surgery. A malignant tumour on the other hand is a serious and often lethal ailment. By definition a malignant tumour has the severe potential of uncontrolled growth and is often prone to metastasis in a distant organ. The major treatment consists of radiation, surgery, chemotherapy or combination of all these three therapies. In addition, some beneficial palliative treatments accompany with the main line treatments.

Solid malignancies form lumps and liquid tumours circulate freely in the blood stream. Cancer can be caused or at least initiated by both external (Carcinogens, tobacco
and radiation) and internal (hormonal effect, inherited mutations or immune deficiency) factors. It can be broadly classified into four classes (Freeman, 1985) as (a) Carcinoma - originated from the cells which cover external or internal body surface as ovarian, lung, colon, breast, cervix, prostate etc. (b) Sarcoma - originated from the cells of supporting or connective tissues as muscle, bone, cartilage etc. (c) Lymphoma - originating from lymphatic nodes and (d) Leukaemia - originated from immature blood cells grown in bone marrow and accumulates in blood stream.

Cancer is the outcome of accumulation of mutations involving any of the tumour suppressor genes, oncogenes and DNA repair genes. This mutation initiates with single nucleotide changes or deletion (or duplication) of normal DNA sequence (Kostova, 2006). This defect in genetic sequence is passed down to daughter cells and subsequent generations proliferate even more rapidly and this anarchist cycle continues to death if left untreated. Cancer cells therefore acquire some special characteristics as (a) growth in absence of growth stimulatory signals, (b) growth in presence of growth inhibitory signals; (c) avoid the programmed cell death (Hanahan and Weinberg, 2000). In addition, cancer cells become angiogenic (formation of new blood vessels) to survive and proliferate. They attract the blood vessels inside the tumour mass to provide essential nutrients, glucose and oxygen uninterrupted and to remove metabolic wastes and CO₂.

The telomeric DNA which resides at the end of the chromosome controls important cellular mechanisms such as (a) frequency of cell growth and division and (b) number of cell cycle before death (www.insidecancer.org/;www.cancer.gov and http://science.cancerresearchuk.org). These specific moieties prevent end to end fusion of chromosomes. The normal cells pass through couples of cycles of growth and division, their telomeric DNA gets shorter and ultimately too short to protect the ends of chromosomal DNA. As a result the fusion of telomeres leads to chromosomal merge and the cell death is induced. To avoid this regular sequence cancer cells turn on telomerase’ (normally expressed only early in embryologic development) and stem cells to a smaller extent. This enzyme keeps the length of telomeres longer and prevents the imminent collapse of cells (Fig.1 -1).
Fig.1-1. Cancer cell proliferation

The unexpected rapidly spreading cells and invasion and or metastasis to different organs other than seed cause most death from human cancer (90%). Invasion takes place by the direct migration and penetration by cancer cells to penetrate into lymphatic and blood vessels, circulate through the blood stream, and then invade normal tissues elsewhere in the body. The cancer cells modify their immediate cellular environment easily by inhibition of growth-halting receptors, over expression of cytokines and proteases, destruction of basement membrane and matrix and ultimately the access to the blood vessels is facilitated. The symptoms of cancer are yet-to-be substantiated though proper documentation of individual patients data which leads to a better diagnosis. Each kind of cancer exhibits variable symptoms inspite of some common indications (a change in a wart or mole; lump or thickening in the breast or testicles; a non-curable cough or coughing blood; a skin core or a persistent sore throat; chronic fatigue, a change in bowel or bladder habits; constant indigestion or trouble swallowing and unusual bleeding or vaginal discharge).

The main handicap to treat cancer is inability to detect it in an early stage. Therefore, regular medical checkups especially for aged people could be the facile key to prevent and treat cancer. For the diagnosis of a cancer usually a sample of the affected
tissue is tested microscopically. With the help of several advanced pathological tests, possible existence of cancer can be anticipated or confirmed. Often a next step is the biopsy, which is the surgical removal of a small piece of tissue for microscopic examination (www.cancer.gov and http://science.cancerresearchuk.org/). In case of leukaemia the blood sample is used for confirmation. Additionally in the post-genomic era, micro arrays may be used to determine specific genes which turned on or off in the sample, or proteomic profiles may be collected for an analysis of protein activity. Therefore, with the help of genomics and proteomics custom-made diagnosis protocol is possible for every patient.

Detailed and careful examination of cancer cells microscopically indicates the different traits. Generally variation in cell size and shape, a large number of irregularly shaped dividing cells, variation in nuclear size and shape, loss of normal tissue organisation, loss of specialised cell features and a poorly defined tumour boundary can be identified. After positive detection of cancer the treatment regime and dosage are determined by medical practitioners. Treatment of each individual can vary with specific type and stage of cancer, though there are certain general procedures to be followed. The main weapons to treat cancer are surgery, radiation and chemotherapy though recently a combined therapy regimen is often followed. Some newer but case-specific techniques are getting more familiar in cancer treatment regimen namely, photodynamic therapy, bone marrow and peripheral bone marrow transplantation therapy, laser treatment, angiogenesis inhibitor therapy, hyperthermia therapy, biological therapy, gene therapy, and targeted therapy (www.insidecancer.org / www. cancer. gov and http://science.cancerresearchuk. org / ).

Chemotherapy uses drugs (organic drugs or metal-containing) to destroy cancer cells often in a non-specific way. These drugs are lethal to healthy fast-growing cells and often induce acute side-effects. Chemotherapy assists to cure, control and ease cancer symptoms. When combined with other modes of treatment chemotherapy can (a) reduce the bulk of tumour lump before surgery or radiation (neo-adjuvant), (b) kill the remaining cancer cells after surgery or radiation (Adjuvant) and (c) destroy recurrent and metastatic
cancer cells. These drugs can be administered using several methods namely injection, intra-arterial (IA), intraperitoneal (IP), intravenous (IV), topically and orally (Hajdu, 2005; Chabner et al., 2005; Hartinger et al., 2006; Hannon, 2007 and Strebhardt et al., 2008).

1.2. Chemotherapy

Chemotherapy has been in medical history from 2000 years back (Hajdu, 2005). Arsenic and mercury concoctions were used in ancient ages as chemotherapeutics and the first book on chemotherapy appeared in the year of 1909, written by Nobel-prize winner Paul Ehrlich. The Saga of chemotherapy started with usage of herbal extracts and animal organs in the prehistoric age and then the turn was of nitrogen mustard and antifolates. The modern era of chemotherapy begins with the approval of alkylating agent, cyclophosphamide in 1959. The first metallodrug, cisplatin, is introduced in medical practice in 1978.

There are several chemotherapeutic agents which can be classified as alkylating agents, proliferation inhibitors, enzyme inhibitors, DNA intercalators and antimetabolites, DNA-synthesis inhibitors and membrane permeability modifiers. Some very common drugs which are widely used in medical practice according to NIH (USA) are doxorubicin, epirubicin, bleomycin, fluorouracil, vinristine, vinblastine, etopside, teniposide, chlorambucil, melphalan, busulfan, carmustine (BCNU), lomustine (CCNU), sterpotozotocin, thiotepa, dacarbazine (DTIC), methotrexate, cytarabine, azaribine, meracaptopurine, thioguanine, actinomycinD, plicamycin, mitomycinC, asparaginase, procarbazine, hydroxyurea, topotecan, irinotecan, gemcitabine, temozolamide, capecitabine, tezacitabine, mechlorethamine, cyclophosphamide, mitoxantrone, and tegafur.

1.2.1. Resistance to chemotherapy

Drug resistance can occur at many levels, including increased drug efflux, drug inactivation, alterations in drug target, processing of drug induced damage and evasion of apoptosis. On the other hand conventional therapy that is based on surgery, radiotherapy, chemotherapy or combination of treatment has various side effects because these drugs do not spare normal cells from their devastating actions generating toxic effects in the patients.
Resistance to chemotherapy limits the effectiveness of anticancer drug treatment. Tumours may be intrinsically drug-resistant or develop resistance to chemotherapy during treatment. Acquired resistance is a particular problem, as tumours not only become resistant to the drugs originally used to treat them, but may also become cross-resistant to other drugs with different mechanisms of action. Resistance to chemotherapy is believed to failure in over 90% of patients with metastatic cancer and resistant micrometastatic tumour cells may also reduce the effectiveness of chemotherapy in the adjuvant setting.

Advances in DNA micro array and proteomic technology and the ongoing development of new targeted therapies have opened up new opportunities to combat drug resistance. This will facilitate the future development of rational combined chemotherapy regimens, in which the newer targeted therapies are used in combination with cytotoxic drugs to enhance chemotherapy activity. The ability to predict response to chemotherapy and to modulate this response with targeted therapies will permit selection of the best treatment for individual patients. Chemotherapy is resistance to antifolates. Hence, the alternative as pterin deaminase.

1.3. Pterin deaminase

Levenberg and Hayaishi (1959) first described the pterin deaminase (E.C.3.5.4.11) - a hydrolase enzyme from the bacteria Alcaligenes metalcaligenes ATCC 132700. The systemic name and synonym of pterin deaminase are 2-amino-4-hydroxypteridine aminohydrolase and acrasinase, respectively. Hydrolases (E.C.3.5) act on carbon-nitrogen bonds (hydrolyze amides, amidines and other C-N bonds) other than peptide bonds, especially on cyclic amidines (E.C.3.5.4). Pterin deaminase catalyzes 2-amino-4-hydroxypteridine in presence of water to produce 2, 4-dihydroxypteridine and ammonia.

Pterin deaminase is widely distributed in both prokaryotes and eukaryotes. This enzyme identified to be involved in the regulation of pteridines in these organisms (Rembold, 1983), also exhibits antitumor (Kusakabe et al., 1979) and antioxidant
activities (Angayarkanni et al., 2010). Pterin deaminase is a water-soluble enzyme (Kusakabe et al., 1974) belonging to the enzyme class hydrolase. The enzyme deaminates the substrate folic acid to products lumazine and ammonia (Kusakabe et al., 1974) (Fig. 1-2).

Systemic name : 2-amino-4-hydroxypteridine aminohydrolase
E.C.No : 3.5.4.11
Synonyms : Acrasinase

![Deamination reaction of pterin deaminase](image)

**Fig.1-2. Deamination reaction of pterin deaminase**

### 1.3.1. Substrates of pterin deaminase

Folic acid, pterin, isoxanthopterin, tetrahydropterin, biopterin, dictyopterin, monapterin, neopterin, 6-methylpterin, acrasin, 6, 7-dimethylpterin, 6-carboxypterin, 6-hydroxymethylpterin, 7-methylpterin, isosepiapterin, sepiapterin, xanthopterin, 2-amino-4-hydroxy pterin.
1.3.2. Microbial source of pterin deaminase

Pterin deaminase is reported in various organisms such as Aspergillus Y8-5 (Kusakabe et al., 1979), Bacillus megaterium (Takikawa et al., 1979; Matsuura et al., 1978), Bacillus subtilis (Tsusue et al., 1978), Alcaligenes faecalis (Mc Nutt, 1963), Rhizopus arrhizus IAM 6052 and Mucor lam prosporus IAM 6114 (Kusakabe et al., 1974). It has also been reported in Dictyostelium discoideum (Wurster et al., 1981), Dictyostelium lacteum (Van Haastert et al., 1982 a, b), Rattus norvegicus (Rembold and Simmersbach, 1969 a), and silkworm (Tsusue, 1967).

1.3.3. Deamination reactions

Four pterin amidohydrolases viz., pterin deaminase and sepiapterin deaminase, dihydropterin deaminase and isoxanthopterin deaminase respectively have been identified to involve in the regulation of pteridines in mammals and Drosophila melanogaster via deamination. Sepiapterin deaminase catalyses the hydrolysis of sepiapterin into dihydroxanthopterin in mammals. Dihydropterin and isoxanthopterin deaminase catalyses the hydrolysis of 7, 8-dihydropterin and isoxanthopterin respectively (Hall et al., 2010; Kim et al., 2009; Rembold, 1983).

Depending on the presence or absence of the different pterin deaminases, the catabolism of pteridines results in either simple forms of pterin or lumazine. Exceptions are the pterin deaminases from rat liver (Rembold and Simmersbach, 1969a) and Drosophila melanogaster (Gyure, 1970) which can catalyse the deamination of both aromatic and reduced pterins. The enzyme is of importance in organisms that are capable of synthesizing their own riboflavin. The properties of pterin deaminase (Rembold, 1983) isolated from various organisms are briefly summarised in Table 1-1.
Table 1-1. Comparison of characteristics of different pterin deaminase

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Optimum pH</th>
<th>Stability</th>
<th>Km</th>
<th>Specificity</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. faecalis pterin deaminase</td>
<td>6.3-6.7</td>
<td>Unstable at pH 7 and 0°C;</td>
<td></td>
<td>Pterin and all 6 - substituted derivatives</td>
<td>p-Chloro mercuri benzoate, NaF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stable at pH 9 and 0°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat liver pterin deaminase</td>
<td>6.5</td>
<td>Unstable at pH 6.5 and</td>
<td>3x10-5</td>
<td>Tetrahydro pterin &gt; pterin</td>
<td>KCN, Lumazine, Azaguanine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>activity decreases 50% in 5 days</td>
<td>50%</td>
<td>&gt;Isoxantho pterin</td>
<td></td>
</tr>
<tr>
<td>P. Americana isoxantho pterin deaminase</td>
<td>6.6</td>
<td>Unstable at pH 7 and 0°C</td>
<td></td>
<td>Isoxantho pterin</td>
<td>KCN, KF, Xanthopterin, Azaguanine</td>
</tr>
<tr>
<td>B. mori isoxantho pterin deaminase</td>
<td>6.6</td>
<td>Unstable at pH 7 and 0°C;</td>
<td>1.2x10-5</td>
<td>Isoxantho pterin</td>
<td>KCN, KF, Xanthopterin, Guanine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>activity decreases 50% in 7 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. mori Sepiapterin deaminase</td>
<td>8.0</td>
<td>stable</td>
<td>5.9x10-4</td>
<td>Sepiapterin deaminase and isosepiapterin</td>
<td>Pterin, Bioterin, Xanthopterin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mol/l</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.3.4. Occurrence and location of pterin deaminase

Pterin deaminase are distributed as intracellular, membrane - bound or extracellular enzyme among the reported organisms. Intracellular pterin deaminase have been reported in various organisms viz., Alcaligenes metalcaligenes and other strains of Alcaligenes (Levenberg and Hayashi, 1959), Rattus norvegicus (Rembold and Simmersbach, 1969a), Apis mellifica (Rembold and Buschmann, 1963; Rembold and Simmersbach, 1969a), Mus musculus (Rembold and Simmersbach, 1969a), Clostridium acidi-urici (Pan and Wurster, 1978; Whitakker and Rabinowitz, 1969). Pseudomonas sp. F01, Pseudomonas sp. F08 (Rappold and Bacher, 1974), Pseudomonas fluorescens UK-1 (Pan and Wurster, 1978; Soini and Backman, 1975), Bacillus megaterium strains IF0 12108 and 63 (Takikawa et al., 1979), Danio rerio (Ziegler et al., 2000).
Chapter I

1.3.5. Pterin deaminase in bacteria and fungi


Pterin deaminases are produced as intracellular, extracellular and membrane bound forms in cellular slime molds Dictyostelium discoideum strains Ne4 and Ax-2, Polysphondylium violaceum (Pan et al., 1975; Pan and Wurster, 1978), Dictyostelium lacteum (Van Haastert et al., 1982). Pterin deaminase is also denoted by researchers as folate deaminase as the enzyme replaces the amino group of folate at the 2-position of the pterin ring with a hydroxyl group (Bernstein and Vandriel, 1980).

Various researchers have also identified other types of pterin deaminases. Levenberg and Hayaishi (1959) have reported guanine deaminase along with pterin deaminase. Mc Nutt (1963) reported isoxanthopterin deaminase, leucopterin deaminase in Alcaligenes faecalis. Tsusue (1967) reported a highly specific sepiapterin deaminase in Bombyx mori. Isoxanthopterin deaminase is also found to be produced by Periplaneta americana. Takikawa et al., (1983) reported the presence of dihydropterin deaminase in Drosophila melanogaster.

1.3.6. Properties of pterin deaminase

Literature review on pterin deaminase signifies that very few works has been attempted to characterize by various researchers. As an overview, pterin deaminase has been found to be highly stable at alkaline pH and at high temperature. The catalytic mechanism of action is irreversible (Levenberg and Hayaishi, 1959). They are highly sensitive to fluorides; activity is enhanced by p-chloromercuribenzoate (Rembold et al., 1969b) and is highly stable against the action of proteases (Bernstein and Vandriel, 1980). Some of the properties of pterin deaminases are briefly described in Table 1-2.
### Table 1-2. Prelude of pterin deaminase properties

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Source organisms</th>
<th>Characteristic features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activating compounds</td>
<td><em>D. discoideum</em></td>
<td>3’ , 5’ –CAMP, folic acid, pterin</td>
<td>Wurster <em>et al.</em>, (1981)</td>
</tr>
<tr>
<td>Molecular weight (kDa)</td>
<td><em>B. megaterium</em>, <em>B. subtilis</em></td>
<td>110</td>
<td>Tsusue <em>et al.</em>, (1979)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Takikawa <em>et al.</em>, (1979)</td>
</tr>
<tr>
<td>Enzyme Isoforms</td>
<td><em>D. discoideum</em></td>
<td>2</td>
<td>Wurster <em>et al.</em>, (1981)</td>
</tr>
<tr>
<td>Temperature Stability (°C)</td>
<td><em>R. norvegicus</em></td>
<td>4</td>
<td>Rembold and Simmersbach, (1969a)</td>
</tr>
<tr>
<td></td>
<td><em>D. lacteum</em></td>
<td>-20</td>
<td>Van Haastert <em>et al.</em>, (1982 a)</td>
</tr>
<tr>
<td>Reaction products (corresponding substrates)</td>
<td><em>B. megaterium</em></td>
<td>Biolumazine (Biopterin)</td>
<td>Klein <em>et al.</em>, (1994)</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas sp.</em>, <em>Aspergillus sp.</em></td>
<td>Lumazine (Folic acid and tetrahydropterin)</td>
<td>Rembold and Simmersbach, (1969a)</td>
</tr>
<tr>
<td></td>
<td><em>B. megaterium</em></td>
<td></td>
<td>Kusakabe <em>et al.</em>, (1979)</td>
</tr>
<tr>
<td></td>
<td><em>R. norvegicus</em></td>
<td></td>
<td>Takikawa <em>et al.</em>, (1979)</td>
</tr>
<tr>
<td></td>
<td><em>D. discoideum</em>, <em>Pseudomonas sp</em></td>
<td>Neolumazine (Neopterin)</td>
<td>Bacher and Rappold, (1980)</td>
</tr>
<tr>
<td></td>
<td><em>D. lacteum</em>, <em>Pseudomonas sp.</em></td>
<td>2-deamino-2-hydroxypterin and water (pterin)</td>
<td>Rembold and Simmersbach, (1969a)</td>
</tr>
<tr>
<td></td>
<td><em>B. megaterium</em></td>
<td></td>
<td>Tsusue <em>et al.</em>, (1967)</td>
</tr>
<tr>
<td></td>
<td><em>B. subtilis</em></td>
<td></td>
<td>Takikawa <em>et al.</em>, (1979)</td>
</tr>
<tr>
<td></td>
<td><em>R. norvegicus</em></td>
<td></td>
<td>Bacher and Rappold (1980), Van Haastert <em>et al.</em>, (1982 b)</td>
</tr>
<tr>
<td>Criteria</td>
<td>Source organisms</td>
<td>Characteristic features</td>
<td>References</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------</td>
<td>----------------------------------------------------------------------------------------</td>
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</tr>
<tr>
<td></td>
<td>Pseudomonos sp.</td>
<td>2-hydroxy-4-oxo-3, 4-dihydropteridine-6 carboxylic acid and ammonia (Pterin-6-carboxylic acid)</td>
<td>Bacher and Rappold (1980)</td>
</tr>
<tr>
<td>Substrate kinetics (Km, mM)</td>
<td>B.megaterium</td>
<td>6-carboxypterin-1.3</td>
<td>Tsusue et al., (1979)</td>
</tr>
<tr>
<td></td>
<td>B.subtilis</td>
<td>Folic acid -0.002, 0.02</td>
<td>Takikawa et al., (1979)</td>
</tr>
<tr>
<td></td>
<td>D.discoideum</td>
<td>Pterin-0.03, 30</td>
<td>Wurster et al., (1981)</td>
</tr>
<tr>
<td></td>
<td>R.norvegicus</td>
<td>Pterin-6-carboxylic acid-0.05</td>
<td>Rembold and Simmersbach, (1969a)</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH Range 5.5 -9</td>
<td>Pseudomonas sp</td>
<td>8.3</td>
<td>Bacher and Rappold (1980)</td>
</tr>
<tr>
<td></td>
<td>B.megaterium</td>
<td>7.3</td>
<td>Takikawa et al., (1979)</td>
</tr>
<tr>
<td></td>
<td>B.subtilis</td>
<td>7.3</td>
<td>Tsusue et al., (1979)</td>
</tr>
<tr>
<td></td>
<td>Aspergillus sp</td>
<td>7.0</td>
<td>Kusakabe et al., (1979)</td>
</tr>
<tr>
<td></td>
<td>Y8-5</td>
<td>6.5</td>
<td>Rembold and Simmersbach, (1969a)</td>
</tr>
<tr>
<td></td>
<td>R.norvegicus</td>
<td></td>
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</tr>
</tbody>
</table>

1.3.7. Application of pterin deaminase

In series of new antitumor drugs (Kusakabe et al., 1974), the anti tumor property exhibited by pterin deaminase was unraveled against leukaemia in cell lines L5178Y. The enzyme exhibit effective antitumor property by completely inactivating folic acid by transforming pterin structure into biologically inactive lumazine. In the year 1976, Kusakabe et al. patented the organisms, production and purification processes of pterin deaminase along with their antitumor property. They have also identified the antitumor property...
activity in vivo conditions using melanoma B16 induced C57BL mice. They have isolated pterin deaminase exhibiting antitumor property from organisms Aspergillus tamari IFO 4287, Aspergillus gymnosaradae IAM 2149, Mucor albo-ater IAM 6141, Mucor lamprosporus IAM 6114 (ATCC No.20410), Rhizopus japonicus IAM 6002 (ATCC No.20409), Rhizopus arrhizus IAM 6052, Aspergillus oryzae var. No.13, Aspergillus Y-8-5 (ATCC No.20413), Aspergillus Y- 43-4 (ATCC No.20414), Penicillium Y- 70-2 (ATCC No.20411) and Penicillium Y- 110-2 (ATCC No.20412).

1.4. Substrates of pterin deaminase

Pteridines are a group of heterocyclic compounds composed of fused pyrimidine and pyrazine rings and with the numbering system (Brown, 1988). There are three main classes of naturally occurring pteridines namely, lumazines, isoalloxazine and pterins. Lumazines and isoalloxazines have oxo-substituents at the 2- and 4-positions with the difference being a phenyl ring annealed in the 6- and 7- position on the isoalloxazine. The most common class of naturally occurring pteridines are the pterins which have an amino group at the 2-position and an oxo-group at the 4-position (Fig.1-3).

![Fig. 1-3. Main classes of naturally occurring pteridines](image)

Pteridines have been known to exist from as early as the 19th century, with the first synthesis and isolation reported in 1857 and 1889, respectively. However, the exact structure of these pteridines were only resolved in 1940 and this discovery subsequently led to a) the isolation, characterisation and synthesis of natural occurring pteridines, (b) the synthesis of derivatives of natural pteridines used as drugs for various diseases.
and (c) the initiation of interdisciplinary collaboration between synthetic chemistry and biological chemistry, giving birth to medicinal chemistry.

1.4.1. Introduction to the natural occurrence of pteridines

Long before their structures were fully resolved, pteridines were identified as yellow pigments in butterflies and insects due to their brightly colored nature. In 1889, two natural pteridines were isolated by Frederick Hopkins from wings of Brimstone butterflies (Brown, 1988). The structure of these pteridines were later resolved to be xanthopterin and leucopterin (Fig. 1-4) further investigation of the Brimstone butterflies pigments led to the isolation of a third pteridine, isoxanthopterin in 1933. However, due to difficulties with elemental analysis, the constitution of all three compounds remained unknown until 1940 when the pteridine nucleus was finally resolved.

![Fig. 1-4. Structure of naturally occurring pteridines isolated from insects](image)

Since then over 50 other naturally occurring pteridines have been isolated and characterised, and the total synthesis of some have been achieved. Naturally occurring pteridines have been isolated from (a) insects, examples include xanthopterin and isoxanthopterin; (b) plants, an example being folic acid; and (c) mammals, an example being neopterin (Brown, 1988). Most of the pteridines isolated belonged to the pterin family and some were realised to be incorporated in a number of redox cofactors.

1.4.2. Non-redox active pterins

Many naturally occurring pterins are incorporated in a number of redox active cofactors and are believed to be redox active themselves, although the mechanism is not fully understood. There are however, pterins that have not been associated with redox
reactions and their biological functions, if any, are largely unknown. In some cases these
pterins have been classified as pigments. Examples of these pigments are xanthopterin
and sepiapterin-C (isolated from insects, Fig. 1-5). More recently xanthopterin, oncopterin
and neopterin have been identified as useful bio-markers for human disease states
(Durdi et al., 2001).

Fig. 1-5. Structure of Non-redox active pterins

Xanthopterin has been found in butterfly wings and in the urine of mammals. It is
the end product of a non-conjugated pteridine compound and inhibits the growth of
lymphocytes produced by concanavalin (Durdi et al., 2001). High levels of xanthopterin
are found in patients with liver diseases and hemolysis and can be used to detect the
presence of these diseases. Sepiapterin-C is found along with biopterin and is believed to
be a by-product in the biopterin metabolic pathway (Sugiura, et al., 1973).

Neopterin has been found to serve as a bio-marker of mammalian cellular immune
system activation (Fuchs et al., 2009). High levels of neopterin are associated with
increased production of reactive oxygen species, commonly found in diseases like HIV,
cancer, arthritis and bacterial infections. Measuring the amount of neopterin in blood
fluids or urine gives an indication on the progression of these diseases.

1.4.3. Redox cofactors incorporating pterins

Pterins are incorporated in a number of redox active cofactors which are essential
to all life; examples include tetrahydrobiopterin (BH4), the molybdenum cofactors
(Moco), flavin and folic acid. All these pterins have a rich redox chemistry which take
place mainly at the pyrazine ring, where the 5, 6 and 7, 8-positions can be reduced and
oxidised to facilitate electron transfer reactions forming new products. Folic acid for an example undergoes reduction at the 7, 8-position to dihydrofolate which can also be reduced at the 5, 6-position to tetrahydrofolate. However, the redox chemistry of most pterins is still not clear as to whether it goes via a 2-electron or 1-electron transfer pathway and this is still being investigated to date (Fig. 1-6).

![Fig. 1-6. Structure of redox cofactors incorporating pterins]

Tetrahydrobiopterin (BH$_4$) is the reduced form of biopterin which is biosynthesised from guanosine triphosphate (GTP) in an enzyme catalysed reaction. Tetrahydrobiopterin functions as the cofactor in enzymes responsible for the production of monoamine neurotransmitters (epinephrine, norepinephrine, dopamine, and serotonin) and nitric oxide production (Moens et al., 2007; Crabtree et al., 2009; Werner-Felmayer, 2002). Conditions such as Alzheimer’s disease, Parkinson’s disease, depression, autism and schizophrenia are believed to arise due to deficiency in BH$_4$. Patients suffering from these diseases are treated with synthetic BH$_4$.

The molybdenum cofactor is biosynthesised from guanosine triphosphate (GTP) and is required in human sulphite oxidase, xanthine oxidase and aldehyde reductase enzymes (Hinton et al., 1990). These enzymes are found in all forms of life except streptomyces and are responsible for the oxidation of toxic substances into less
toxic and easily excretable compounds. Sulfite oxidase is responsible for the oxidation of sulphites into sulphates which are easily excreted. Absence of Moco results in accumulation of toxic levels of sulphites and these results in accumulation of toxic levels of sulphites and these results in death within months of birth, underlying the vital role of the pterin. People suffering from molybdenum cofactor deficiency have recently been treated with a daily dose of cyclic pyranopterin monophosphate (cPMP) which is the precursor in the biosynthesis of Moco.

Folic acid, which is not biosynthesised by humans, is an essential vitamin responsible for mediating the transfer of one-carbon units. It acts as both the acceptor and donor of one-carbon units in a variety of reactions critical in the metabolism of nucleic acids and amino acids (Bailey et al., 1999; Choi et al., 2000). In nucleic acid metabolism, folic acid is vital for the synthesis of DNA from its precursors, thymidine and purines, which is also involved in the methylation of various sites within DNA and RNA. Folic acid is by far the most widely studied and understood pteridine to date and its metabolic pathway is well understood.

1.5. Folate

The name originates from folium (= leaf), because folate was first isolated from spinach leaves in 1941. However, already in 1931 Lucy wills found that anaemic pregnant Indian women could be treated with yeast extract. The active component was later identified as folate.

1.5.1. Chemical structure

Folates are compounds consisting of a pteridine ring, p-aminobenzoic acid and glutamate units (Fig.1-7). The synthetic form, folic acid, is a monoglutamate (Pteroylmono glutamic acid, PGA), while foods mainly contain polyglutamates. Bioactive folates, dihydrofolates (DHF) and tetrahydrofolates (THF), are reduced forms of folic acid with additional glutamate units, dihydropteroyl glutamate (H2PteGlun) or tetrahydropteroyl glutamate (H4PteGlun). The structure of folates vary depending on the oxidation state of the pteridine ring, one-carbon constituents at different positions and the number of glutamate residues (Baugh et al., 1971; Filer et al., 1996).
1.5.2. Biochemistry of folic acid

The chemistry of folic acid is as follows: Folic acid or folate is converted to dihydrofolate (DHF) in the presence of Nicotinamide adenine dinucleotide phosphate (NADPH); dihydrofolate is then converted to tetrahydrofolate by an enzyme dihydrofolate reductase (DHFR). Tetrahydrofolate (FH₄) is the most active form of the folate family and is responsible for the production and maintenance of new cells, for DNA synthesis and RNA synthesis (Fig.1-12) (Bailey et al., 1999; Choi et al., 2000; Klareskog et al., 2004). Tetrahydrofolate is a substrate in a number of single-carbon-transfer reactions. FH₄ is converted to methylene -FH4 by the addition of a methylene group from one of three carbon donors; formaldehyde, serine or glycine. The methylene-FH₄ is then converted to methyl -FH₄ by reduction with NADPH. The methyl group is then transferred to vitamin -B12 cobalamin which in turn is transferred to another acceptor, homocysteine, generating methionine which is an amino acid. The methylene -FH₄ is also used to methylate the pyrimidine base uracil to thymine which is essential for DNA synthesis (Fig. 1-8).

Fig. 1-7. Chemical structure of folic acid
FH4 is very essential in DNA and aminoacid synthesis and its concentration is regulated by the presence of folate and also the activity of DHFR. Folate deficiency (FD) results in many health problems such as neural tube defects in developing embryos and also cancer formation due to lack of nucleotides required for DNA synthesis and repair. During early stages of pregnancy, FH4 is required for proper development of the brain, skull and spinal cord.

The association of neural birth defects with FD have long been realised and folic acid supplements are given to pregnant women, and women who plan to get pregnant are advised to take folic acid supplements three months in advance. Research studies have shown a sharp decrease in birth defects in women who took folic acid supplements during or before pregnancy when compared to the control (Milunsky et al., 1989; Steegers-Theunissen et al., 2009). The success of these studies led to treatment of other diseases associated with FD.
Folate deficiency can be avoided by taking folic acid supplements along with the daily diet, however this is limited to first world countries as many people in developing countries cannot afford to buy these supplements or folate rich food products. Another approach to avoiding FD is by fortification, where folic acid is added to flour in order to increase the levels of folic acid in the diet. Studies have shown that people who eat folate fortified food generally have an increased level of folic acid and are less likely to suffer from diseases associated with FD (Smith et al., 2008; Steegers-Theunissen et al., 2009; LeBlanc et al., 2010). Although fortification of flour and or grains has proved success in a number of countries, not for all countries are performing this practice.

The practice of fortification is so far limited to first world countries, primarily due to cost implications, but other countries are refusing to fortify their food products as there are concerns which link cancer to high level of folic acid. Studies have shown that different race groups absorb folic acid differently and as a result high levels of folic acid are found in their blood streams (Bailey et al., 2010). Although there is no conclusive evidence, high levels of folic acid have been shown to worsen the vitamin B12 deficiency (Baggott et al., 1992). Other studies have also shown that high levels of folic acid in late pregnant women resulted in an increase number of babies developing childhood asthma.

Because folic acid or folate is essential for cell growth and development, diseases such as cancer, bacterial infection and viral infections also require folate to grow and spread. Unlike humans, micro-organisms and plants have a biosynthetic pathway for folate and it came as no surprise that the synthetic compounds to inhibit the production of folate were some of the first therapeutic antibacterial agents.

1.6. Folate and disease

Since folate is necessary for normal cell division, the vitamin is especially important during growth periods and to rapidly dividing cells such as blood cells and tumor cells. Folate deficiency may lead to megaloblastic anemia, characterised by large erythrocytes. Folate requirement is increased during pregnancy, and low intakes have been associated with several adverse birth outcomes. The association between folic acid supplementation before conception and reduced occurrence of neural tube defects is often considered to be one of the
most important findings relating vitamins to disease (Czeizel et al., 1992; Rush, 1994). The mechanism behind this finding is however unknown, but it probably concerns DNA methylation. Recently it was suggested that changes in methylation of the insulin-like growth factor gene are involved (Steegers-Theunissen et al., 2009). Folate may also protect against cardiovascular disease (Ueland et al., 2000; Voutilainen et al., 2004). In addition, it has been related to several other disorders including Alzheimers disease (Seshadri et al., 2002) and bone fractures (Gjesdal et al., 2006). Concerning its potential value in cancer development, research has mainly focused on colorectal cancer (Giovannucci et al., 2002) but studies has also suggested associations with cancers at other sites including cervix, breast, prostate, esophageal and pancreas (Eichholzer et al., 2001, Larsson et al., 2006b, Stevens et al., 2006; Van Guelpen, 2007).

1.6.1. Folate metabolism and cancer

Folate intake of dietary one-carbon sources (e.g. serine, methionine, choline and betaine) may influence carcinogenesis through their involvement in DNA metabolism (Choi et al., 2000). Folate is a coenzyme that carries one carbon units and is thereby of great importance in DNA metabolism (Stover, 2004).

1.6.2. DNA synthesis and repair

5, 10 methylene THF acts as a methyl donor when the pyrimidine thymine is synthesized from uracil. Folate deficiency may lead to changes in DNA, such as misincorporation of uracil, subsequent chromosome breaks and disruption of DNA repair. Unrepaired double strand breaks enhance cellular transformation, and may contribute to an increased risk of cancer (Blount et al., 1997) 5, 10-methylene THF can also be converted to 10-formyl THF and thereby act as a methyl donor in purine synthesis (Lamprecht et al., 2003).

1.6.3. DNA- methylation

5, 10-methylene THF is also converted to 5-methyl THF (the main form of folate in blood). 5-methyl THF functions as a methyl donor when homocysteine is converted into methionone. Diminishing levels of methionine lead to decreased formation of S-adenosylmethionine (SAM), the most important methyl donor in biological reactions.
including DNA methylation (Choi et al., 2000). Changes in DNA – methylation patterns are early events in carcinogenesis and may influence the expression of proto-oncogenes and tumor suppressor genes (Duthie et al., 2004; Kim, 2005).

Changes in DNA methylation patterns involve global genomic hypomethylation, but the changes can also be site specific, such as hypermethylation at cytosine-guanine rich areas (CpG islands) in promoter regions of tumor suppressor genes, causing silencing of these genes (Wajed et al., 2001). Methyl deficiency may increase the activity of DNA methyltransferase, and the enzyme may facilitate methylation of CpG sites (Zhu et al., 1998; Zhu, 2003; Ergul et al., 2003; Semenza et al., 2003). Disturbed DNA methylation may also involve hypometylation of noncoding DNA sequences. Hypomethylation may activate these regions and lead to inappropriate recombination, with subsequent spread of the normally non-coding DNA and chromosomal instability (Lamprecht et al., 2003). Since SAM is a universal methyl donor, another possible mechanism for the involvement of folate in carcinogenesis, is its effect on non-inherited methylation of for example proteins and lipids (Lamprecht et al., 2003).

1.6.4. Dual role of folate in carcinogenesis

Depending on site, timing and dose, folate seems to have differing effects (Kim, 2006 a,b). It is possible that folate intake inhibits the initiation of cancer. On the other hand, folate seems to stimulate the progression and growth of already existing pre-stages of cancer (Song et al., 2000a). Drugs that interfere with folate are therefore often used in cancer treatment. The antifolate methotrexate is for example used to treat cancer and other diseases with rapidly dividing cells, because it inhibits the conversion of dihydrofolate into tetrahydrofolate and thereby DNA synthesis.

1.6.5. MTHFR polymorphisms, folate metabolism and cancer

Single nucleotide polymorphisms (SNPs) are variants in a single base pair that occur with a frequency of above 1% in a population (Adami et al., 2002). SNPs in genes encoding for enzymes in folate metabolism may alter relations between folate and carcinogenesis. Methylenetetrahydrofolate reductase (MTHFR) is an enzyme that catalyses the irreversible conversion of 5, 10-methylene THF to 5- methyl THF.
The MTHFR gene is located at chromosome 1, on the short arm at position 36.3 (1p36.3). Particularly two polymorphisms are associated with changes in the activity of MTHFR. MTHFR 677C>T results in substitution of alanine by valine at position 222 in the amino acid sequence. 1298A>C results in substitution of glutamate by alanine at position 429 in the amino acid sequence. The two polymorphisms are in total linkage disequilibrium, i.e., variants never occur on the same chromosome. The variants have been related to reduced enzyme activity, and subsequent decrease in the conversion of 5,10-methylene THF to 5-methyl THF. Thereby methyl groups for DNA methylation are directed towards DNA synthesis/repair (Chen et al., 2005). The heterozygote 677 CT-variant has about 65% of the enzyme activity of the homozygote wild type, and the homozygote 677 TT may be reduced to only about 30% of the activity of the homozygous 677 CC wild-type (Kang et al., 1991; Frosst, et al., 1995; Engbersen et al., 1995).

The variant has been connected to lower levels of plasma folate (Ma et al., 1996). The 1298 C allele has also been associated with reduced enzyme MTHFR activity (Chango et al., 2000). In addition to genetic influence of the MTHFR activity, concentrations of different metabolites in the folate cycles affect the activity (Guenther et al., 1999; Nijhout et al., 2006; Smulders et al., 2007). The frequency of 677 TT homozygotes in Nordic populations is 5-8% (Nordic Nutrition, 2004). This is somewhat lower than in other European populations (Levine et al., 2000; Sharp et al., 2002). The frequency of the TT-genotype is about 10% among European Whites, but higher in the southern countries (Chambers et al., 2000; Wilcken et al., 2003). Worldwide the frequency range between 3 and 32% and seem to depend on ethnicity. The frequency seems to be highest for groups of Hispanic origin, and lowest for those of African origin (Wilcken et al., 2003).

In a recent American study from the Cancer Prevention Nutrition Cohort, tendencies towards an increased postmenopausal breast cancer risk was observed for the 677T allele, and women with variant alleles from both the 677C>T and 1298A>C were at Higher risk (Stevens et al., 2007). However, most studies have not observed any associations between the 1298A>C SNP and breast cancer (Lissowska et al., 2007; Cheng et al., 2007) and a meta-analysis did not support any overall associations.
(Lissowska et al., 2007). Among women at high risk due to MHT, the 677TT genotype has been associated with 40% lower breast cancer risk (Le Marchand et al., 2004), and among women at high risk due to the BRCA1 gene the 677T allele has been associated with increased breast cancer risk among women below 50 y of age (Jakubowska et al., 2007), whereas the 1298C allele was associated with a decreased risk.

1.7. Folate and cancer

The nature of folates influence upon tumorigenesis was realised in the decade immediately following the vitamin’s structural discovery. As folate’s biochemical role in growth and development became clear, folate presented itself as an important requisite for the proliferation of neoplastic lesions. Subsequently, synthetic folate analogues were developed as antiproliferative agents which became the first cancer chemotherapies (Hoffbrand and Weir, 2001). Notably, the antimetabolite and dihydrofolate reductase inhibitor methotrexate (4-amino-10-methylpteroylglutamic acid), which was developed almost 60 years ago, is still used today conjunction with other chemotherapies to treat cancers such as childhood acute lymphoblastic leukaemia (ALL) and non-hodgkin’s lymphoma (Assaraf, 2007; Ifergan and Assaraf, 2008).

Although the disruption of folate metabolism can be beneficial in limiting tumour growth, folate depletion can also deleteriously affect DNA integrity, invoking a propensity towards a malignant cell phenotype. Low levels of cellular 5, 10-methylene-THF impede thymidylate synthases ability to generate thymidine from uridine, resulting in inadequate amounts of the former and excessive concentrations of the latter. As these two species are chemically similar, uracil becomes a substitute for thymine during DNA synthesis (Olinski et al., 2010). While the cell has mechanisms for excising and replacing misincorporated bases, excessive uracil substitution (up to ten fold) can occur under depleted folate conditions, overwhelming corrective machinery (Blount et al., 1997). This leads to DNA strand branching or breaks, which fragment chromosomes and produce micronuclei (Blount and Ames, 1995; Blount et al., 1997; Duthie et al., 2002). In turn, these chromosomal aberrations manifest in the malignant transformation of affected cell (Melnyk et al., 1999; Crott et al., 2007).
Given that folate depletion can invoke such genetic damage, it is unsurprising that numerous observational studies have associated changes in folate status with a modified risk for a wide variety of human cancers. For the most part, a high folate status is protective and a low folate intake increases the risk of contracting the disease; this is true of breast (Zhang et al., 1999; Zhang et al., 2003; Ericson et al., 2007), ovarian (Kelemen et al., 2004; Larsson et al., 2004) and pancreatic (Larsson et al., 2006c) malignancies. In some tumour types the association is weak, for example lung cancer (Cho et al., 2006), or not significant, as with gastric (Larsson et al., 2006a; Vollset et al., 2007), or prostate cancers (Stevens et al., 2006), although often such observational studies are hampered by a lack of statistical power.

The relationship between folate status and cancer risk is not invariably linear and folate is an important component of the biosynthetic pathways responsible for generating DNA and methylating multiple components of the cell. If a large folate intake may in fact facilitate accelerated tumour growth (Ulrich and Potter, 2007). Thus, excessive folate intake may negate a lower relative risk afforded by a moderate folate status. Furthermore, some studies have reported an increased risk with high folate status as a result of supplementation, reinforcing folates role as a facilitator of cell proliferation (Stolzenberg-Solomon et al., 2006; Ebbing et al., 2009).

This seemingly bimodal relationship between folate and carcinogenesis is exemplified in studies documenting colorectal neoplastic, probably the most thoroughly investigated of all cancers with respect to folate status (Duthie 2010). Collectively, human epidemiological studies that an adequate folate status or folic acid supplementation (Sanjoaquin et al., 2005; Kim, 2007a, b, 2008). There is some evidence of low folate levels conferring a decreased risk, however (Van Guelpen et al., 2006). Large prospective cohorts-approximately 16,000 women followed over 16 years, and over 88,000 women assessed over 14 years, for example-add considerable weight to the supposition that high folate status is protective (Giovannucci et al., 1998; Fuchs et al., 2002). However, recent substantial randomised controlled trials attempting to reduce adenoma recurrence through folic acid supplementation have been conflicting. One study observed a lower incidence
of polyps following supplementation when baseline folate status was low, but found no benefit when participant’s blood folate was already adequate (Wu et al., 2009). This result was not observed in other studies-folate supplementation either had no effect (Logan et al., 2008), or was in fact detrimental, increasing the frequency of advanced lesions found in follow-up endoscopy examinations (Cole et al., 2007).

It has been suggested that the timing of folic acid administration may be important in determining whether the vitamins effects are favourable or harmful (Eichholzer et al., 2006; Kim, 2007b; Ulrich and Potter, 2007). Whereas folate deficiency may predispose tissues to neoplastic transformation and an adequate folate status is protective against this event, neoplasm’s that have already developed may have their growth inhibited by a lower folate status and supplementation may instead increase their proliferative rate. This hypothesis is borne out by animal studies. In colorectal cancer mouse models, folate supplementation suppressed tumour formation if administered prior to the development of aberrant foci, the earliest precursor of cancer. After the development of polyps, tumour progression was enhanced when folate status was high (Kim, 2004; Song et al., 2000a; Song et al., 2000b). Similar observations have been made in rat mammary tumours (Baggott et al., 1992; Kotsopoulos et al., 2003; Lindzon et al., 2009).

1.7.1. Folate targeting

Folic acid (FA, folate or vitamin B9), is a vital nutrient required by all living cells for nucleotide biosynthesis and for the proper metabolic maintenance of 1-carbon pathways (Clifford et al., 1998). Aside from its cofactor role for intracellular enzymes, FA also displays high affinity for the folate receptor (FR), a glycosylphosphatidylinositol-linked protein that captures its ligands from the extra cellular milieu and transports them inside the cell via a non-destructive, recycling endosomal pathway (Kamen et al., 1986; Luhrs et al., 1989). The FR is also a recognised tumor antigen / biomarker (Coney et al., 1991; Campbell et al., 1991; Weitman et al., 1994). Because of this, diagnostic and therapeutic methods which exploit the FRs function are being developed for cancer.

From a mechanistic perspective, the FR functions to concentrate exogenous legends (e.g.folates and folate-drug conjugates) into the cell cytosol by endocytosis
The term endocytosis refers to the process whereby the plasma membrane invaginates and eventually forms a distinct intracellular compartment. The endocytic vesicles (endosomes) rapidly become acidified to allow the FR to release its ligand (Lee et al., 1996). Afterwards, the empty FR returns to the cell surface where it can participate in another round of ligand-mediated endocytosis (Kamen et al., 1988).

1.7.2. FR-positive cancer

Elevated expression of the FR occurs in many human malignancies, especially when associated with aggressively growing cancers (Campbell et al., 1991; Toffoli et al., 1997; Toffoli et al., 1998; Hartmann et al., 2007). Recently, it was proposed that this relationship may possibly be used for prognostic purposes (Hartmann et al., 2007). Non-mucinous ovarian cancer (the majority of ovarian cancers) was the first tumor type to be associated with FR”’over expression’’ (Miotti et al., 1987; Veggian et al., 1989; Campbell et al., 1991) and it was later shown that this antigen was identical to that found on KB tumor cells and in placental tissue (Coney et al., 1991; Campbell et al., 1991). Several studies confirmed that 80-90% of ovarian tumors over-express the FR (Toffoli et al., 1997; Wu et al., 1999; Parker et al., 2005). Other gynaecological cancers also over express the receptor (Wu et al., 1999; Maziarz et al., 1999; Allard et al., 2007; Dainty et al., 2007) as well as pediatric ependymal brain tumors, mesothelioma, and breast, colon, renal and lung tumors (Parker et al., 2005). The FR may also be found associated with cancer, particularly when related to myeloid leukemia and perhaps head and neck carcinomas (Ross et al., 1994; Ross et al., 1999). Taken together, the total number of tumors that express the FR is very large; therefore, FR-targeted strategies could have significant impact on cancer treatment for patients diagnosed with FR-positive disease.

1.8. Antifolates

Antifolates or antifols are compounds commonly used to treat various forms of cancer. They act as antitumour agents by suppressing the effects of folic acid and its derivatives on cellular processes. Antifolate drugs produce an intracellular state of folic acid deficiency in order to inhibit folate-dependant enzymes along the folate metabolic pathway.
The first antifolate drug, aminopterin, is a folic acid analog (4-aminofolic acid) that inhibits dihydrofolate reductase, preventing the reduction of folic acid and dihydrofolic acid to THF.

Methotrexate (MTX) is another analog of folic acid still in use as an antitumour chemotherapeutic that directly inhibits dihydrofolate reductase, as well as inhibiting thymidylate synthase. In light of developed drug resistance to methotrexate, many synthetic compounds that inhibit dihydrofolate reductase and or thymidylate synthase have been screened.

Newer classical antifolates (folate structural analogs) used in cancer chemotherapy include trimetrexate, permetrexed and raltitrexed, while non classical antifolates have also been investigated for functionality as antitumour and antibacterial agents. At some level antifolates hinder the folate metabolic pathway. At the molecular level, the substrates along this pathway are forced to transform into tight-binding inhibitors of the enzyme DHFR. This occurs because of the structural difference of the antifolate.

1.8.1. Structure of antifolate compounds

Instead of having a hydroxyl at the 4-position of the pteridine ring, antifolates have an amino group at this location, changing the way upon which the substrate binds to the enzymes active site. The enzyme involved in this reaction, DHFR is the one that maintains the production of tetrahydrofolates, which are the reduced forms of folate within the cell. These reduced folates become depleted by the presence of antifolates which in fact, use the reduced folate carrier route into the cell to take up the intracellular folates.

Tetrahydrofolates play a crucial part in the formation of the DNA molecule since they are the cofactors that donate a carbon atom in the enzymatic formation of thymidylate and purine nucleotides, essential precursors for the synthesis of DNA. Therefore, the interference of the production of tetrahydrofolates by antifolates inhibits the biosynthesis of essential nucleotides for DNA synthesis. Therefore, when DHFR is reduced by the interference of an antifolate, the amount of intracellular folates is also
reduced, which contribute significantly to the prevention of the formation of nucleotide precursors needed for the synthesis of DNA. The impediment of DNA production eventually leads to cell death, hence causing the antitumour effect of the antifol.

The first generation of drugs targeting the folic acid pathway was the sulphonamides, which were the first synthetic (Fig.1-9) anti-bacterial drugs (Hayes et al., 2007). The sulphonamides inhibit the synthesis of folic acid in bacteria thus hindering its growth. These drugs act by competing with para-aminobenzoic acid (PABA), effectively blocking the addition of L-glutamate which is the final step in the formation of folate. The sulphonamides are still used till this date and over 1000 variations of sulphonamides have been prepared and are used for bacterial infections.

![sulphonamides](image)

**Fig.1-9. Structure of antifolate compound sulphonamides**

The sulphonamides were in effective in treatment of cancer since cancer cells are almost identical to normal human cells and do not have a folate biosynthetic pathway. Cancer cells differ from normal cells in that they have a high rate of cell division and growth, and these require relatively large quantities of folate. Down regulation of folate in these cells will result in reduced cell growth and division. The second generation of drugs targeting the folic acid pathway were the anti folates which were targeting the conversion of DHF to the active FH4 and are commonly known as dihydrofolate reductase inhibitors (DHFR inhibitors).

### 1.8.2. Interference of antifolate drugs in cell cycle

When using antifolate drugs, the cell-cycle must be taken into consideration to ensure its maximum efficacy. Many of the same enzymes and proteins that are involved with folate metabolism fluctuate in accordance with the cell cycle. Infact, the folate-dependant enzymes, such as DHFR, increase during the S-phase of mitosis. Cells in the
resting (Go) phase are less affected by the same amount of antifolate drug than are cells in other stages. Therefore, antifolates are most effective when there are relatively few cells, as in the Go phase. Another important factor is that when using a folate antagonist, the synthesis of DNA in both normal and cancerous cells will be hindered. However, RNA and protein synthesis will take place within the cell. If folates are not replenished, megaloblasts (giant cells) will form and cell death will then increase.

1.9. Zinc finger protein

Zfra (Zinc finger like protein that regulates apoptosis) is a naturally occurring short peptide consisting of 31 aminoacids, which regulates tumor necrosis factor (TNF)-mediated cell death by interacting with receptor adaptor protein TRADD (TNF receptor associated death domain protein) and downstream JNK (c-Jun N-terminal kinase), NF-kB (Nuclear factor kappaB) and WWOX / WOX1 (WWdomain-containing oxidoreductase). Cytochrome c release is generally considered as a pivotal step in apoptosis.

![Zfra in mitochondrial pathway of apoptosis](Fig.1-10_Zfra_in_mitochondrial_pathway_of_apoptosis)
Remarkably, over expressed Zfra induces apoptosis via the mitochondrial pathway, which involves suppression of Bcl-2 and Bcl-xL expression (without causing cytochrome c release), counteracting the apoptotic function of tumor suppressor p53 and WWOX, and dissipation of mitochondrial membrane potential for ultimately leading to cell death (Fig.1-10). So, the zinc finger technology could be used to investigate regulatory regions for mtDNA replication and transcription.

1.9.1. A role of Zfra in the nucleus

Many C2H2 zinc finger proteins are involved in the regulation of gene transcription, growth suppression, and or apoptosis (Furukawa et al., 2001; Kim et al., 2003; Huang et al., 2004). Nuclear localization of this protein is essential for their functions. Supporting data shows that Zfra targets both nuclei and mitochondria for controlling cell growth and apoptosis (Hsu et al., 2005; Hong et al., 2007). For example, UV irradiation up regulates the expression of Zfra, and the protein becomes phosphorylated at Ser8 and then relocates to the nucleus (Hong et al., 2007; Hsu et al., 2008). That is, phosphor-Zfra is found accumulated in the nucleus. Ser8 phosphorylated-Zfra is essential in inducing apoptosis probably starting at the nuclear level. Without Ser8 phosphorylation, no apoptosis occurs (Hsu et al., 2005; Hong et al., 2007; Hsu et al., 2008).

The specific threonine/serine kinase(s), which phosphorylates Zfra, is unknown and remains to be identified. A likely candidate for phosphorylating Zfra is JNK1. JNK1 plays a central role in the MAPK signalling, and it integrates many routes of signalling pathways (Huang et al., 2009). TNF and UV light, for instance, causes JNK1 activation and induces the complex formation of Zfra and JNK1. Whether activated JNK1 phosphorylates ZFra remains to be determined. Alternatively, Zfra may be able to stabilize and induce constitutive JNK1 activation, or cause rapid JNK1 turnover.

Interestingly, phosphor-Zfra undergoes rapid de-phosphorylation and degradation, suggesting that Zfra may affect the functional activation and turnover of its binding proteins. During UV irradiation, Zfra is shown to physically interact with activated p53
and WOX1. That is, UV induces the denovo formation of the Zfra-p53-WOX1 complex for relocating to the nuclei. Whether the endogenous Zfra blocks the apoptotic function of p53 and WOX1 remains to be determined.

1.10. Pterin deaminase as alternative antifolate and therapeutic enzyme in medicine in future

The above mentioned available antifolate, folate targeted chemotherapy and therapeutic enzymes drawbacks stimulated the search for new antitumour chemotherapeutic agents. In January 1976, U.S. Patent No.3, 930,955 was awarded to Kusakabe et al. (1976) for the process of producing pterin deaminases having antitumour activity. These extracellular enzyme produced by Aspergillus species catalyse the hydrolytic deamination of pterin, pteroic and folic acid yielding the corresponding 4-dihydroxycompounds (lumazines) which are incompatible for the DNA synthesis. This enzyme holds much promise in cancer therapy because it will induce a condition of folate deficiency by directly acting on folic acid and its action is independent of the level of DHFR. It also inhibited the growth of L5117Y mouse leukemic cells invitro and walker 256 carcinoma invivo (Kusakabe et al., 1979).
1.1. Reference


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Www.cancer.gov ; Www.insidecancer.org/; Www.who.int; www.cancer.gov