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Preclinical Toxicology of Antimetabolite Drugs Used in Cancer Therapy – Literary Review

A rákterápiában használt antimetabolit gyógyszerek preklinikai toxikológiai vizsgálata - Irodalmi áttekintés

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Abstract

This paper discusses the preclinical toxicology of some of the most commonly used antimetabolite drugs in cancer therapies, from their history up to their current use. The classes of antimetabolite drugs included in this discussion are purine antagonists, pyrimidine antagonists, folic acid antagonists, thymidylate synthase (TS) inhibitors, and phosphoribosylglycinamide formyltransferase (GARFT) inhibitors. These classes of drugs were designed to disrupt the synthesis of DNA, halt the cell cycle, and prevent the further growth of tumours. Purine antagonists used in cancer therapy include mercaptopurine, fludarabine, cladribine, clofarabine, nelarabine, and thioguanine. Their action is to compete with their respective purine analogues for incorporation into DNA synthesis. Pyrimidine antagonists, such as cytarabine, gemcitabine, azacitidine, and decitabine, are similarly competing with their pyrimidine analogues for incorporation into DNA and RNA. The incorporation of these analogues leads to impaired synthesis and, thereby, reduced proliferation of tumour cells. GARFT inhibitors, such as AG2034, dihydrofolate reductase inhibitors, such as methotrexate and pemetrexed, and TS inhibitors, such as fluorouracil and capecitabine, target the enzymes responsible for the *de novo* synthesis of DNA. By inhibiting these enzymes, the creation of key molecules required for the synthesis of DNA is prevented. Some of the drugs in this group of antimetabolites have secondary mechanisms leading to an overall increase in cytotoxic effects and anticancer activity. The clinical efficacy of these drugs is promising in cancer therapy due to the increased proliferation of tumour cells, which makes them a good target. However, most of the drugs currently in use are not specific to tumour cells and can cause adverse side effects and toxicities in normal tissues.

Absztrakt

Ez a cikk a rákterápiákban leggyakrabban használt antimetabolit gyógyszerek preklinikai toxikológiáját tárgyalja, a történetüktől a jelenlegi használatukig. Az antimetabolit gyógyszerek ebben a tárgyalásban a purin antagonisták, pirimidin antagonisták, folsav antagonisták, timidilát-szintáz inhibitorok és GARFT inhibitorok. Ezeket a gyógyszercsoportokat azzal a céllal tervezték, hogy megzavarják a DNS szintézisét, leállítsák a sejtciklust és megakadályozzák a daganatok további növekedését. A rákterápiában használt purin antagonisták közé tartozik a merkaptopurin, fludarabin, kladribin, klofarabin, nelarabin

és tioguanin. Tevékenységük az, hogy versenyeznek a megfelelő purin analógjaikkal a DNS-szintézisbe való beépülésért. A pirimidin antagonisták, mint például a citarabin, gemcitabin, azacitidin és decitabin, hasonlóan versengenek pirimidin analógjaikkal a DNS-be és RNS-be való beépülésért. Ezen analógok beépülése a szintézis károsodásához és ezáltal a tumorsejtek proliferációjának csökkenéséhez vezet. A GARFT-inhibitorok, például az AG2034, a DHFR-gátlók, például a metotrexát és a pemetrexed, valamint a TS-gátlók, például a fluorouracil és a capecitabin a DNS *de novo* szintéziséért felelős enzimeket célozzák. Ezen enzimek gátlása megakadályozza a DNS-szintéziséhez szükséges kulcsmolekulák létrejöttét. Az antimetabolitok ebbe a csoportjába tartozó egyes gyógyszerek másodlagos mechanizmusokkal rendelkeznek, amelyek a citotoxikus hatások és a rákellenes hatás általános növekedéséhez vezetnek. Ezeknek a gyógyszereknek a klinikai hatékonysága ígéretes a rákterápiában a daganatsejtek fokozott proliferációja miatt, ami jó célponttá teszi őket. A jelenleg használt gyógyszerek többsége azonban nem specifikus a tumorsejtekre, és káros mellékhatásokat és toxicitást okozhat a normál szövetekben.

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Abbreviations

dCK - deoxycytidine kinase

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DNA - deoxyribonucleic acid

RNA - ribonucleic acid

dATP - deoxyadenosine triphosphate

dCTP - deoxycytidine triphosphate

dUMP - deoxyuridine triphosphate

dTMP - deoxythymidine triphosphate

FDA - Food and drug administration (USA)

EU - European Union

FR - folate receptor

TS - thymidylate synthase

AICARFT - 5-aminoimidazole-4-carboxamide formyltransferase

DHFR - dihydrofolate reductase

THF - tetrahydrofolate

RFC - reduced folate carrier

PCFT - proton coupled folate transporter

FPGS - folylpolyglutamate synthase

GARFT - phosphoribosylglycinamide formyltransferase

5NT - 5’ nucleotidase

HGPRT - hypoxanthine guanine phosphoribosyltransferase

INTRODUCTION

The field of preclinical toxicology involves many disciplines and collaborative efforts in research [1]. This method of study began, historically, with Paracelsus in 1493 and further developed through the years. Procedures, like the ones we use today, came into practice between the 1940s and 1960s [2]. Any new drug or active substance must be tested on different species to determine its toxicities, effectiveness, and safety before clinical use in humans [1]. Toxicities can be discerned using *in vitro* methods (with cells or cell lines) and/or *in vivo* methods with animals [2]. Cell lines and/or animals should be chosen as such so that their results could be compared to targeted diseases in humans [1]. This is an important step, as this data, along with surveillance and understanding by an expert pharmacologist, is the basis of a successful phase 1 clinical trial [3].

Today, worldwide, cancer is one of the leading diseases affecting the human population [4]. Currently, there are many treatment options for cancer. The most common ones include surgical intervention, radiation therapy, and chemotherapy [5]. However, these current treatments are not specific to neoplastic cells and often attack healthy cells of the patient, resulting in toxic or adverse side effects [4]. Commonly seen side effects include myelosuppression, anaemia, diarrhoea and vomiting, alopecia, weight loss, and fatigue [6, 7]. Carcinogenicity, infertility, and drug resistance are some effects that can be seen in patients undergoing long-term treatment [8]. Further understanding of the molecular biology of tumours, along with improvements in diagnostics, can improve the treatment of cancer. There is ongoing research into finding strategies to increase survival and quality of life for cancer patients [4].

A drug that hinders the regular metabolic pathways of the cells is described as an antimetabolite [9]. Interaction with DNA, which regulates the proliferation of cells, can produce a range of anti-tumour effects [5]. The synthesis of purines and pyrimidines is an essential step in the DNA replication of tumour cells. This provides us with targets that can be used in the therapy of cancer, such as dihydrofolate reductase and thymidylate synthase [9]. As a result, the use of these drugs has been very successful in the creation of modern anti-cancer therapies.

This literature review will describe five main classes of antimetabolites, namely purine antagonists, pyrimidine antagonists, folic acid antagonists, thymidylate synthase (TS) inhibitors, and phosphoribosylglycinamide formyltransferase (GARFT) inhibitors, with a focus on their toxicities recorded in preclinical studies.

The aim of this review is to compile the data from published papers on the basic pharmacology and toxicities of these drugs with a focus on the preclinical evaluations.

LITERATURE REVIEW

1. PURINE ANTAGONISTS (ANALOGUES)

Purines, adenine, and guanine are part of the basic building blocks of the nucleotides of DNA. Evidence of high concentrations of these metabolites in tumour cells prompted the development of purine antimetabolite drugs for anticancer therapy. The intense proliferation of cells is a trademark of cancer, and this has allowed us to target their DNA synthesis pathway. However, these drugs cannot differentiate between healthy and cancerous cells, resulting in toxic effects [10].

The most commonly used antineoplastic drugs within this group today are mercaptopurine, fludarabine, nelarabine and thioguanine, and they will be discussed in this review.

Other drugs that belong to this group but are less commonly used are: cladribine, clofarabine and pentostatin.

1.1. Mercaptopurine

Elion was responsible for the synthesis of 6-mercaptopurine (MCP), which is a transitional substance in the purine synthesis pathway [5]. It is a prodrug of purine analogue hypoxanthine showing good immunosuppressive and antineoplastic effects [11, 12]. It was discovered, through clinical trials, that MCP was highly active and extremely safe in the treatment of acute lymphoblastic leukaemia and has since become a critical part of its treatment [5, 11, 13]. MCP has also received approval for treatment of acute lymphoblastic anaemia from the FDA [11]. It can be used as combination therapy with antifolate drugs, as it does not show any cross-resistance [5]. MCP use is wide in both human and veterinary medicine. Some of the other diseases that can be treated include rheumatoid arthritis, psoriasis, systemic lupus erythematosus, and non-Hodgkin lymphoma [11, 12]. Metabolism of the drug via the purine salvage pathway occurs in the liver and gastrointestinal tract and is further taken up by lymphocytes. Hypoxanthine guanine phosphoribosyltransferase (HGPRT) converts MCP to thioinosine monophosphate (TIMP). This is further converted to thioguanine nucleotides (6TGN) by inosine-5-monophosphate dehydrogenase and then guanine monophosphate synthetase. The hydrolysis of thioinosine triphosphate by inosine

triphosphate converts it to TIMP, in turn increasing 6TGN levels. Another pathway produces inactivated methylated bases, 6-methyl-MCP and 6-methyl-TIMP, by the action of the enzyme thiopurine methyltransferase (TPMT). 6-methyl-TIMP efficiently inhibits purine *de novo* synthesis and lessens the amount of proteins that can be incorporated in the synthesis of DNA [11, 12]. MCP works as an antagonist to the endogenous purine required for DNA replication during the S phase of the cell cycle and further inhibits the synthesis of RNA and proteins. Synthetic thiopurine nucleotides substitute endogenous purines, which halts the synthesis of DNA, RNA, and protein due to intracellular purine deficiency. Without the synthesis of these molecules, the proliferative ability of cells is reduced, and cytotoxic effects are seen [11]. Cytotoxicities caused by short- and long-term treatment with MCP, such as myeloid, hepatic, and renal, are linked to the accumulation of its metabolites, 6TGN and 6-methyl-MCP, within the cells, leading to incorporation within DNA, compromised synthesis of DNA, and cell death [12]. Two studies were done in Wistar rats to determine cytotoxic effects: an acute *oral* toxicity study with single doses and a subacute oral toxicity study over 28 days and a range of doses. In the acute study, three rats were given a single dose of 300 mg/kg, resulting in one mortality. A repeated test was done, and these results were confirmed. The rats were then given a higher dose of 2000 mg/kg, which resulted in three mortalities. During the necropsy of these animals, there were no abnormalities seen in the organs [12]. Doses for the subacute test were determined based on the equivalent of human doses in animals. The selected doses, low, mid, and high, correspond to therapeutic, supratherapeutic, and toxic doses (three times more than therapeutic), respectively. MCP toxicities showed dose-dependent severity. Haematological parameters remained in the normal range during the administration of low doses. The main toxicities seen at high doses are those of the bone marrow, liver, and kidney. Some general effects include reduced appetite, body weight, and dehydration. High doses in the subacute study demonstrated severe central vein and sinusoidal space congestion in the liver, along with increased levels of the enzymes alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALKP), and other liver parameters. The accumulation of MCP metabolites in renal tubules results in architectural changes and can cause internal hydronephrosis. Severe congestion, degeneration of the tubular epithelium, and glomerular atrophy are seen at high doses. Mid-doses show lower levels of degeneration and atrophy. Other hematologic parameters affected by high doses are total erythrocyte count, haemoglobin, and packed cell volume, all of which show a decreased amount [12]. The safety and efficacy of intrathecal (IT) administration of MCP were tested *in vitro* using cell lines from human leukaemia and lymphoma [13]. The

behaviour of MCP demonstrated a dose- and schedule-dependent nature across all three cell lines. Using pharmacokinetic parameters from rhesus monkeys, we derived the dose that would be required to reach desired concentrations in cerebrospinal fluid (CSF) in humans. No immediate signs of neurotoxicity were seen in animals after IT administration [13]. Above MCP concentrations of 10 µmol, *in vitro* data show no increase in cytotoxicity. Increasing the dose demonstrates clinical activity and will increase the risk of irreversible or fatal neurotoxicity [13]. Overall, this method of administration exhibits good activity and shows promise for the treatment of heavily treated meningeal malignancies, such as meningeal leukaemia [13]. A study with Swiss male mice receiving mercaptopurine in two dosing schedules starting from 1 mg/kg/day (single or five successive daily intraperitoneal injections) until observance of lethal effects, to determine the LD_{50} value. Mercaptopurine demonstrated a minor cumulative effect after five daily injections as seen by the lethal dose (LD_{50}) of single dose (275 mg/kg/day) being only slightly higher than that of multiple doses (100 mg/kg/day) [14]. Female Swiss mice and Wistar male rats given a single lethal dose of MCP (500 mg/kg), survived around 5 and 2-3 days on average, respectively. Necropsies of mice and rats demonstrated hepatic necrosis, epithelial lesions of the intestine and depletion of bone marrow. However, some findings were only seen in the autopsy of rats in the thoracic (protein rich fluid) and abdominal spaces (ascites, fibrinous adhesions, peritonitis). Alveolar septal thrombosis, pulmonary oedema and haemorrhage are seen more often in necropsies of those rats sacrificed in the later stages of intoxication. This is a species pulmonary disturbance seen as dyspnoea increasing in severity until death. When dogs were given MCP, all doses administered produced anorexia, loss of weight, diarrhoea (becoming haemorrhagic at fatal doses), jaundice and leukopenia. On necropsy, lesions were seen in liver (microscopic necrotic foci occasionally), bile ducts (distended due to thick bile, with some calcified material) and small intestine (denudation and regenerative changes of epithelial surface, capillary dilation and congestion of villus tips, glandular nuclei swelling and atypia, mucosal lymphocytic infiltration) [14].

In another study involving different mammal species, such as mice (Swiss Webster), rats (male Wistar), dogs (adult mongrel) and cats, the toxicology of MCP was investigated [15]. Blood samples from rodents were from abdominal aorta after sacrifice and bone marrow samples from femur and sternum. Blood samples from dogs were collected from the jugular vein and bone marrow samples from vertebrae and ribs. The findings of these studies are described below.

In mice implanted with sarcoma 180 cells, the doses were given as seven successive daily doses of 50, 100 or 125 mg/kg/day starting 24 hours after implantation of tumours. During treatment, in those mice given doses 100 or 125 mg/kg/day, weight gain and survival time were reduced. Testing of blood revealed granulocytopenia and reticulocytopenia, and evaluation of bone marrow samples revealed hypoplasia, with cellularity depletion ranging from 20-70%. Animals were sacrificed 24 hours after the end of treatment, necropsies performed, and thymic involution was seen. At doses >50 mg/kg, gastrointestinal tract glandular epithelial nuclei swelling was also seen [15].

A total of 150 healthy mice were given doses (250 or 500 mg/kg) of MCP through either oral or intraperitoneal (IP) route. Within 4-7 days after administration, 58% (87) of animals treated died, out of which 8 (5%) were in the first 72 hours. There were 14 (9%) further deaths reported in the second week. The first 6 hours after IP administration of the fatal dose (500 mg/kg) did not yield any significant changes. However, 24-48 hours later, all mice were weak, their appearance was dishevelled, and severe body weight loss was observed. These symptoms progressed in severity and decrease in body weight was found to be 30-40%, along with occurrence of diarrhoea in the terminal animals. Mice surviving post treatment with 125 or 250 mg/kg displayed weight loss in week 1 with recovery in week 2 [15].

Small group of male mice were given 500 mg/kg by oral intubation and then sacrificed in groups of 5 at time points of 24, 48 and 72 hours. Cell counts from blood of animals sacrificed at 72 hours revealed distinct reticulocytopenia and moderate haematocrit elevation. Necropsies at all time points showed no macroscopic tissue lesions. However, on microscopic study at 72 hours demonstrated lesions in sternal bone marrow (hypoplasia, 30-40% cellularity decrease), thymus (involution), small and large intestine (nuclei swelling of glandular and surface epithelium, oedema, congestion, mucosal leukocyte infiltration), with these effects being less prominent in the large intestine [15].

When rats were treated with MCP, they developed major fatalities within 24-48 hours (intraperitoneal injection) and within 72-96 hours (oral). Similar to mice, rats displayed progressing weakness and loss of body weight however the appearance and continuation of dyspnoea from 24 hours after injection was a finding specific to rats. A total of 24 rats were treated with various doses of MCP and sacrificed at different times: group 1 (12 given 2 successive daily doses of 100 mg/kg/day IP and sacrificed at 48 hours), group 2 (6 given same dose but sacrificed at 96 hours), group 3 (6 given 500 mg/kg dose orally and sacrificed at 72 hours). Each animal displayed severe dyspnoea associated with pleural effusions. Out of the 24 animals treated with the above doses, only 6 were found to have lesions in the lungs (5

with scattered grey-yellow areas of consolidation, 1 with oedema and 1 with atelectasis). Necropsies of animals of group 2 and 3 also displayed marked oedema of the thymus and dark red and fluid bone marrow expelled from the femora. Liver lobes of rats in group 3 were rounded and displayed multiple superficial areas of reddish yellow discolouration. Rats of group 1 and 2, given IP injections, had accumulation of peritoneal fluid and adhesions among the organs, which is evidence of peritonitis, which was later confirmed by microscopic observation. Peritonitis was not observed in group 3 (oral administration) and this lesion is likely due to IP mode of administration. Haematological changes such as a depletion of lymphocytes and reticulocytes as well as hyper segmentation of granulocytes was seen. The sternal bone samples taken displayed hypoplasia with a 20-75% decrease in cellularity, and thymic involution was also seen in all three groups. Lesions of the large and small intestine were seen in all rats, being most prominent in those of group 2. The characteristics of the lesions were similar to those seen in mice given the 500 mg/kg dose by oral intubation mentioned above. Hepatic damage was seen in 58% rats in group 1, and all rats in groups 2 and were most prominently seen in group 3 rats. Areas of tissue necrosis mainly in the subcapsular regions involving adjacent lobules with diffuse damage were seen, with necrotic areas seen to be infiltrated by polymorphonuclear leukocyte cells on histological examination. Other organ toxicities were seen in the lung (perivascular and peribronchial tissue oedema) and heart (acute myocarditis, 83% of rats group 3) [15].

When two pairs of cats were given intravenous injections of 50 or 100 mg/kg MCP, there were no acute manifestations noted. Both pairs survived in the following week however, the pair receiving a dose of 100 mg/kg lost weight, became anorexic and appeared unhealthy [15].

The effects of MCP were studied in 10 dogs receiving daily intravenous injections. 2 dogs received a dose of 50 mg/kg/day. Emesis was observed after 3 days, starting from 5 hours after the first dose and continuing sporadically. A decrease in food intake and a resulting weight loss was also seen in both animals. By day 4 of treatment, bloody diarrhoea was exhibited in both cases with one succumbing by the end of this day and the other in poor health was sacrificed for pathological examination. A blood sample of the latter dog taken just before sacrifice displayed a pronounced haemoconcentration, with haematocrit value of 65% as compared to a pretreatment value of 45%. Bright yellow plasma with high concentration of bilirubin was also seen. On necropsy, the primary findings included changes in the kidney (yellow discolouration of medulla), gastrointestinal tract (dark, bloody fluid in small and large intestine; haemorrhages in colon mucosa) and mesenteric lymph nodes

(haemorrhage). In another group of 4 dogs given a lower dose of 25 mg/kg/day for 4 successive days, anorexia and emesis were seen in the first week in 50% of dogs, however there was no development of haemorrhagic diarrhoea. Changes observed in all 4 dogs included weight loss, haemoconcentration, hypochloraemia, hyperbilirubinemia, and increased retention of sodium sulfobromophthalein dye. Dark brown urine (choluria) and yellow colour of buccal and scleral mucosa were also seen. 2 weeks after the start of treatment, 75% demonstrated recovery and continued to improve in the subsequent 2-week observation period. However, 1 dog was severely intoxicated and was sacrificed on day 7, with autopsy revealing yellow discoloration of liver, kidney, and aorta, along with bloody fluid in the small intestine and tarry, hardened stool in the colon. Finally, a group of 4 dogs was given doses of 10 mg/kg/day 9 times over a period of 10 days. All animals in this group survived treatment and displayed only moderate weight loss. A moderate decrease in the levels of granulocytes (38-58% of initial) and lymphocytes were seen in 50% dogs. Haematocrit levels of all animals also decreased by 15-27% of the initial level. Two animals from this group were sacrificed for histopathological study. The findings revealed lesions of the gastrointestinal tract, in the small intestine (widespread denudation of surface epithelium, congested and dilated capillaries on denuded villi tips, glandular nuclei swelling and atypia) and in the colon (moderate glandular atypia). Other histological alterations were seen in only one of the above dogs and included a moderate cellular depletion of bone marrow and small areas of focal hepatic necrosis [15].

1.2. Fludarabine

The success of pyrimidine analogue cytarabine in chemotherapy prompted the search and development of other effective analogues, and this led us to fludarabine monophosphate, a halogenated purine analogue [16]. Fludarabine is created by the addition of fluorine to the 2' location of adenine creating 2-fluoroadenosine (F-ara-A), which demonstrates resistance to deamination [16, 17]. Fludarabine itself is water insoluble, the addition of monophosphate group makes it more soluble allowing for intravenous administration [16, 17]. Therapy with fludarabine currently shows promising results and has major impact in the treatment of B cell malignancies and different leukaemias, such as acute and chronic lymphocytic leukaemia, and singular therapy might be effective in follicular lymphomas [16, 17]. However, the effectiveness in treatment of solid tumours, such as colorectal and ovarian tumours as well as

soft tissue and osseous sarcomas, is not adequate [16]. Due to the negative change of fludarabine it is unable to enter cells, therefore it must be dephosphorylated to F-ara-A. Dephosphorylation mechanism is quick. This can be credited to activity of 5' nucleotidase (5NT) enzyme activity in endothelial cells, erythrocytes, and vascular linings of organs. Uptake of F-ara-A into the cell is facilitated by nucleoside transport systems. Mouse intestinal crypt cells exhibit kinetics in line with low affinity transporters whereas leukaemia cell lines exhibit kinetics in line with both low and high affinity transporters mechanisms [17]. Its distribution in the body is swift followed by monophasic elimination, with an intermediate and prolonged terminal phase. Impaired renal function significantly reduces the elimination, likely resulting in increased toxicity [16, 17]. After administration, fludarabine is dephosphorylated in serum to 9-beta-D-arabinofuranosyl-2-fluoroadenine (F-ara-A), to allow it to be taken up into the cell. Once in the cell, F-ara-A is converted to F-ara-ATP, the pharmacologically active form, through phosphorylation with deoxycytidine kinase (dCK) [17, 18]. This conversion is the rate limiting step in the formation of triphosphate active metabolites. F-ara-ATP, the active metabolite, competes with dATP for incorporation into DNA [17]. Once this form is incorporated into nucleic acids, it inhibits ribonucleotide reductase, which causes a deficiency of deoxynucleotides in intracellular pools [17]. It also causes inhibition of DNA polymerases, primase and ligase thereby causing repression of DNA polymerisation and strong inhibition of DNA synthesis, which is the cause of its cytotoxic effect [16, 17]. A secondary mechanism unique to F-ara-A seen in cell lines, is its incorporation into RNA and triggering the inhibition of its synthesis and transcription [19]. This mechanism is not specific to tumour cells and results in toxicities [18]. Testing of F-ara-A performed on human dermal microvascular endothelial cell lines (HMEC), such as bronchial, alveolar, and gastrointestinal lines, yielded different results. The alveolar cell lines demonstrated apoptosis whereas the gastrointestinal and bronchial epithelial cell lines showed no apoptosis, suggesting that they are resistant to its effect [20]. F-ara induced apoptosis was also seen in about 40% of peripheral blood mononuclear cells (PBMC) collected from healthy human blood [20]. In acute myeloid leukaemia (AML) cells, up to 80% of observed cell death was due to F-ara induced necrosis rather than apoptosis [20]. From these results we can summate the effects of F-ara in activating and damaging endothelial and epithelial cells, with toxicity seen in the epithelial cells of the alveoli but not the bronchi, suggesting that the effect is compartment specific [20]. Although the exact mechanism of the apoptotic effect is not known, it is likely caused due to the integration of F-ara into DNA causing mutations and

ultimately cell death. Another proposed mechanism is the cooperation with cytochrome c and apoptosis protein activating factor 1 to trigger the caspase apoptosis pathway [20].

In a study, 8 groups of healthy male BD2F1 mice were treated with intraperitoneal injection of fludarabine with either single (75-1000 mg/kg) or multiple dose schedule (20-150 mg/kg/dose; every day for 5 days) [21]. Mortalities due to toxicity after a single dose were seen within 7 days after injection and at 2-3 weeks post treatment no delayed toxicities were observed. Weight loss of survivors of toxic single doses was maximum after 7-10 days, followed by an increase in body weight. After a single dose of 41 mg/kg, the bone marrow and mucosa were maximally inhibited to 35-40% on control values by 2 hours but neither showed progress to recovery in the following 22 hours [21].

In another study with healthy male BD2F1 mice, 8 groups were given single doses of fludarabine ranging from 1000-2500 mg/kg/dose, by the intraperitoneal route [22]. The maximum tolerated dose was established at 1485 mg/kg. Most mice died by the 3-day time point and the remainder died by day 7, no delayed toxicities were observed. Weight loss of mice that survived toxic doses was maximal between 7 and 10 days and increasing after [22].

Preclinical toxicology studies with fludarabine phosphate have been conducted in CD2F1 mice and beagle dogs [23]. Animals were administered the drug through an intravenous route on either a single or multiple (daily for 5 days) dose schedule. The mouse lethal dose (LD_{10}) for single (2623 mg/m^2/day) and multiple (1118 mg/m^2/day) was determined by toxicological evaluation. Mice treated were seen to have decreased activity, dehydration, and myelosuppression at toxic doses. Beagle dogs displayed reversible dose related toxicities when administered doses 4 times the LD_{10} for mice. Myelosuppression was similarly seen with severe reticulocytopenia, lymphocytopenia and neutropenia. The organ toxicities included the liver (elevated ALKP and total bilirubin, moderately increased serum transaminases) and gastrointestinal tract, demonstrated by decreased appetite, anorexia, emesis, and diarrhoea. Evidence of delayed toxicity, such as renal or neurological, were not seen on either schedule [23].

15-week-old male BXSB mice were also investigated using IP injection with a dose of either 25 mg/kg (low) or 100 mg/kg (high) [24]. It was administered as a saline and fludarabine solution for 4 weeks, with mice receiving a total of 12 doses. There were no treatment related deaths reported and animals were sacrificed two weeks after the termination of treatment. None of the lymphocyte populations examined were significantly altered in mice receiving either high or low dose fludarabine [24].

1.3. Nelarabine

Nelarabine was designed as a prodrug of D-arabinofuranosyl guanine (ara-G), which is an analogue of the purine guanine [25]. Nelarabine treatment is currently FDA approved for non-responsive and/or relapsed T cell acute lymphoblastic leukaemia (ALL) and T cell lymphoblastic lymphoma cases [26]. Elimination half-life of nelarabine and its active metabolite, ara-GTP could not be established, due to its prolonged intracellular retention. Excretion of ara-G is through the renal system and its clearance in cases of mild or moderate renal impairment is lower [26]. Nelarabine is converted by adenosine deaminase (AD) to a deoxyguanosine analogue, ara-G, which is the active form. dGK and dCK then promptly phosphorylate ara-G to ara-guanosine triphosphate (ara-GTP) in the intracellular space. Exposure to ara-GTP in the intracellular space is higher than that of its previous forms. Ara-GTP then competes with GTP for incorporation into DNA. Substitution in replication of DNA results in inhibition of synthesis and ultimately cytotoxicity, especially to T cells and other rapidly proliferating cells [26]. *In vitro* testing in the progenitor cell line of human bone marrow shows increased cytotoxicity of nelarabine to malignant T cells as compared to malignant B cells, this is shown to be due to accumulation of ara-GTP [26]. Majority of toxicology studies were performed in the monkey due to other tested animal species, such as rabbits, dogs, mice, having very rapid conversion to ara-g after intravenous administration [26]. It demonstrates dose dependent toxicity, with little to no effects with low doses, but at higher doses, a deepening loss of consciousness is observed caused by an irreversible neurotoxicity. Shallow respiration, flaccid limbs, minimal jaw tone and loss of palpebral reflex are symptoms seen in monkeys that eventually died from acute toxicity. This could be due to disruption of RNA synthesis caused by ara-GTP accumulation [26]. Monkeys treated at lower doses for 30 days displayed tremors, convulsions, and body weight decrease. On histopathology, lesions were seen in the brain (cerebellar degeneration) and in the spine (myelopathy), both of which are irreversible in survivors [26]. Toxicities in other species are milder; in mice, shallow breathing and decreased activity, in rabbits, reduced appetite and body weight. No toxicity is observed in dogs due to rapid elimination [26].

A series of investigations were conducted by the FDA in multiple species, and it was found that the elimination of nelarabine in mice, rats and dogs was too rapid and thus they were not suitable models to test the toxicity. Thus, the toxicity studies were performed in the monkey species [27].

When female monkeys under anaesthesia were given a single intravenous dose of nelarabine in the range of 100-500 mg/kg, a deepening of anaesthesia was seen at doses of 400 mg/kg and 500 mg/kg. Within 24 hours, all monkeys recovered and were clinically normal. Doses ≤300 mg/kg did not show any observable effects [27]. One female monkey was given a continuous intravenous dose of 500 mg/kg for 15 minutes for a period of 2 days. No treatment-related signs were seen after the first dose, however similar to the previous study, the anaesthesia deepened after the second dose and the animal died within four hours [27]. When one female monkey was given a single 1-minute intravenous dose of 400 mg/kg, the monkey displayed no palpebral reflex, shallow breathing, flaccid limbs, minimal jaw tone and death within 5 hours. This protocol was then modified to a lower dose, 300 mg/kg for 10 minutes daily for a period of 4 days to treat another monkey. In this case, the monkey displayed some emesis and deepening of anaesthesia, but recovery was seen within 4-5 hours after dosing [27].

No treatment-related changes were seen when white rabbits were treated with doses of 4-61 mg/kg of nelarabine intravenously for 10-20 minutes over a period of 13-16 days [27]. For a period of 13 days, a dose of 64 mg/kg given intravenously demonstrated slight tremors during dosing (75%, 3/4 rabbits) but no other effects were seen. Similarly, dosing with 128 mg/kg did not demonstrate any treatment related effects [27]. Another study was performed in white rabbits with daily intravenous doses of 30 mg/kg for 8 hours or 360 mg/kg for 24 hours, over a period of 2 weeks. The body weight loss for rabbits in both groups was 6% and 11%, respectively. Reduction in white blood cells (neutrophils, lymphocytes), red blood cells, haemoglobin, haematocrit, and reticulocytes was seen in both groups. The severity of these decreases was dose-dependent. A decrease in appetite, food intake and faeces production were seen [27]. Based on the data from this study, an intravenous dose of 300 mg/kg/day was given to pregnant 20-21-week-old rabbits and caused decreased body weight gain and food consumption, laboured breathing, abortion, and deaths of the does. The toxicity to the foetus was not lethal and manifested as reduced foetal weights. When doses of 30 mg/kg/day (low) and 100 mg/kg/day (mid) were given to pregnant does, there was delayed ossification of the foetuses seen. This effect was also seen in the high dose group (300 mg/kg/day). The incidence of foetal abnormalities was as such, absent gallbladder, and accessory lung lobes, fused or extra vertebrae and cleft palate (only seen in high dose group) [27].

1.4. Thioguanine

Thioguanine (2-amino-6-mercaptopurine) is a purine analogue of guanine. It is derived from the parent compound mercaptopurine [28]. The treatment of acute and chronic myelogenous leukemias with thioguanine (TG) has been approved since 1966 in the US. Due to its added anti-inflammatory effect, it is used off-label in the treatment of autoimmune diseases [28]. Metabolism of TG to its mono-, di- and triphosphate derivatives is done with the help of HGPRT. This process is necessary for the incorporation of its metabolites into DNA and RNA. This leads to inhibition of protein synthesis and eventual cytotoxic action [28, 29]. The metabolite, 6-thioguanine monophosphate (TGMP), inhibits several enzymes in the purine biosynthesis pathway by high levels of accumulation after administration of therapeutic doses. Guanine nucleotide formation is inhibited at different points that together create an obstruction causing reduced levels of guanine in intracellular nucleotide pools. These points are PRPP amidotransferase (for which it is a pseudo feedback inhibitor), IMP dehydrogenase, ATP-GMP phosphotransferase and HGPRT [29]. Cytotoxicity of TG on human cervical carcinoma (HeLa) cell lines showed a dose dependent effect on the viability and proliferation of cells. It was also shown to be dependent on the malignancy and the TPMT expression of the cells [30]. In other evaluations done in transgenic HGPRT deficient mice, the lethal dose was found to be extremely elevated as compared to that of wild type mice [31]. Biochemical analysis of the serum of wild type mice showed typically increased levels of liver enzymes, suggesting liver damage, that is expected on treatment with TG. However, the serum levels of HGPRT deficient mice were in normal range [31]. These differences could also be observed on histopathological examination, where wild type mice showed loss of hematopoietic cells from bone marrow, severe loss of gastrointestinal epithelial cells, liver atrophy and necrosis at low doses in comparison to little to no changes in the same organs even at the higher doses in deficient mice [31].

6-thioguanine was given to Swiss male mice to determine the LD_{50} value on 2 dosing schedules with doses starting from 1 mg/kg/day until demonstration of lethal effects. They were given as either single or five successive daily intraperitoneal injections, and the LD_{50} for multiple injections (2 mg/kg) was determined to be lower than that of single dose (99 mg/kg) demonstrating that thioguanine has enhanced and cumulative activity [14]. When single intraperitoneal (IP) doses of 250-500 mg/kg were given to mice or rats, they produced ataxia, weakness, and dyspnoea within the first few hours after injection. These symptoms increased

in severity and death occurred within 12 hours due to respiratory failure. Other lesions found in Wistar rats given IP injections of TG are peritonitis, ascites, and fibrinous adhesions of abdominal viscera. Fatal doses in dogs and rodents do not cause significant alterations in intestinal epithelium. Chronic administration of lethal doses in dogs reveal early destruction of myeloid and nucleated erythroid elements, resulting in an overall myelosuppression (reticulocytopenia, anaemia, thrombocytopenia and granulocytopenia). After the development of agranulocytosis, dogs ultimately become febrile and die, with occasional exhibition of pharyngitis. Usage of antibiotics can allow for maintenance of body temperature, but animals ultimately succumb due to generalised haemorrhage [14].

Six groups of 6-10-week-old male or female wild type (WT), hypoxanthine phosphoribosyl transferase (Hprt) deficient and Winnie C57BI/6 mice, were intragastrically gavaged daily with doses ≤5 mg/kg/day for periods between 3 and 28 days with 6-thioguanine [32]. Animals were monitored during treatment for signs of weight loss and were sacrificed if it fell more than 20% compared to day 0. Once blood was collected for biochemistry and blood count examinations, animals were sacrificed by the method of cervical dislocation. Samples were taken from liver and bone marrow. When 6-thioguanine was given at a dose of 0.5 mg/kg/day, 100% survival rate was observed. When given at ≥1 mg/kg/day, the dose was fatal. Body weight, liver weight, cellularity of bone marrow and blood cell count (lymphocytes, leukocytes, haemoglobin) were decreased with increasing doses of 6-thioguanine over 28 days [32].

Healthy male BD2F1 mice were administered 6-thioguanine with doses ranging from 0.04-25 μM through 12 hours subcutaneous infusion to determine the dose producing toxicity [33]. From this study, the 50% lethal dose was determined to be approximately 0.8-1 μmol/kg/min. During the treatment period, weight loss, ruffled appearance and facial swelling was seen in the 4-7 days post infusion. Necropsy of the swelling demonstrated septicaemia and inflammation of salivary glands. On occasion, rectal bleeding was also seen [33].

The toxicity of 6-TG was assessed *in vivo* in CD-1 heterozygous nude male mice with single and repeated dose schedules, with intraperitoneal administration (IP) [34]. The single dose given was 42.5 μmol/kg and the two multiple dose schedules were 8.5 μmol/kg or 21.25 μmol/kg, administered for 3 consecutive days for either one or two (repeat dose after five days) cycles. There was a total of 12 mice, 4 in each group studied. Urine was collected 24 hours before initiation of treatment and at every 24 hours after initiation. Blood samples were taken from all animals through cardiac puncture for blood count examination and calculation of myeloid:erythroid (M:E) ratios, and bone marrow smears were prepared from the right

femur. Mice were sacrificed 24 hours after the end of treatment and samples from internal organs were collected for examination. Examination of blood and organs of mice treated with 42.5 μmol/kg did not reveal any significant changes. Mice treated with 21.25 μmol/kg for two cycles demonstrated a reduction in white blood cell and red blood cell counts compared to those parameters in control animals. M:E ratios calculated from all mice treated with 6-thioguanine were seen to be substantially higher than that of control animals. Mice treated with 21.25 μmol/kg for two cycles displayed a two-fold and a seven-fold higher M:E ratio as compared to the same dose given in one cycle and lower dose (8.5 μmol/kg) given for two cycles. Macroscopic examination of organs did not show any signs of inflammation, however on histopathological evaluation of the small intestine of the treated group, shrunken globular hypereosinophilic crypt epithelial cells containing condensed pyknotic nuclei were seen. Mice treated with both doses (8.5 μmol/kg and 21.25 μmol/kg) on both cycle schedules, displayed a dose-dependent crypt-cell apoptosis in the small intestine and to a smaller extent in the large intestine. No lesions were detected in the stomach and kidneys [34].

2. PYRIMIDINE ANTAGONISTS (ANALOGUES)

Pyrimidine derivatives, cytosine, thymine, and uracil, form the other halves of the purines in DNA and RNA molecules. Without them, the molecules themselves cannot be complete and coupled with the increased proliferation of cancer cells, analogues of pyrimidines are a good choice of anticancer drug. However, selectivity of these drugs to tumour cells is not optimum and can lead to adverse effects on healthy tissues. The most commonly used antineoplastic drugs within this group today are cytarabine, gemcitabine, azacitidine and decitabine. We will focus on these drugs in this review.

2.1. Cytarabine

A nucleoside compound containing arabinose sugar was found by researchers in *Cryptotethya crypta*, a species of sponge [35, 36]. Having similar molecular weight and structure as cytidine, it behaves as a deoxyribose sugar due to its 2' hydroxyl group being in the trans conformation [37]. This compound came to be known as cytarabine and it belongs to the

category of drugs known as anthracyclines. It is also referred to as arabinosylcytosine (Ara-C). It is an analogue of pyrimidine and therefore can be used as an antimetabolite drug [35, 36]. Currently, cytarabine in humans is primarily used in the treatment of leukemias, such as acute lymphoblastic (ALL) and acute myeloid leukaemia (AML), for which it is a cornerstone, lymphomas, such as non-Hodgkin's and primary nervous system lymphomas and other haematological malignancies [16, 35, 36]. However, it is also used off label in the treatment of refractory cases, such as those of chronic lymphocytic leukaemia and Hodgkin disease [35]. It can also be used in combination with other antimetabolite antineoplastic agents such as methotrexate (MTX) and fluorouracil (FU) in solid tumour chemotherapy regimens [37]. The use of cytarabine in domestic animals is for the treatment of lymphoproliferative disease and meningoencephalitis of unknown aetiology [38]. Its clinical *in vivo* application in solid tumours is impeded due to poor membrane permeability of tumour cells, limited phosphorylation or rapid deamination and deactivation [36]. Cytarabine can achieve high bioavailability when administered subcutaneously, intravenously, or intrathecally (in case of neural neoplasias). It has a high volume of distribution due to its low level of plasma protein binding [35], and it does not bind to the blood and tissues [37]. The off-label use in nervous system lymphomas is due to the ability to cross the blood brain barrier [35]. For example, in the case of stage five CNS lymphoma in canines despite its efficacy as a mono therapy against canine lymphomas being suboptimal [38]. Cytarabine is transported into the cell through carrier mediated processes. Cytarabine is then phosphorylated through consecutive reactions, into its active form, cytarabine-5-triphosphate (ara-CTP) within the cell [37, 38]. Upon entering into the systemic circulation, cytarabine is rapidly converted to its inactive form, uracil arabinoside, by the enzyme cytidine or deoxycytidine deaminase predominantly in the liver [35, 37, 38]. Once in this form, it can be eliminated from the body via the renal system [35]. About 5-8% of administered ara-C can be found in its original form in urine, while the remaining 95% is found as uracil arabinoside [39]. Cytarabine displays a biphasic elimination from systemic circulation, with an initial half-life of 7-20 minutes and a terminal half-life of 2-3 hours with intravenous administration [37], and 1 hour with subcutaneous administration [38]. As seen above, the overall duration of activity of cytarabine in the body is short and as a consequence, treatment with cytarabine must be with repeated administration or continuous rate infusion to allow for maintenance of steady plasma concentration [37, 38]. However, when cytarabine is administered intrathecally, its elimination from the body is seen to be slower with a half-life of 3-4 hours. This is likely due to the low activity of cytidine deaminase in the CNS and consequently,

lower rates of conversion to inactive uracil arabinoside [37]. Ara-CTP competes with cytidine for incorporation into DNA. Once it is added onto the growing strand of DNA, the addition of the next deoxynucleotide becomes slow [40]. This causes replication to halt in the S phase, which is the stage of the cell cycle where rapid division occurs [35]. As a result, the effect of the drug can only be seen when cells are in this phase [37, 38]. Another mechanism of action is through the inhibition of DNA polymerases resulting in termination of chain elongation and subsequently inhibiting the synthesis of DNA [37, 38]. This also halts replication and repair processes of DNA thereby resulting in cell death [35, 37]. Though the exact process of cell death remains unclear, it is known that concentration and duration of exposure are needed for cytotoxic effects to be seen [37, 38]. To attain maximum affectivity, the cells must be exposed to a cytotoxic concentration of the drug for extended periods of time. A concentration of ≥0.1mg/L maintained for ≥24 hours can achieve maximum cytotoxicity as seen in vitro mammalian studies [37, 39]. The property of cytarabine affecting rapidly dividing cells allows us to target the tumours due their rapid cellular proliferation [35]. However, it is not a specific target and can also lead to the occurrence of toxicities in other rapidly dividing cell types, such as gastrointestinal tract and bone marrow. The action of the drug must be over a period of time of one cell cycle to allow for effective inhibition of tumour cell growth [35]. If cytarabine is administered through constant rate infusion (CRI), it allows for the maintenance of effective drug concentration thereby improving therapeutic index and can also increase the chance of killing tumour cells in the correct phase of cell cycle, making it the preferred method of administration [38]. Cytarabine was administered orally to healthy mice in order to determine systemic toxicity. No obvious histopathological lesions were seen in the major organs, such as heart, liver, spleen etc, stained with haematoxylin-eosin (HE) [36]. Inflammation, redness, and erythema is seen on the fore and hind paws after treatment with Ara-C. After ten days, wasting of the corners of the mouth was observed and after fifteen days, oral mucositis worsened. Decreased serum albumin concentration is one of the most common toxicities seen in rats [36].

In a retrospective study of canine lymphoma patients, with either bone marrow or CNS involvement, treated with 100mg/ml cytarabine as part of chemotherapeutic protocol, the predominant toxicities were of the gastrointestinal tract and bone marrow [38]. The gastrointestinal toxicities, including vomiting, anorexia, diarrhoea, colitis, melena, were seen in 65% of patients of which a quarter experienced a high-grade toxicity. This correlates with the expectation of extended cytarabine exposure to destroy the mucosal barrier, due to enterocyte apoptosis and villus atrophy. The hematologic toxicities included mild neutropenia

(35%) and severe thrombocytopenia (11%). Toxicities were also observed in other organ systems such as liver (lymphocytic infiltration, direct hepatotoxicity; 15%) and kidney (urinary tract infection, glycosuria, acute tubular damage; 11%). There were adverse reactions related to infusion or anaphylaxis during treatment [38].

In a study done with two-day-old rats, given intraperitoneal injection of 4 mg/kg cytarabine for five successive days, the toxicities were measured during treatment and over the 28 days after the termination of treatment [41]. Post-mortem examinations were carried out at time points of 5, 12, 19 and 33 days from the start of treatment. The signs of toxicity such as weight loss, delays in hair growth, unsteady gait, and slower development of dermis, was seen from day 10 onwards. However, the first evidence of toxicity could be seen earlier at the 5-day necropsy, showing microcyst formation in renal cortical tubules (delayed nephrogenesis) and retinal dysplasia. Nephrogenesis returned to normal for the rest of the study however retinal dysplasia remained till the end point. Post-mortem evidence of unsteady gait was seen as cerebellar hypoplasia and dysfunction [41].

To assess the developmental toxicity of cytarabine, an investigation was done using pregnant Swiss mice given different dosages at days 6-15 of gestation [40]. They were divided into 4 groups with dosages 0, 0.5, 2 or 8 mg/kg/day. The evaluation of toxicities was performed by observing the clinical signs during treatment and post-mortem examination of organs and uterine contents, including foetuses, after euthanasia at gestational day 18. A substantial decrease in body weight gain was seen at the higher doses (2 and 8 mg/kg), the rate of which was directly dose dependent. This was coupled with an overall reduction in food intake over the treatment period. An increase in early and late resorptions resulting in decrease in size of litters seen at 8 mg/kg dose, as well as the reduced body weight of the remaining live foetuses seen at all dosages. The developmental abnormalities of the foetuses included increased occurrence of cleft palate, renoureteral agenesis or hypoplasia and poly- or oligodactyly, seen at 2 mg/kg. Upon administration of higher dose (8 mg/kg), the dilation of cerebral ventricles was also seen. Among the skeletal abnormalities, the maturation of the skeleton was stunted from 0.5 mg/kg upwards. In the 8 mg/kg group, severe malformations, such as incomplete ossification of skull bones, partial and complete absence of limbs, fused and fragmented ribs, were seen [40].

2.2. Gemcitabine

Gemcitabine is a synthetic nucleoside analogue, 2', 2'-difluoro-deoxycytidine (2FdC) [42, 43]. It is an analogue of deoxyribose sugar, deoxycytidine and has a molecular weight of 299.66 [44]. It is one of the most utilised drugs in chemotherapy, both in solid and recently increasing in hematologic human malignancies [43, 45]. However, their activity in veterinary oncology has only recently been explored [46]. It first demonstrated effectiveness against pancreatic ductal adenocarcinoma (PDA) in 1997 and has since become a cornerstone in its therapy [42, 44]. Since then, it has become one of the most commonly used drugs in clinical oncology and shows prominent effects in many types of tumours, some of which are ovarian, bladder, breast and in lung neoplasias like non-small cell lung cancer, squamous cell carcinoma and nasopharyngeal carcinoma [42, 43, 47]. It might also be a good candidate for combination therapy of acute myelogenous leukaemia [43]. Due to the hydrophilic nature of 2FdC, its uptake and transport across cellular lipid membranes is controlled by nucleoside transporters [42]. As a prodrug, the activation of 2FdC is essential for its effect to be seen. Activation to its triphosphate form, 2F-deoxycytidine triphosphate (2FdCTP), is through phosphorylation brought about by deoxycytidine kinase (dCK) upon entry into the cell [42, 43, 44]. The primary mechanism of effect is through the incorporation of active metabolite into DNA, setting off a "masked chain reaction" [43], which results in the cell cycle coming to a halt in the S phase, causing a cytotoxic effect and inhibition of DNA synthesis [42, 44]. Apoptosis of cells in G1, G2 and M phase may be possible at higher dosages [42]. The metabolites can also inhibit other metabolic enzymes, such as ribonucleotide reductase which causes self-potentiation of cytotoxic activity that is unique to gemcitabine [42, 43, 44]. This causes a dCTP deficiency causing an increased activity of dCK thereby enhancing 2FdC activation as well as increasing the incorporation of 2FdCTP [42, 43, 44]. It also causes a dATP deficiency which halts DNA repair [43]. Another mechanism by which gemcitabine acts is the inhibition of dCMP deaminase and CTP synthetase, which decreased the breakdown of 2FdC and potentiate incorporation of 2FdCTP into RNA and DNA [43, 44]. The pharmacokinetics of intravenously administered gemcitabine in humans, is linear and can be described by a two-compartment model. The volume of distribution and half-life of the drug are considerably influenced by the duration of infusion [44]. In nude mice implanted with squamous cell carcinoma of the head and neck (SCCHN) xenografts, 2FdC shows powerful antitumor activity. This effect is schedule dependent as seen by the reduced activity of the drug when given daily as compared to when it is given at three-day intervals [45]. This effect is also seen when leukaemia cells are exposed to 2FdC for a limited period of time. The schedule dependency could be explained by the time required for the drug to reach a

threshold level, thereby allowing it to effectively compete with deoxycytidine for dCK activation [45].

Another study was performed proving the tolerability and effectiveness of systemically administered gemcitabine in cases of metastatic transitional cell carcinoma of the bladder. Results of this study showed favourable outcomes for previously treated and untreated patients. Using the clinical data obtained from the above study, an investigation into the safety and organ specific toxicity of intravesical administration of gemcitabine was undertaken [44]. Twelve beagle dogs were chosen for this study due to the histological structural similarities between the human and canine bladder. Dogs were divided into two groups and given one of three dosages (100 mg, 350 mg, 1 g) through catheterization for one hour. One dog was given three scheduled doses of 1g at the start of the investigation, however it was seen to have very high serum level of the drug and displayed clinical toxicity in the form of severe leukopenia and thrombocytopenia. Due to this the dosages administered to the remainder of the dogs was either 100 mg or 350 mg. The schedule of administration was one dose, three times a week for four weeks. Prior to draining the bladder, blood samples were collected for cell counts and gemcitabine assays. During the treatment, there were no clinically significant side effects, and it was generally well tolerated. There was a slight leukopenia and thrombocytopenia, without corresponding changes in the bone marrow, seen in the 350-mg group. This change was not seen in the group dosed with 100 mg. The white blood cell and platelet counts were at their lowest at the 4th week of treatment, however their numbers increased after the termination of treatment. At the end of treatment period, one dog from each dosage group was euthanized at two time points, 1 day and 14 days post treatment. Necropsy of both groups revealed no toxicities, in particular of the bladder tissue and bone marrow. Although the necropsy revealed no toxicities, there was an appearance of toxic effects as one of the dogs died two days after the termination of treatment due to the development of small intestinal haemorrhage. Similarly, another was euthanized five days after termination, due to intravenous fluid and antibiotic unresponsive febrile neutropenia. The necropsies of these two animals displayed toxicities in multiple organs such as bone marrow (severe hyperplasia), small intestine (transmural haemorrhage, necrosis) and bladder (severe ulcerative and haemorrhagic cystitis) [44].

A similar study was performed in swine due to the similarity of the urogenital tract to that of humans [48]. Two groups of pigs received only a single dose of either 175 mg or 350 mg of gemcitabine through catheterization for 2 hours. A third group received weekly doses of 350 mg for 6 weeks, with blood samples collected for blood counts. 24 hours after last treatment,

all animals were euthanized, and investigation of the bladder wall histology was completed. At all doses and schedules, treatment with gemcitabine was well tolerated in all animals with no signs of deterioration or immunosuppression. Histological examination also yielded normal results, except for some cases of mild signs of infection seen in the third group after 6 doses, such as subepithelial leukocyte infiltration and epithelial cellular and nuclear enlargement [48].

To evaluate the immediate and delayed toxicity of gemcitabine administered through isolated lung perfusion (ILP), as well as establish dose limiting toxicity (DLT) and maximum tolerated dose (MTD), a dose escalation study in pigs was set up [49]. Under anaesthesia, 23 3-month-old pigs were given doses from 40 µg/ml initially, doubling at the next level up to a dose of 1280 µg/ml. Administration of gemcitabine was successful, with 66% mortality only seen at the end of perfusion with 80 µg/ml. In the follow up period however, further mortalities were seen in the 320 µg/ml (100%) and 640 µg/ml (60%) in the 24 hours after treatment. Mortality due to hypoxia after lung recirculation were seen in all pigs given a dose of 1280 µg/ml (100%). At the end of the follow up period (1 month) after treatment, 48% of animals survived. At concentrations of 640 µg/ml and above, major pulmonary toxicities were observed in the form of interstitial and alveolar infiltration, the extent of which increased with the dose. These effects were seen at the end of treatment and follow up period. There were no significant toxicities seen in other organ systems (liver, kidney). As a result, the DLT was established at 640 µg/ml and MTD was established at 320 µg/ml [49].

The safety of aerosol delivery of gemcitabine was evaluated in pet dogs with osteosarcoma [46]. Metastasis to the lung is one of the common treatment failures of osteosarcoma cases of humans and dogs. The control of pulmonary metastasis with systemic drugs is limited by the diminished drug concentration in the lungs as a result of blood volume dilution. These observations prompted the strategy of aerosol administration. A total of 20 dogs were treated, with doses ranging from 5-50mg/dog, over 2 days in a week, increasing the dose by 5mg every week. The treatment was well tolerated, with the exception of one dog each in the 5mg and 10mg group whose condition deteriorated rapidly and the owners made a decision for euthanasia, before the dose escalation could not be performed. Eleven dogs were in the 25-mg group and 7 dogs in the 50-mg group. Thrombocytopenia and anaemia were not seen on the regular blood count examinations, and neither body weight nor blood gas parameters varied from the baseline. Owners reported no gastrointestinal toxicity. Upon histological examination of samples taken from distal trachea, main stem bronchi and lungs, there were slight toxicities seen. Minimal to mild submucosal expansion, with small papillary

projections protruding into airway lumen was seen in the large airways of 4 dogs. These signs were not seen in untreated animals and were attributed to gemcitabine treatment. Alveoli in both treated and untreated remained normal. Regionally extensive to generalised villous pleuritis was seen in 12 animals with pleural effusion or metastasis (5 of them had both). Hypertrophic osteophyte was identified antemortem and confirmed on post-mortem examination of 3 dogs with pulmonary and/or pleural metastasis. Lumina of alveoli adjacent to metastatic foci, were seen to have hemosiderin pigment within the cytoplasm of alveolar macrophages which were also increased in number. The hemosiderin pigment is indicative of haemorrhage [46].

2.3. Azacitidine

5-azacytidine (Aza-C) is an analogue of cytidine with nitrogen instead of carbon at the 5' place of the pyrimidine ring [14, 50]. It was synthesised in 1964 and clinical studies began in 1967 in Europe and 1970 in the US [50]. Aza-C shows good antitumor activity in previously resistant or refractory cases of acute myelogenous leukaemia both as single and combination therapy. Preclinical models of infant rearranged acute lymphoblastic leukaemia show promising results with azacytidine treatment [51]. It has also received approval for the treatment of high-risk myelodysplastic syndrome [44, 52]. However, clinical results are not encouraging for treatment of solid tumours [50]. Aza-C undergoes rapid hydrolysis in both basic and neutral environments as shown by *in vitro* studies [50]. Pharmacokinetics of the drug were investigated in mice using C-labelled Aza-C. The radioactivity of the parent drug and/or metabolite dropped rapidly after administration and calculated half-life was around 4 hours. There was an increased level of radioactivity seen in lymphatic organs for prolonged periods of time. The quick drop in radioactivity corresponds to the initial excretion of the drug in urine [50]. There were slight differences in the pharmacokinetics of Aza-C in humans, evaluated using radioactively labelled drugs with intravenous and subcutaneous administration. The noted half-life in this case was 3.5 and 4.2 hours after intravenous and subcutaneous administration respectively [50]. 90% of total radioactivity was excreted in the urine within 24 hours. Although no active transport mechanism is seen, the drug was taken up into tumour tissue and radioactivity was always greater inside than in the surrounding healthy tissue [50]. Elimination pharmacokinetics, using radioactive labelled drugs, shows the same results in dogs, humans, and rodents [50]. Aza-C is phosphorylated by enzyme uridine kinase

(UK) into its active form [50]. There are multiple proposed mechanisms of antineoplastic effect. First, is incorporation of phosphorylated Aza-C into DNA and RNA polynucleotides [50, 51]. Once incorporated into DNA, the symmetrical triazine molecule of Aza-C spontaneously degrades, making DNA more liable to chain breakage [50]. However, with incorporation into RNA, it creates disruptions in translation and faulty messenger RNA (mRNA) is produced [50, 51]. This faulty mRNA is not able to code for protein synthesis thereby causing inhibition. Transfer RNA is also modified and plays a role in protein synthesis inhibition [50]. Another mechanism by which Aza-C acts is the competition with uridine and cytidine bases for the UK enzyme. UK is responsible for the phosphorylation of uridine and cytidine, which is the rate limiting step of the salvage pathway of pyrimidine synthesis [50]. And the final mechanism of action is through the interference with orotidylic acid decarboxylase, responsible for the synthesis of pyrimidines through the *de novo* pathway [50]. The toxic effect of Aza-C, as seen in individual hamster fibroblasts, seems to be specific to the S phase of cell cycle, especially at low concentrations. It was also seen that non proliferating mouse leukaemia cell lines are relatively impervious to Aza-C effect. This confirms the specificity of Aza-c to diving cells [50]. Out of the four animal species (hamsters, rhesus monkeys, mice, and beagles) that were studied for dose toxicities, the beagles were the most sensitive [50]. A widespread necrosis of lymphatic organs coupled with decreased level of circulating leukocytes was seen. Single doses of Aza-C showed hepatic damage in the form of elevated ALKP, serum pyruvate transaminase and prothrombin time. Hepatic cell necrosis was seen occasionally. There was also accumulation of eosinophilia globular material in the renal tubule lumens, with sporadic increase of blood urea nitrogen levels. Overall, the observed toxicities in beagles were dose related and reversible in most cases [50]. The disparity between clinical efficacy in leukaemia and solid tumours can be seen in both preclinical (animal) and clinical (human) evaluations, with good antitumor activity against leukaemia versus poor activity against solid tumours [50]. The beagle model was accurate in predicting hepatic and bone marrow toxicity but overestimated its renal toxicity in humans. Unfortunately, the symptoms of nausea and vomiting seen in clinical trials have not been replicated in any of the animal species tested [50]. The differences between species in the activities of kinases and deaminases are likely the reason for the increased tolerability of humans to Aza-C. The adverse effects such as severe nausea, vomiting and dose limiting myelosuppression seen with systemic administration of azacytidine have impaired its clinical effectiveness [52, 53].

Pulmonary toxicity is not seen with this systemic route of administration and thus a study was performed to determine the safety and efficacy of aerosol delivery of azacytidine in mice [52]. The basis for this was the laboratory findings proving the effect of aerosolized azacytidine in reducing promoter demethylation of tumour suppressor genes (TSGs) and increasing the expression of proteins when tested on human orthotopic lung cancer xenograft models in mice. This form was also better tolerated than the intravenous administration [52]. Harlan mice of both sexes were divided into two groups and administered one of two doses (0.83 mg/kg or 2.5 mg/kg daily) of azacytidine via aerosolized route for seven days. After termination of treatment, mice were euthanized at two time points (3 weeks and 6 weeks) and their organs were preserved and stained in the appropriate methods. Evaluation of organ toxicity was performed through histological and pathological methods. During the treatment period, blood samples collected showed no significant decrease in the white blood cell counts at either dose. The examination of the bone marrow also did not yield evidence of toxicity. In contrast, mice given a dose of 2.5 mg/kg intravenously demonstrated a 30-50% decrease of white blood cell counts. Blood chemistry tests performed, with particular attention to liver and kidney function, demonstrated that they remained unaffected in mice treated at the lower dose. The liver and kidney did not show any histological or pathological signs of toxicity either. When the lungs of the two groups were examined, there was no sign of toxicity in the lower dose group at either time points. However, in mice euthanized at 3 weeks in the higher dose group, there were signs of toxicity. The lesions observed were accumulation of fibrin along the alveolar walls, increased infiltrates in alveolar macrophages and perivascular lymphoplasmacytic cuffing, which are all typical signs of pneumonitis. The necropsies of mice from the week six time point did not demonstrate these pulmonary lesions, indicating that the toxicity seen at 2.5 mg/kg dose is reversible [52].

Nineteen dogs, with invasive urothelial carcinoma, were included in a study to examine the effects of subcutaneous administration of azacitidine, with dosages ranging from 0.1-0.3 mg/kg/day, for 5 consecutive days [54]. They were divided into 2 groups, a 28-day group with 11 dogs (treated on days 1-5) and a 14-day group with 8 dogs (treated on days 1-5 and 15-19). Treatment with azacitidine was well tolerated, with the expected myelosuppressive and gastrointestinal toxicities usually seen with this drug.

The maximum tolerated dose (MTD) observed in the 28-day group was 0.2 mg/kg, with doses above resulting in high grade neutropenia in 4 of 6 (66%) dogs. 1 dog developed high grade anorexia and nausea on treatment with 0.3 mg/kg. The MTD for the 14-day group was 0.1 mg/kg, similarly, showing neutropenia in 2 of 3 (66%) dogs treated at higher doses. 2

dogs in this group developed high grade anorexia and nausea after 2 treatment cycles with 0.3 mg/kg. There were no treatment related deaths seen in either dosing schedules [54].

In a study performed on 10-16-week-old Syrian hamsters, given 10 mg/kg of azacytidine intraperitoneally twice a week for 2 months, demonstrated major hematologic toxicities [55]. Out of the 15 hamsters on this schedule, 12 died 1-2 months after initiation of treatment and the remaining 3 were sacrificed due to dismal health status at the 35-45-day mark. Necropsies of all hamsters were performed. Samples were collected from all the organs, as well as from the femur and other abnormal areas, sectioned and stained with haematoxylin-eosin (HE). At the start of treatment, decreased weight gain was seen in all hamsters. As treatment continued, scoliosis, hair loss and severe weight loss were seen. Blood collected and examined showed severe leukopenia, granulocytopenia and hypoalbuminemia in 2 hamsters. Alkaline phosphatase (ALKP) and blood urea nitrogen (BUN) values decreased but remained within the reference range. Pathologic changes were seen in organs such as spleen (2 - congestion), liver (1 - granuloma), kidney (2 - congestion, 1 - benign hyperplasia, 3 - chronic pyelonephritis), adrenal gland (1 - benign hyperplasia). 5 hamsters showed signs of agonal congestion of spleen and kidney [55].

2.4. Decitabine

The initial development of a 2'-deoxycytidine analogue compound began in 1984. The 5' carbon was replaced with nitrogen and so we achieved decitabine or 5'-aza-deoxycytidine (Aza-dC) [56]. It is the deoxy- derivative of the previously mentioned drug, azacytidine [44]. Current treatment in acute myeloblastic leukaemia (AML), low risk myelodysplastic syndrome (MDS) [52], and chronic myeloblastic leukaemia, as a complementary to already existing therapy, in elderly patients and those with poor prognosis, displays good clinical efficacy [56]. The bioavailability of Aza-dC of the drug was around 10% due its considerable first pass metabolism and elimination brought about by cytidine deaminase (CDA) enzyme [57]. Activation of Aza-dC, is through phosphorylation with enzyme dCK, resulting in the formation of monophosphate (Aza-dCMP), then rapid conversion to active triphosphate metabolite (Aza-dCTP). If Aza-dC or its monophosphate form is acted upon by deaminase enzyme, its activity is lost [56]. Aza-dCTP is a good substrate for DNA polymerase alpha enzyme and incorporates itself into the DNA chain, in the S phase of the cell cycle due to its specificity [56, 58]. Upon incorporation, Aza-dCTP inhibits methylation at 5' position by

trapping and degrading enzyme DNA methyltransferase (DNMT) by forming a covalent complex with it [58]. Aza-dC can exert its effect through cytotoxicity induced cell death but also through promotion of differentiation and active cell maturation by hypo- or demethylation [56]. The interval schedule of dosing was found to be more effective than daily schedule in an evaluation with SCCHN xenograft bearing mice [45]. Aza-dC in human mouse cell hybrid was found to induce the expression of HGPRT enzyme, which is positively correlated to the demethylation of DNA [56]. Irreversible dose dependent concentration of haemoglobin and morphological differentiation of cells was seen after treatment of human erythroleukemic cell line [56]. Methylation of DNA has been proposed, in preclinical studies, to be due to inhibitory action of Aza-dC on DNMT and induce differentiation and apoptosis of cancer cells [57].

Investigation of hematopoietic toxicities of decitabine were performed in healthy male mice and dogs displaying normal marrow function [59]. 10-week-old CD2F1 male mice were given a total dose of 20 mg/kg through an intravenous infusion for 12 hours, with a flow rate of 0.16 ml/hour. Femur (bone marrow) samples were collected at different time points following the termination of treatment. The analysis of the samples demonstrated a drastic reduction (50%) in white blood cell counts on day 2. White blood cells continued to be depressed until day 19 and showed recovery and values similar to those of the control group by day 22-25. Femur cell counts demonstrated a harsher decrease with values hitting 18% (day 2) and 10% (day 4) of controls, however the recovery was faster as cell counts returned to 75% of controls by day 8 and complete recovery seen by day 21. 3-4-month beagle dogs were similarly administered decitabine through 12-hour intravenous infusion at doses ranging from 3-7 mg/kg, with dose rate of 4-5 ml/hour. In this study, blood samples collected during the post treatment period were used for complete blood count testing. Similar to the mouse study, the treatment produced an overall reduction of all cell types (strong leukopenia, anaemia, granulocytopenia and thrombocytopenia). Lowest values for granulocytes were seen with 5 mg/kg dose on days 7-16 and lowest values for thrombocytes were seen with 7 mg/kg dose on days 9-16. Both parameters showed recovery by day 19-21 and thrombocyte counts surpassed control range on days 23-27 [59].

Toxic effects were also investigated in 9-week-old CD2F1 mice, a research animal model, when decitabine is administered as a 12-hour continuous rate infusion (CRI) with doses ranging from 11.1-44.4 mg/kg in males and 11-30.7 mg/kg in females [60]. Necropsies were performed at 5 and 26 days after infusion, with data collection and analysis of pathological lesions done using an automated approach. At the highest dose of 44.4 (male) and 30.7

(female), showed a loss of more than 30% of their body weight and survived only 7-8 days as compared to lower doses, whose mean survival time was 7.7 and 7.9 days in male and female mice, respectively. Leukopenia was seen in mice given 27.8 mg/kg and 14.4 mg/kg at days 2-9 following infusion. Partial recovery was seen on the following day but surviving animals continued to be leukopenic even 43 days after termination of treatment. A marked decrease in platelet count was seen on days 5-9 with a rebound effect seen on day 15, where values were higher than controls. Histopathology performed on day 7 demonstrated hypoplasia of the bone marrow and atrophy of thymus seen in 66% of males given 29.2 mg/kg and 14% of females given 18 mg/kg. Necrosis of small intestinal mucosa and hyperplasia were also seen in 90% of males and 28% of females, which is likely the cause of reduced body weights seen throughout treatment. Repeated examination of bone marrow and small intestine on day 28 revealed a return to normal in all except one male. Day 28 examination also revealed an atrophy of the testis of all males which was not seen on day 7 [60].

3. FOLIC ACID ANTAGONISTS (ANTIFOLATES)

The application of antifolates is used because folate receptors (FR) have been found in very high amounts in many different kinds of solid tumours, especially in those originating from the epithelium such as adenocarcinomas of the uterus and ovaries [61]. FR is a membrane glycoprotein that has a good effect of folate binding [61]. Another reason for the production of antifolates was found in a phase 1 clinical trial, conducted by Farber, which determined that the addition of folate sped up the progress of leukaemia in children [5]. These findings have promoted the research and formulation of drugs that antagonise folic acid as well as selectively target FR, without affecting other mechanisms of folate transport [61]. This class of drugs can be divided into two types based on whether polyglutamylation reaction occurs. Polyglutamylation reaction is the addition of glutamates as conjugated peptide chains on glutamate residues [62]. Polyglutamylation alters its target specificity by converting polyglutamates into potent inhibitors of TS and AICARFT in addition to potent inhibition of DHFR [61]. Polyglutamate derivatives can accumulate to a larger amount in tumour cells as compared to normal cells, like gastrointestinal tract or bone marrow. This quality is an

important selective advantage for any antifolate drug that can form polyglutamate derivatives in cells [63].

The most commonly used antineoplastic drugs within this group today are methotrexate, piritrexim, pemetrexed and pralatrexate, and they will be discussed in this review.

3.1. Methotrexate

Methotrexate (MTX), also known as amethopterin, was first introduced into a clinical setting in 1948. Further animal testing in 1956 showed that its therapeutic index was better and its toxicity lower than that of aminopterin. As a result, the use of aminopterin was discontinued in favour of methotrexate in clinical therapies [61, 63]. The first time MTX demonstrated promising clinical activity was in the treatment of rheumatoid arthritis in 1985 [64]. Along with its antirheumatic effect, it is an important component of the treatment of lymphoproliferative disorders, such as paediatric acute lymphoblastic leukaemia, lymphomas, osteosarcomas, choriocarcinomas, squamous cell and hepatocarcinoma, gastric and breast cancers. [63, 65, 66]. Absorption of MTX occurs primarily from the gastrointestinal tract with the help of the reduced folate carrier (RFC) [64]. This leads to the drug administration most commonly being orally, but the development of adverse reactions seems to correlate with this form of administration. Parenteral administration, like intramuscularly, could support reduction of these adverse effects and increase bioavailability by avoiding the gastrointestinal system [64]. The protein drug binding is low and distribution in the tissues is high. Its half-life was found to be approximately 6 to 8 hours after administration. MTX can accumulate in the extravascular pool [64]. The main extracellular metabolite is 7-hydroxy-MTX, which metabolises from MTX in the liver [66]. MTX blocks the synthesis of purines and pyrimidines by effectively inhibiting the actions of enzymes such as dihydrofolate reductase (DHFR), responsible for conversion of dihydrofolate to tetrahydrofolate (THF). THF is required by tumour cells as it provides the building blocks for the biosynthetic reactions that are responsible for the creation of purines and pyrimidines. MTX also shows inhibitory activity on other enzymes, thymidylate synthase (TS) and 5-aminoimidazole-4-carboxamide formyltransferase (AICARFT) through its polyglutamated metabolites [64]. This conversion allows for high levels of polyglutamate retention within cells and prolonged cytotoxic effect due their inability to be transported out of the cell [61, 63]. Polyglutamylation also causes a change in the spectrum of MTX. Its monoglutamate form is

a potent inhibitor of DHFR, but once polyglutamylated, it accumulates in the cell and can also inhibit the activity of AICARFT and TS enzymes [63, 66]. This action on multiple enzymes responsible for biosynthesis increases its overall efficacy as a chemotherapeutic agent [63]. Another mechanism by which MTX attacks is its ability to induce apoptosis through activation of nuclear factor *kappa* B (NFkB), p38 and caspase-dependent and -independent pathways. These trigger fragmentation of DNA and are responsible for anticarcinogenic effects [66]. Male Wistar rats were used to investigate the basis of toxicity induced by MTX, in the liver, kidney and heart. Liver toxicities presented as hepatocyte necrosis, increased activity of ALT, AST and ALKP [66]. Hepatocyte necrosis is due to the reduction of folic acid caused by increase in polyglutamate levels in the cells. The inhibition of DHFR also caused an impairment and damage of the cell membrane thereby increasing the activities of liver enzymes [66]. Renal toxicities presented as renal failure, nephrotoxicity and increased levels of serum urea, creatinine, and uric acid. This is due to the precipitation of MTX within the renal tubules leading to a reduction in glomerular filtration rate [66]. Cardiac toxicity was seen in the form of impairment of cardiac tissue caused by an increase in activity of enzyme creatine kinase (CK) and troponin [66]. An increase in oxidative stress (OS) is also seen during MTX treatment. This is caused by the generation of free oxygen and nitrogen radicals, starting a process of lipid peroxidation causing cell membrane and tissue damage as well as mitochondrial dysfunction, which is associated with OS. The increase in lipid peroxidation can be seen by measuring the amount of malondialdehyde (MDA), and its levels are seen to directly correlate to the amount of lipid peroxidation [66]. The increase in cellular damage also leads to inflammation, necrosis, and fibrosis, which is seen by increased levels of NF kappa B and TNF alpha in the tested organs [66]. In another study, irreversible or fatal neurotoxicities were seen in patients administered with intrathecal doses of MTX based on body surface area and were seen to correlate with elevated drug concentration in cerebrospinal fluid [13].

Toxicity of MTX was evaluated in Sprague-Dawley rats over a period of 23 months [67]. During this time, groups of 40 rats of each sex were administered MTX through the diet for 5 consecutive days followed by drug free chow for 9 days. This 14-day cycle continued for the whole treatment period. The doses given to the treatment group were 0.1, 0.2 or 0.4 mg/kg, and the control group of 40 animals were given basic rat chow. Animals were examined daily, complete haematology performed bimonthly for the first 6 months and then at 3-month intervals. At the end of each dosing period, body weights measured, and drug amount added to feed was modified to maintain the correct dose. At the 6-month mark, 20 rats were

sacrificed from each group for an interim evaluation. At the 23-month mark, 10 animals from each group were sacrificed and bone marrow samples collected for evaluation. These samples were processed and examined for chromosomal abnormalities. All animals that perished during the treatment period as well as those sacrificed were examined for gross and microscopic aberrations in all organ systems as well as sampling any other neoplastic or abnormal areas. Animals in all groups treated with MTX did not develop any treatment related effects on body weight or weights of organs. At lower doses the mortality between sexes did not vary significantly however at higher doses, the mortality in females was higher at the 18- and 23- month mark when compared to controls. By the 23-month mark, the survival of the males and females in all groups ranged between 46.7-55.2% and 41.4-75% respectively, as compared to survival of controls which was 68.9% and 59.2% for males and females, respectively. Blood samples taken during the first 6-7 months of treatment showed leukocyte counts to be comparable to those of controls. However, samples taken after the 7-month mark, showed a decreased white blood cell count (leukopenia) in both males and females given high dose, with the lowest count seen at the time of sacrifice. Pathological investigation revealed lesions in the lungs (focal interstitial fibrosis) and bone marrow (myeloid and erythroid hypoplasia) in both sexes, with the severity increasing with the dose given. However, the prevalence of liver (reduced glycogen content, cloudy swelling of hepatocytes) and spleen (increased hemosiderin deposition) lesions were seen only in males receiving high doses [67].

In a study involving male Wistar rats, the maximum tolerated dose for intravenous administration of MTX was determined [68]. In this study, the maximum tolerated dose was defined as the dose at which 90% of animals survive chronic dosing. The doses of MTX given as bolus plus continuous infusion were in the range of 1.8-11.3 g/kg. First, rats were given a bolus infusion of MTX at 1 g/kg dose for 10 minutes, followed by continuous administration of 30 ml/kg volume of MTX with varying concentrations for the remaining 110 minutes. Blood samples were collected at several time points and after the 2-hour treatment period completed, animals were administered NaHCO3 and NaCl solution to prevent dehydration. Laparotomy and exsanguination were performed on all animals that survived to the 8-hour time point. The maximum tolerated dose of MTX was determined to be between 3-5 g/kg. Rats receiving doses of 7.6 and 11.3 g/kg did not survive past 3 hours. ECG was also used to monitor the heart rate and rhythm of rats in the study, and the evaluation of animals given a dose of ≥5 g/kg displayed one or more of the following signs:

partial atrioventricular (AV) block, atrial flutter, atrial fibrillation, second degree AV block before a total third-degree AV block and sequences of sinus arrhythmia [68].

Tumour free Wistar rats were also used for the evaluation of the toxic effects of MTX when given as a metronomic oral dose over a period of 45 days [69]. A total of 18 6-week-old male rats were given a dose of 0.08 mg/kg through oral route, and were monitored daily for signs of clinical alterations, changes in body weight, intake of food and water. The amount of drug given was modified according to the weight of the animal. Haematological, biochemical, bone marrow (metaphyseal femoral region) and histopathological analysis of all animals were performed at 30, 45 and 60 days from the start of treatment. No spontaneous deaths or withdrawal of animals was seen during the experimental period. Nasal discharge and crackles were seen from day 5, increasing in severity until day 45 and persisting after termination of treatment. Moderate chromodacryorrhea (red tears) and blepharospasm were seen throughout the treatment, however, became significant only after day 16 of treatment and persisted till day 60. No changes in body weight or food and water intake were seen as compared to the control group studied. MTX treated animals demonstrated a pronounced increase in white blood cell counts, with a decrease in platelets, haemoglobin and mean corpuscular haemoglobin concentration (MCHC) at day 30. Slight but significant decrease was seen in MCHC at day 45 and augmentation of neutrophils was seen at day 60. They also demonstrated a pronounced decrease in plasma cells and immature myeloid populations at day 30, followed by the reduction of lymphocytes and immature myeloid populations and elevated mature myeloid cells at day 60. The pathological changes seen over the course of treatment were mainly of the lung and spleen, with increased incidence of lesions in other organs in the post treatment period. At the 30-day-time-point, the incidence of lesions was as such chronic interstitial pneumonia (83%), moderate to severe splenic hypoplasia (50%), mild to moderate hepatic alterations including congestion and hepatocellular degeneration (50%), mild acute renal tubular degeneration (16%) and mild myocardial fibre degeneration (33%). At the 45-day-time-point, the incidence of lesions were seen in multiple organs such as the lung [mild to severe compromise (83%) with foamy macrophages (16%), focal haemorrhage (16%), moderate congestion (16%), chronic interstitial pneumonia (16%), acute fibrinopurulent bronchopneumonia (16%)], liver [moderate congestion (16%) and hydropic degeneration (33%)], kidney [mild renal congestion (33%) and acute tubular necrosis (33%)], brain [discrete focal haemorrhage (16%)], heart [myocardial fibre degeneration (16%)], and spleen [mild to moderate splenic cellular depletion (66%)]. At the 60-day-time-point, which is 15 days after the termination of treatment, the incidence of lesions was severe pulmonary

compromise (100%) with congestion and chronic interstitial pneumonia, moderate hepatic congestion (50%), hepatocellular degeneration (50%) and moderate to severe splenic hypoplasia (50%) [69].

In a study involving Wistar rats, the toxico-pathological effects of MTX were evaluated [70]. A total of 48 (7-8-week-old) albino Wistar rats were divided into 4 groups with an equal number of male and female rats in each group. Three groups received either low (0.062 mg/kg), mid (0.125 mg/kg) and high (0.25 mg/kg) dose, with the fourth group receiving distilled water and forming the control group. The administration of MTX was through oral gavage and lasted for a period of 28 days, after which blood was collected and all rats were euthanized by high dose of anaesthetic. Haematological and biochemical parameters were measured and after euthanasia, necropsy was performed, gross lesions recorded, and tissue samples collected for histopathological examination. A reduction of multiple parameters, such as body weight, feed consumption, red blood cell count, total leukocyte and neutrophil count, haemoglobin (Hb) etc, were observed in all animals treated with MTX and the severity of these changes were seen to be dose-dependent. Animals treated at the high dose (0.25 mg/kg) demonstrated an increase in liver enzymes, creatinine, and blood urea nitrogen (BUN). The principal organs affected by MTX were liver, kidney, lungs, and testes with lesions characterised by degeneration, necrosis, congestion, haemorrhage, and vascular changes. Diarrhoea was seen in 25% of animals from the mid (0.125 mg/kg) dose group in the last week of treatment, in 16% of animals from the high (0.25 mg/kg) dose group in the third week of treatment. The remaining 84% from the high (0.25 mg/kg) dose similarly showed diarrhoea in the last week of treatment. Dullness and lethargy were seen in 58% of rats in the high (0.25 mg/kg) dose group. No significant weight loss was seen in rats treated with low (0.062 mg/kg) dose, however, those treated with mid (0.125 mg/kg) and high (0.25 mg/kg) dose, weight loss was observed from the seventh day of treatment onwards. Haematological analysis demonstrated a pronounced reduction in red and white blood cell counts, neutrophils and lymphocytes in males treated with high (0.25 mg/kg) doses. Males treated with mid (0.125 mg/kg) dose showed an increase in white blood cell and neutrophil counts. High (0.25 mg/kg) dose females demonstrated a significant decrease in red and white blood cell count, with an increase in neutrophil and lymphocyte count. Females treated with mid (0.125 mg/kg) dose only showed an increase in white blood cell count. At a low (0.062 mg/kg) dose, the only pathologic lesion seen was an enlargement of the liver. At mid dose (0.125 mg/kg), the liver was pale and fatty, congestion on the surface with mild to moderate hepatocyte granularity. Severe congestion, vacuolar degeneration of tubular epithelium and

haemorrhages in between the tubules was also seen in the kidneys of these rats. Mild to moderate congestion and emphysema was seen in the lungs of rats treated in this group, with alveolar dilation and haemorrhage. And at high dose (0.25 mg/kg), the liver was pale and fatty with mild to moderate surface congestion with signs of vacuolar degeneration, central vein dilation and congestion along with scattered appearance of haemorrhages. Mild to moderate renal tubular necrosis was seen along with vacuolar degeneration of tubular cell cytoplasm, as well as haemorrhages amidst the tubules. Congestion and emphysema were also seen in the rats of this group with thickening and mononuclear cell infiltration of interstitial space as well as focal haemorrhages. An alteration seen only at high dose was that of the testes, epididymis, spleen, and intestine. Testes displayed swollen nature and bulging cut surface on macroscopic examination with histological examination displaying mild atrophy of seminiferous tubules and mild to moderate germ cell shedding. The epididymis showed a reduced concentration of sperm within the tubules. Enlargement and congestion of the spleen was seen with differing levels of haemosiderin deposition. Intestinal lesions included mild mucosal congestion, characterized by desquamation and denudation of villi surface epithelium further leading to erosions and necrosis with neutrophilic infiltration [70].

Adult male Wistar albino rats were used in a study to discern the histomorphological and cellular toxicity of 2-month intraperitoneal administration of MTX [71]. A total of 30 rats were divided into 3 groups, 1 control and 2 experimental, containing 10 animals each. The two experimental doses were 25 µg and 50 µg, whereas the control group was given normal saline. After the termination of treatment, animals were sacrificed, testes were collected, processed, and stained with HE-staining. Microscopic study of the seminiferous tubules, interstitial spaces, primary spermatocytes, and spermatids were performed. Examination revealed discontinuity of the basement membrane of seminiferous tubule, reduction in the number and diameter of spermatocytes and disrupted morphology of Leydig cells. Both experimental groups also showed a decrease in diameter of seminiferous tubules, increase in interstitial space and changes in cellular proliferation. All the above effects were seen to have a dose dependent increase in severity [71].

Young mice were used to determine whether chronic administration of MTX would cause suppression of skeletal growth [72]. 12 Balb/c male mice, 3 weeks of age (rapid growth phase) were divided into 2 groups containing 6 animals each. One group received intraperitoneal MTX at a dose of 3.5 mg/kg every second day, and the other group received physiological saline at the same time points and served as the control group. During the treatment period, animals were weighed every second day to appropriately dose them.

Animals were sacrificed two days after the last treatment was administered, blood was collected by cardiac puncture, femurs and tibias from both sides were collected, measured, and examined by X-ray. Histological tissue samples were also collected from the bones for the measurement of height of the distal femoral and proximal tibial growth plates. A marked decrease in the lengths of the femur and tibia with a similar decrease in the height of the growth plates were seen in the experimental group, with histological examination showing the effect of MTX was mainly in the hypertrophic proliferative zone of chondrocytes. Blood samples also showed an increased level of plasma MTX and decreased levels of erythrocyte folates. When examining the body weights of these animals, the difference in weights when compared to the control group was significant at week 2 and 3 [72].

3.2. Piritrexim

BW301, or more commonly known as piritrexim, a demethoxy analogue of MTX, is a lipid soluble second-generation inhibitor of DHFR [73, 74]. An advantage of piritrexim, over its predecessor methotrexate, is the reduced side effects due to lack of affinity to histamine-N-methyltransferase enzyme [74]. Piritrexim has demonstrated good antineoplastic activity in clinical trials of carcinomas, sarcomas, leukaemia, melanomas, lung, colon, head, and neck cancer [74]. Another indication for its use is in the treatment of psoriasis and secondary infections, caused by *Pneumocystis* and *Toxoplasma* species, in AIDS patients [74]. Due to their lipophilic nature, they do not require active transport and passively diffuse into the cell. The molecule also lacks a glutamate side chain which means they do not need to be activated [74]. Antitumor activity of piritrexim and its methyl substituents was evaluated in a large number of human cell lines including leukaemia, melanoma, lung and colon cancer, among others [74]. The activity was evaluated using the growth inhibition power (GI_{50}). An increase in potency was seen by increasing the methyl substituents at 5' position. The highest activity was seen against leukaemia cell lines, followed by colon and non-small cell lung cancer lines [74].

An evaluation of the toxicity of piritrexim was performed in rats and beagle dogs [75]. Four dose schedules were tested - 5 daily *oral* doses, 90 daily *oral* doses, single intravenous dose and 5 daily intravenous doses.

For the single oral dose study, male and female rats were given a combined mean dose of either 764 mg/kg, 1168 mg/kg, or 1572 mg/kg, through gavage. These doses correspond to

the lethal dose seen for 10% (LD_{10}), 50% (LD_{50}) and 90% (LD_{90}) of animals, respectively. Observations during the treatment period displayed weight loss, nasal discharge, soft faeces and urine stained genital area, due to leakage of urine, as part of the clinical signs of toxicity. For the 5 daily oral dose study, male and female rats were given a dose of either 60 mg/kg, 120 mg/kg, and 180 mg/kg. Blood samples taken after the first dose from rats receiving 60 mg/kg for 5 days showed a decrease in white blood cell and lymphocyte counts as well as total serum protein, albumin, and globulin. In addition to this, rats in the groups receiving doses of 120 mg/kg and 180 mg/kg for 5 days, showed reduction in the quantity of absolute neutrophils and alkaline phosphatase levels after a single dose. In females from the mid dose (120 mg/kg) group surviving to day 15 from the start of treatment, a reduction in red blood cell count, haemoglobin and haematocrit and an associated increase in nucleated increase in nucleated red blood cell, platelet, white blood cell, neutrophil and lymphocyte counts, and mean cell volume (MCV) were seen along with the appearance of erythrocytes polychroma. Histopathological examination was performed on both animals that died during treatment as well as those sacrificed 29 days after start of treatment. Lesions in those that died during treatment were seen in the bone marrow (hypoplasia, congestion, haemorrhage), intestine (maturation arrest, ulcerative enteritis most severe in the cecum, caecal ulcerations), spleen and thymus (involution or atrophy) as well as the liver (subacute or chronic pericholangitis). The lesions seen at 29 days were noted to be absent or healing suggesting that the effects of piritrexim were reversible [75]. For the 90-day oral dose study, the rats were given a dose of 5 mg/kg/day, 15 mg/kg/day or 45 mg/kg/day through oral gavage. No drug-related effects were seen in the low dose group, however clinical signs and examinations from animals treated in the mid and high dose group were comparable to those seen in rats treated for 5 consecutive days [75]. The intravenous studies in rats were limited to the requirement of high volume of infusion which could not be appropriately performed. The highest doses that could be given (50 mg/kg for a single dose, 40 mg/kg/day for 5 consecutive days) were less than the dose lethal to 10% of animals (LD_{10}) [75].

A single oral dose of either 24 mg/kg, 240 mg/kg or 480 mg/kg was given to 3 groups of 4 beagle dogs each (2 male and 2 female) in the form of gelatine capsules. 25% of dogs that received a dose of 24 mg/kg displayed a decreased food intake and resulting weight loss, however these changes were modest and could be reversed. Vomiting and changes in consistency of faeces as well as a similar reversible decrease in food consumption was seen in all animals receiving 240 mg/kg. 50% (2 female) of dogs receiving 480 mg/kg either died or were sacrificed due to moribund status by the 7th day after treatment. All dogs in this

group also showed similar signs to the previous dose groups with additional appearance of dehydration and lethargy [75]. The doses given for 5 consecutive days were 2.5 mg/kg/day, 12.5 mg/kg/day and 25 mg/kg/day and were given orally in gelatine capsules to 3 groups of 4 dogs each, as seen above. Dogs in the low dose group were noted to have diarrhoea and weight loss (seen only in males) and blood analysis demonstrated minimal decrease in total white blood cell and absolute neutrophil counts. Similar to the single dose study, these effects were seen to be reversible. In addition to the above symptoms, emesis, anorexia, and decreased activity were also seen in dogs treated with mid dose (12.5 mg/kg/day). Both male dogs in the high dose (25 mg/kg/day) group died 8 days after the start of treatment. All dogs in this group exhibited the same symptoms as the mid dose group [75]. Histopathological lesions were comparable to those of the 90-day group described below.

Groups of 10 dogs each (5 male and 5 female) were treated with an oral dose of 0.1 mg/kg/day, 0.5 mg/kg/day or 2.5 mg/kg/day for 91-92 consecutive days. Animals in the low dose group displayed emesis but no other treated related histopathological or clinical signs. Similarly, animals treated with mid (0.5 mg/kg/day) did not display any adverse effects during treatment period, however, blood analysis of females (20%) displayed a decrease in red blood cell count, haemoglobin, and packed cell volume (PCV) on treatment day 86 with recovery of these parameters seen in the 29-day post-treatment period. Other lesions seen in the 4 dogs surviving till post treatment period were maturation arrest of myeloid series (50%) and moderate thymic atrophy (25%). Thymic atrophy was also noted in 16% of animals killed at the end of dosing period [75]. Out of 10 dogs treated with high dose (2.5 mg/kg/day), 70% of animals died or were killed due to moribund health status during the period of 14 to 86 days from the start of treatment. Vomiting, diarrhoea, and dehydration were seen preceding these natural or induced deaths. An average weight loss of 24% (15-33%) was seen in 90% of dogs in this group. Haematological changes included a decrease in red blood cell and white blood cell counts, haemoglobin and packed cell volume seen in all animals. Histopathological effects seen included maturation arrest of the oesophageal, intestinal and gall bladder epithelium. Hematopoietic maturation arrest and atrophy of the testes was seen in one male dog (20%) [75]. The single dose intravenous study was not able to be performed in dogs similarly due to the high level of infusion volume required, however, it was determined that single 5 mg/kg doses of 0.2 mg/ml drug solution did not demonstrate any toxic effects [75]. The 5 daily *intravenous* dose study was performed in groups of 8 dogs with equal numbers of each sex at a dose of either 1.25 mg/kg/day or 5 mg/kg/day. Mild drug related effects were seen in a minority of animals treated with the low dose and these effects

were seen to be reversible. Dogs in the high dose group displayed adverse effects comparable to those seen in dogs given a subchronic dose of piritrexim orally for 5 days. These effects were also reversible as seen in the 2 months after termination of treatment [75].

3.3. Pemetrexed

In an effort to develop new inhibitors of THF cofactor dependent enzymes, a team from Princeton and Eli Lilly explored compounds related to predecessor lometrexol. In their efforts, they replaced the 5-deaza-pteridine ring of lometrexol with a pyrrolopyrimidine ring and synthesised a new generation antifolate that came to be known as pemetrexed (PTX) [63]. PTX is currently used in the treatment of pleural mesothelioma and non-small cell lung cancers. Combined therapy of PTX and other chemotherapeutic agents have displayed good activity in the treatment of solid tumours such as breast, colorectal, gastric, and cervical tumours [63]. Large amounts (about 80%) of PTX are eliminated rapidly in the urine, a few hours after administration, in early clinical models [76]. Transport of PTX into the cell is rapid and facilitated by 3 transporters, reduced folate carrier (RFC), folate receptor alpha and the proton coupled folate transporter (PCFT), a unidirectional symporter. PTX displays high affinity for all of them [61, 63]. This means that if the transport with one of them is impaired, then the overall activity of PTX remains the same as it can be transported by other mechanisms. Transport via PCFT is a requirement for the absorption of folates from the gastrointestinal tract. Individuals with hereditary folate malabsorption are found to have a mutation in this transporter [63]. PTX primarily targets the enzyme TS, whose action produces thymine nucleotide, a building block of DNA [61]. Upon entry into the cell, it is rapidly converted by folylpolyglutamate synthase (FPGS), for which it has high affinity [76], into its active metabolite. The polyglutamated metabolite can be retained within the cell for long periods throughout which it produces a high level of suppression of TS and THF cofactor-dependent reactions [63]. It also displays an increased sensitivity to the levels of folates in cellular pools. PTX was found to produce a secondary inhibition on GARFT enzymes through cell culture experiments and enzymology [63, 76]. Its effect on GARFT was much less potent than its effect on TS. This recognition of the ability of PTX to inhibit multiple targets resulted in its naming initially, as multitargeted antifolate (MTA) [63].

Male Sprague-Dawley rats were used in a study to determine the safety of intrathecal administration of pemetrexed [77]. Three groups of 15 rats each were given a dose of 0.3

mg/kg (low), 1 mg/kg (mid) or 3 mg/kg (high), 2 times a week for a period of 2 weeks (4 total doses). The drug solution was administered over a period of 20 minutes. Animals were monitored every day during treatment and for up to 7 days after end of treatment and evaluated for the presence of clinical or pathological neurotoxicity. All rats showing signs of toxicity as well as 1 randomly chosen rat from each group were euthanized and pathological examination of the entire neuroaxis and representative samples of the brain and thoracic level spinal cord were performed. No drug related neurotoxicity was seen in the low and mid dose groups, however a mortality rate of 33% (5/15) was seen in animals treated with high dose. Clinical symptoms seen in these five animals included hyperactive and abnormal movements (turning, screaming) within 10 minutes after first injection in 4 rats and after second injection in 1 rat. These symptoms, however, did not show any corresponding pathological lesions such as neuronal loss, ventriculitis, demyelination etc. [77].

An oral toxicity study was performed on 7- to 8-week-old male athymic nude mice previously inoculated with human lung cancer (A549) cells [78]. Mice were treated on a metronomic schedule with a dose of 5 mg/kg daily for a period of one month and monitored regularly for signs of adverse effects and body weight changes. Blood and organ samples were collected for analysis after the termination of treatment. There were no significant apoptotic regions seen in the individual gastrointestinal tract (stomach, duodenum, jejunum, ileum, colon) samples when compared to control. Histological analysis of other organs such as liver, spleen, and kidney, also did not yield any abnormalities. Similarly, haematological analysis revealed no significant changes in comparison to the control group [78].

A series of toxicological studies were performed by the FDA in beagle dogs and the results of these studies are described below [79]. Two groups of twelve dogs each were given 4 weekly intravenous injections of 10 mg/kg (low) or 25 mg/kg (high), followed by a 3-week period of recovery. Dogs in the high dose group were noted to have infrequent emesis and foamy *vomitus* on days 1-3 of treatment. Redness and flaky skin on the ventral abdomen were seen in low dose (8%) and high dose (50%) groups, which resolved by the end of recovery period. Dose dependent decreases in body weight and changes in faecal matter were seen in both groups. Blood samples taken from both groups demonstrated slight to moderate reduction in neutrophil, lymphocyte, reticulocyte, and thrombocyte count. These parameters showed recovery by the end of the recovery period, except the thrombocyte count of dogs in the high dose group. Pathological examination demonstrated reduced liver weight (without morphological changes), minimal to slight enteropathy of the gastrointestinal tract (mucosal

haemorrhages, inflammation, oedema, crypt necrosis). The enteropathic lesions were still present in minimal amounts in some dogs at the end of recovery period [79].

Two groups of 8 dogs each were given the same doses as above, at first one time per week up to day 77, and further administration was once every 3 weeks. 25% of dogs in the high dose group were euthanized at days 108 and 115, due to severe thrombocytopenia, neutropenia, generalised haemorrhages and significant hypocellularity of the bone marrow. 12% of dogs in the low dose group were euthanized on day 84. The three above dogs all displayed hypoactivity, inappetence and persistent adverse skin lesions. Clinical signs in the surviving dogs during treatment period were skin lesions (erythema, desquamation, excoriation) in the abdominal and urogenital regions, and abnormal faeces (mucoid, watery). Decreases in body weight were observed to be 17-20% within 2-4 days after each dose. This is correlated with the concurrent decrease in food consumption, these were transient and limited to 2-4 days after each dose. The three dogs that were euthanized, however, displayed severe decreases in food consumption (≥75%) before euthanasia. Twelve percentage of dogs in low dose and 37% in high dose displayed severe hematotoxicity (decrease in white blood cell, lymphocyte, neutrophil, reticulocyte, and thrombocyte counts). Pathological lesions were seen in organs such as oesophagus and bone marrow (tissue alteration), stomach (erosion), small and large intestine (focal reddening of mucosa, inflammation), kidney (hydronephrosis), urinary bladder (haemorrhage) and liver, prostate and thymus all decreased in size [79].

The reproductive toxicity of pemetrexed was investigated in 90 male CD1 mice (30 per dose group) given doses of 0.1 mg/kg, 1 mg/kg, and 10 mg/kg intraperitoneally for 6 weeks. The males were mated with untreated females, evidence of which was seen by the inspection of copulatory plugs and detection of sperm presence by vaginal lavage. On day 13 post-mating, pregnant females were euthanized, and uterine contents were removed and examined. One male from each group died without clinical signs. Pathology revealed a pronounced decrease in testis and slight decrease in epididymis weight in all treatment groups. This correlated with the histological examination which displayed severe testicular atrophy, collapsed seminiferous tubules, minimal to slight vacuolation of epididymal epithelium and marked hypospermia [79].

A second reproductive study was performed in 90 female CD1 mice (30 per dose group) at doses of 0.2 mg/kg (low), 1 mg/kg (mid) and 5 mg/kg (high). The dose was given *intravenously* on days 6-15 of gestation. The female mice were mated, and pregnancy was confirmed with the same procedure mentioned above. One female was excluded from the low dose group due to the presence of a swollen and firm vulva seen on gestational day 5. All

other females were sacrificed 18 days after mating and their necropsies performed. A decrease of 16% in body weight gain of females was seen in the high dose group. There was also a dose-dependent decrease in foetal weights, increased incidences of malformations (incomplete ossification of skull bones), cleft palate (85% in high dose group, <2% in other groups) [79].

Six adult rabbits were used to determine the ocular toxicity of pemetrexed. A gel with the dose of 30 mg in 0.1ml into the right conjunctival sac, and the left eye served as control. Iritis was seen in 16% and conjunctivitis (redness, swelling, discharge) was seen in 100% of rabbits within 1 hour after treatment. The iritis resolved within 24 hours whereas the conjunctivitis diminished over the test period and resolved completely after 7 days [79].

The dermal toxicity of pemetrexed was investigated in 10 adult rabbits with a single dose of 1000 mg/kg on shaved dorsal trunk. Clinical signs seen during treatment were soft stools, faecal staining, dermal irritation, and oedema at the drug application site [79].

3.4. Pralatrexate

Pralatrexate (PDX) is an inhibitor of DHFR and displays a more potent decrease in cell viability, as compared to that of its predecessor MTX [61, 80]. PDX was previously used in the treatment of relapsed T cell lymphomas [80]. Transport of PDX is facilitated by the reduced folate carrier (RFC). A 10-fold increase in affinity to RFC, compared to MTX, was achieved by targeting it while designing the compound [80]. As a result, PDX exhibits improved transport and cellular retention [61]. Sensitivity of tumour to PDX is demonstrated to be directly linked to the expression of RFC within the cells [80]. PDX has a high affinity for FPGS, which moderates its polyglutamylation. Its metabolites have prolonged intracellular retention and effect [80]. Cell models testing effects of antifolate drugs show that the tumour growth inhibition, in particular that of T cell malignancies, of PDX is higher than the other antifolates [80]. Treatment with PDX block cells in the S phase of cell cycles as seen in sensitive myeloma cell lines. These cell lines were able to initiate synthesis of DNA but could not progress past the S phase. This effect was varied based on concentration of drug and time of exposure [80]. Although multiple myeloma is not considered to be sensitive to antifolates, we have used cell lines for it in preclinical studies due to evidence suggesting otherwise. Previous non consideration could be due to the fact that results have been poor with previous analogues, such as MTX. However, PDX is shown to be a better anticancer

drug and data collected on myeloma cells studied, show that they are either highly sensitive or resistant to both agents, MTX and PDX [80]. A concentration dependent induction of apoptosis was seen in preclinical cytotoxicity testing in myeloma cell lines. Concomitant administration of leucovorin in both preclinical and clinical tests did not affect the activity of PDX [80].

Sprague-Dawley rats treated with single intravenous doses of 5 mg/kg, 10 mg/kg and 25 mg/kg did not result in neurotoxicity [81]. Single intravenous administration of 0.3-1 mg/kg in beagles lead to emesis and soft stools whereas multiple dosing resulted in weight loss and gastrointestinal toxicities. Reduced red blood cell, lymphocyte and absolute basophil counts, haematocrit and haemoglobin concentration were seen in pralatrexate treated dogs [81].

The Australian department of health and ageing performed an assessment report for pralatrexate in 2013 [82]. The single dose intravenous study performed in beagle dogs reported that 3 mg/kg dose resulted in 100% mortality, which is twice the intended clinical dose. All animals in the study died or were euthanized prematurely (<6 days after dosing). Severe gastrointestinal disturbances (bloody diarrhoea, hematemesis) were seen before death [82].

Beagle dogs were administered intravenous pralatrexate doses of 0.1 mg/kg, 0.3 mg/kg, and 0.7 mg/kg once a week for a period of 6 weeks, followed up by a week with no treatment. This cycle continued for a duration of 9 months. The maximum tolerated dose was determined to be 0.3 mg/kg. Above this dose, erythropoiesis and hypocellularity of bone marrow, resulting in anaemia. As a compensation, an increased level of reticulocytes was also seen. Clinical signs seen during treatment were reduced weight gain, decrease in food consumption, appetite loss, emesis, and diarrhoea. Pathological examination of gastrointestinal tract demonstration minimal to moderate diffuse villus fusion in the small intestine, infrequent dilation, and crypt epithelial necrosis. These gastrointestinal lesions were the cause of euthanasia in some animals and thus was considered a dose limiting toxicity. White blood cell effects and thymic atrophy were seen at a dose of 0.7 mg/kg in 60% (3/5) of females. Intravenous injections also showed signs of irritation with reddish discolouration [82].

SD rats were administered pralatrexate doses of 5 mg/kg, 10 mg/kg, and 25 mg/kg, *intravenously* on the same schedule as the dogs, however, the cycle continued for 6 months.

Rats given doses ≥5 mg/kg displayed similar effects on blood and bone marrow, the resulting anaemia, however, was megaloblastic. In addition to the above-mentioned gastrointestinal effects, splenomegaly and increased haematopoiesis was seen in the liver and spleen. The

maximum tolerated dose was determined to be 25 mg/kg. Treatment with a dose of 25 mg/kg demonstrated white blood cell effects and a pronounced decrease in testis weights [82].
The reproductive toxicity of intravenous pralatrexate was also tested on SD rats and NZW rabbits. The doses tested in rats were 0.01 mg/kg/day, 0.03 mg/kg/day and 0.06 mg/kg/day. When pregnant rats were given ≥0.06 mg/kg, there was an increase in post-implantation loss and reduction in the number of viable implants and live foetuses. Foetuses of females treated with above dose showed growth retardation and malformations such as hydrocephalus, syn- or brachydactyly of forelimb and hindlimb, in 50% (2/4) of foetuses. When a dose of ≥0.1 mg/kg/day was given, a reduction in total litter size was seen [82].
The doses (0.03 mg/kg, 0.1 mg/kg, and 1 mg/kg) tested in rabbits showed similar negative effects on viability of foetuses along with total litter loss at doses ≥1 mg/kg [82].

4. THYMIDYLATE SYNTHASE INHIBITORS

Thymidylate synthase (TS) is an important enzyme in the *de novo* pathway of DNA synthesis and repair. It acts on deoxyuridine monophosphate (dUMP) to form deoxythymidine monophosphate (dTMP), by the transfer of a methylene group from cofactor tetrahydrofolate (THF) converting itself to dihydrofolate (DHF). TS is an ideal target for cancer treatment and efforts to develop dUMP and tetrahydrofolate analogues have been undertaken [83, 84]. However, a common side effect of these agents are the toxicities of the proliferating organs, such as gastrointestinal tract and bone marrow [85].
The most commonly used drugs within this group today are the followings: fluorouracil, raltitrexed, capecitabine and plevitrexed.
Other drugs that belong to this group but are less commonly used are: carmofur, tegafur uracil, nolatrexed and BGC 945.

4.1. Fluorouracil

5-fluorouracil (FU), a heterocyclic aromatic compound that mimics the pyrimidine ring of both DNA and RNA, is a potent inhibitor of TS and shows good antitumor activity [5, 86]. Scientists were encouraged by the clinical success of FU and began testing of other

pyrimidines and their nucleosides [5]. 5FU is currently part of the treatment regimens for breast cancer, especially in those individuals who have developed resistance against tamoxifen. Combination treatment with other chemotherapeutics can provide promising results in drug resistant cancers [86]. Preoperative therapy in locally advanced rectal cancer cases, show improved response and tumour downstaging [87]. It is also commonly used in the clinical treatment of stomach, head, and neck cancer [88]. 5FU is rapidly metabolised in the liver by dihydropyrimidine dehydrogenase (DPD) to dihydro-5-fluorouracil [76, 88]. This poses a major obstacle during treatment. Intracellular conversion to its active metabolites, 5-fluoro-uridine-triphosphate (FUTP) and 5F-deoxyuridine monophosphate (FdUMP) [63]. FUTP causes direct RNA damage by incorporating itself into RNA in the place of UTP. FdUMP causes the inhibition of TS preventing the conversion of dUMP to dTMP, which is an important part of DNA synthesis [63]. It may also provide "counterfeit" bases for incorporation into both DNA and RNA [76]. The anticancer effects of FU are attributed to the above mechanisms, however on administration with leucovorin, the predominant mechanism is TS inhibition [63]. 5FU causes the development of abnormally shaped spermatids and vacuolar degeneration of Sertoli cells but does not cause damage to the spermatogonia [89].

The effect of 5-fluorouracil on cellular proliferation of bone marrow and autophagy was investigated in adult female C3H mice [90]. Animals were administered a dose of 100 mg/kg of 5 FU through intraperitoneal injection. Animals were sacrificed after 10 days of treatment; samples were collected from tissues and a cellular suspension of bone marrow was made from the femurs of the mice. The bone marrow from the treated animals displayed a reduction in the erythroid cell populations at all maturation stages from colony forming cell to late reticulocyte and finally the mature erythrocyte stage. There is a strong change seen in the bone marrow, that is either focal or peripheral, during the period of 1-6 days of treatment. From day 7 onwards, a replacement of bone marrow cells can be seen by the evidence of numerous centres of blood forming cells. All mice in this study showed recovery by the time of sacrifice, with repopulation of myeloid, erythroid, lymphoid, and megakaryocytic cell lineages [90].

The ototoxicity of 5FU after intratympanic administration was investigated in Wistar rats [91]. A total of 7 male rats were given doses of 7.5-10 mg/rat, depending on the individual bulla volume, administered to the right eardrum in the amount of 0.1 ml. All rats were sacrificed 7 days after administration of treatment and their right cochleas were dissected, stained with H&E (haematoxylin eosin) and histopathologically examined. The left untreated cochlea of 3 rats was used as a control. The treated cochleas displayed oedema, cytoplasmic

changes and resulted in an increase in thickness of the stria vascularis. The most severe changes were seen in the spiral ganglion (minimal to moderate oedema, increase in nuclear pyknosis and acidophilia) and limbus (lipid accumulation and vacuolization, minimal oedema) [91].

Twenty six cases of accidental poisoning with 5 FU in dogs during a period of 2 years (1987, 1988) were reviewed for this study [92]. Of these cases, 46% (12/26) were confirmed and 50% (13/26) were suspected of toxicosis. The most common clinical signs, observed within 45-60 minutes after exposure, included generalised (grand mal) seizures, emesis, and diarrhoea (with or without blood), tremors and respiratory distress or cyanosis. 31% (8/26) of dogs were euthanized due to presence of uncontrolled seizures or severe gastrointestinal haemorrhages. Due to most of these cases occurring due to accidental ingestion, the doses of 5-FU were estimated based on known quantities of topical creams containing the drug. Based on the information provided from owners, the ingested doses were estimated to be in the range of 6-271 mg/kg, with mortality seen in every case with an ingestion dose >43 mg/kg. Blood analysis was performed in 2 surviving dogs, and one displayed only mild elevations in ALT, ALKP enzymes and creatinine activity 2 days after exposure. These values returned to normal when repeat blood analysis was performed 12 days later. Pathological lesions were reported in the necropsy examination of one dog, such as diffuse pulmonary oedema, intestinal crypt cell necrosis, lymphoid necrosis of Peyer's patches and oedema in the white matter of the cerebellum [92].

A group of 5 adult male albino rats were given a dose of 10 mg/kg/day through intraperitoneal route for a period of 2 weeks [93]. Rats were anaesthetized at the end of treatment period and blood samples were collected for evaluation. A marked decrease in the counts of white blood cells (lymphocytes, neutrophils, eosinophils, monocytes), red blood cells, platelets, haematocrit, and haemoglobin (Hb) content were reported in the animals treated [93].

Male Wistar rats were used to evaluate the effect of FU on the liver, kidneys, and lungs [94]. 14 rats were divided equally into control and treatment groups, with intraperitoneal administration of 15 mg/kg for 4 consecutive days followed by a reduced dose of 6 mg/kg for 4 alternate days and finally a return to 15 mg/kg on the 14th day from the first dose. Euthanasia of all rats was performed on the 15th day, followed by the collection and examination of blood, liver, lung, and kidney samples. There was a significant increase in the hepatocyte surface area, nuclear area, and diameter of sinusoidal capillaries. 28% (2/7) of animals displayed moderate steatosis, while 71.4% (5/7) displayed severe steatosis. Portal

inflammatory infiltration was seen in all animals with varying levels of severity, 15% (severe), 70% (moderate), 15% (mild). Mild to moderate inflammation of the lobular region was also seen. As for the lung, its weight was significantly higher as compared to control with further examination showing a marked decrease in the amount of type 1 and 3 collagens. An increased number of haemorrhagic foci, perivascular focal infiltrates, diffuse infiltrates of parenchyma, oedema, thickness of alveolar septum and decrease in alveolar area were also seen. The kidney corpuscle parameters, such as corpuscle area, visceral layer area and bowman space, were increased with a decrease in density of the renal corpuscle itself. The distal tubules displayed an increase in the external and internal areas as well as thickness, whereas the proximal tubules remained largely unaffected [94].

The dermal toxicity of FU was evaluated in hairless (20), hairy black and Swiss albino (20) mice [95]. For the hairy mice, an area on the dorsal surface of animals was shaved prior to the application of drug ointment. 5% ointment was applied once daily to the dorsal surface of animals, with the number of doses (either one, two or seven) given to 30 hairy and hairless mice chosen at random. The remaining 10 were used as control. Samples were collected from the dorsal surface prior to drug application, at second, fifth, fourteenth day and two months after application. Light microscopic examination was performed on all the samples mentioned above whereas electron microscopy was performed only on samples taken before application and fourteenth day. Hairy mice did not show any changes when treated with FU, but prominent changes were seen in the majority of hairless mice. 40% mice given one dose demonstrated slight erosion at 2-3 days, crust formation at 4-5 days and pigmentation at 10-14 days after application. 100% that received two doses displayed the same lesions but at earlier time points, erosion (1-2 days), crust formation (3-4 days) and pigmentation (7-10 days). The peak of pigmentation was seen at 2-3 weeks and gradually faded back to normal by 3 months. Samples from this group taken at 2 days showed tissue separation of epidermis and connective tissue, loss of epidermis following necrosis and mild inflammatory cell infiltration of the upper part of dermis. Crust formation with basophilic degeneration and necrosis of the upper part of dermis was seen in the samples taken at five days. The fourteen-day samples revealed acanthosis of epidermis with scattered pigment granules (increased number of melanocytes) in the basal layer. These findings were not seen in the samples taken at 2 months after treatment, with melanocyte numbers returning to normal. 100% mice receiving seven doses exhibited ulceration on the 3-5th application with crust formation (3-4 days), skin hyperplasia (10-14 days) and slight pigmentation (starting at 3 weeks, lasting until 6 months after application). Samples from this group taken at 2 days showed exhibited loss of

epidermis and necrosis of upper dermis with severe inflammatory cell infiltrate. Crust formation with acanthosis of epidermis was seen in the samples taken at five days. The fourteen-day samples revealed marked hyperplasia and irregular downward proliferation of epidermis and an increased number of pseudohorn cysts. The cyst walls contained a small number of melanocytes while larger numbers were seen throughout the dermis, predominantly in the upper part. Unlike with two applications, the samples taken at 2 months after seven doses exhibited scar formation, thin epidermis, and increased number of fibroblasts in upper dermis [95].

LD_{50} in different species is known to be 100 mg/kg (rats), 500 mg/kg (mice) and 20 mg/kg (dogs) [96]. In a case of accidental ingestion of 5% FU ointment by a dog, vomiting and seizures were seen shortly after ingestion, followed by comatose state and death within a few hours. These are the most common effects seen with FU toxicosis in dogs, with onset of symptoms beginning at 30 minutes and death within 7 hours after ingestion. Cats are also seen to be sensitive to FU [96].

A group of nine BALB/c male mice were divided into 3 groups with two groups given an intraperitoneal (IP) dose of either 100 mg/kg (low) or 200 mg/kg (high) and the remaining group was used as control [97]. Weight and diarrhoea scores were measured daily and after termination of treatment, all animals were sacrificed, and organ samples collected. Significant reduction of body weight was seen as early as one day after administration, with a higher rate of reduction seen in the high dose group. Spleen weight was notably decreased with no difference seen in the weight of liver or length of intestines. The cumulative diarrhoea score was seen to be increased 3 days after treatment [97].

Male C57BL/6J mice were given a single IP injection of 50 mg/kg for four days, and 24 hours after termination of treatment, animals were sacrificed, and gastrointestinal tract (jejunum, ileum, and colon) were removed [98]. By the 3-4th day of administration, body weight of mice showed a marked decrease and diarrhoea scores increased. The upregulation of inflammatory cytokines in the colon was seen on day 5 [98].

Six male Wistar rats were given an IP dose of 15 mg/kg for 4 successive days, followed by 6 mg/kg for 4 alternating days and a last dose of 15 mg/kg on the 14th day [99]. After termination of treatment, animals were fasted for 12 hours, euthanised and distal ileum was collected for analysis. Width of villus, depth of crypts and thickness of submucosa was increased, and the height of villus was decreased [99].

Male Sprague Dawley rats were divided into four groups, a control and three treatment groups given a dose of 25 mg/kg for 1 or 2 days, 3 days and 4 or 5 days [100]. These rats had

previously undergone intestinal anastomosis surgery and this study was performed to determine the effect of FU on the anastomosis. All animals that died or were sacrificed at 12 days were examined. Anastomotic leaks leading to death within 3-6 days after operation, were seen in groups of rats given treatment for 4 or 5 days (89%) and 3 days (63%), in comparison to the 1 or 2 days (28%) and control (19%) group. Spontaneous disruption of abdominal wounds was not seen in this study [100].

A group of 6 male rats were given three IP injections of 20 mg/kg FU on alternate days [101]. Body weights were measured daily, and sacrifice was done at the end of treatment on day 7. Cerebellar tissue was collected for staining and immunohistochemical evaluation. No significant differences were seen in survival time, food, or water intake during treatment. Cerebellar sections studied revealed shrunken size of granule cells, and slight degenerative changes with loose and separated structure. Immunohistochemical analysis of the cerebral cortex suggested that FU was capable of stimulating astrocyte activation or proliferation. These histological findings indicate that 5-FU induced a mild neurotoxic effect on granule cells *in vivo* [101].

Nine male C57BL/6 mice were given a dose of 35 mg/kg once daily for a period of 5 days through IP administration [102]. Animals were monitored during treatment and 24 hours after final dose, animals were euthanized, and samples (blood, muscle) were collected. A body weight loss of 2-8%, compared to controls, was seen between 2 and 5 days of treatment. The overall average daily food intake was also decreased by 20.5%. The muscle samples taken, such as plantaris, gastrocnemius, quadriceps etc, showed no differences when compared to controls. Weight of the spleen was decreased by 22.6% with concurrent 46.4% decrease of leukocytes in circulation, specifically lymphocytes (33.3%) and neutrophils (83.5%). Other blood parameters also showed decreased levels such as red blood cells (16.8%), haemoglobin (20.8%), haematocrit (18.8%) and platelets (62.6%) [102].

The lowest dose that caused adverse signs and lowest lethal dose in dogs was determined to be 8.6 mg/kg and 20 mg/kg respectively, by the ASPCA APP [103]. In a retrospective study of 73 cases of accidental ingestion of FU in the period between 1989 and 1998, a mortality of 63% was seen (48% died and 15% were euthanized). Onset of clinical signs such as vomiting, seizures, respiratory depression and cardiac arrhythmias occurred 30 minutes after ingestion with death occurring within 7 hours. Bone marrow suppression is not commonly seen after FU ingestion due to quick death, however in animals surviving more than 4-7 days, leukopenia (750 cells/µl), depression and hyperthermia are seen [103].

Nephrotoxicity of FU was evaluated in 10 male CD1 mice given a dose of 80 mg/kg/day IP for a period of 4 weeks [104]. Animals were monitored during study and 24 hours after the end of treatment, animals were sacrificed, and renal tissue was collected for analysis. Parietal cell erosion of the Bowman's capsules (fusion and destruction of foot processes of podocytes), urinary space widening, disruption of glomerular capillaries and haemorrhage was seen on examination of samples. Vacuolar degeneration and coagulative necrosis were seen in the proximal and distal tubules, with the nuclei of these cells displaying pyknosis and karyolysis. Proximal tubules also displayed microvilli destruction and degeneration of mitochondria. Distal tubules demonstrated destruction of basal infoldings and mitochondria as well as fragmentation of the rough endoplasmic reticulum [104].

A group of 30 ICR female mice were used to assess the reproductive toxicity after administration of 50 mg/kg/day for four days [105]. After treatment period, mice were divided into 3 groups - 9 animals were placed in group 1 (super ovulated 48 hours after last treatment and sacrificed 14 hours after hCG injection, another 9 animals were allowed to recover for one week after treatment followed by superovulation and sacrifice in the same method above and the remaining 12 females were caged with males and recording of number and weight of offspring were performed after delivery. Ovarian size of group 1 mice was significantly decreased as compared to control. Ovarian function tests were performed on ovaries harvested after treatment, which showed no enlargement of ampulla, no mature oocyte and decreased number of *corpus luteum* as compared to enlarged ampullas seen in control mice. The continual body weight decline of treated mice was also noticed during treatment. Group 2 mice were noted to have normal ovarian sizes and comparable numbers of luteal bodies as compared to control mice. Enlarged ampullas and mature oocytes were also detected after superovulation in this group. Litter sizes of group 3 mice were smaller than those of the control group, but no other significant effects were seen. The body weight of female mice in the group was significantly decreased but there was no body weight decrease seen in the offspring. During the recovery period, the body weight of female mice returned to normal after 7 days [105].

A group of 22 male Sprague Dawley rats were used to determine the chemotherapy induced oral mucositis (CIOM) after intraperitoneal administration of 160 mg/kg (low), 200 mg/kg (mid) or 240 mg/kg (high) [106]. Ulcer formation was stimulated by the addition of acetic acid at day two, after which its progression was monitored. After euthanasia, buccal mucosa containing ulcer and underlying muscle was obtained at day four and seven after administration. Six rats were placed in the control group with the remaining two groups (with

(G1) or without ulcer formation (G2)) containing eight rats each. White blood cell counts were measured at administration, 2 days, and 4 days after administration. Along with the weights of rats, it was used to evaluate the myelosuppressive status and CIOM-induced distress. All rats in the low and mid dose group survived till day 7, however 50% of rats in the high dose group died at day 2-3. Rats of all dose groups surviving till day 7 demonstrated ulcers, however cheek necrosis was only seen in rats administered with low and mid dose. As a result, the single dose responsible for producing CIOM was determined to be 200 mg/kg (mid), as it is the dose at which rats survived and ulcers lasted till day 7. Significant decrease in weight of rats was seen in G1 from day 2, with G2 rats showing decreased body weight from day 4. White blood cell counts in both experimental groups were decreased at day 2, compared to control, with relative decrease lasting until day 4. Three rats from the control group and four rats from each experimental group (total eight), were euthanized and buccal mucosa was collected for histological evaluation on day 4 and 7. Large granulation with lymphocyte infiltration was seen around ulcer in G1, whereas G2 had preserved squamous epithelium seen on day 4. On day 7, ulcers were seen to be healing (decreased granulation) but squamous epithelium was not fully recovered in G1 [106].

When male Wistar rats were given a dose of FU orally for 3-4 days, a change in body weight was seen [107]. Rats treated with 15 mg/kg once daily, showed only slight decrease whereas those treated with 30 mg/kg showed significant decrease compared to weight of control [107].

4.2. Capecitabine

Capecitabine is formulated as an oral fluoropyrimidine, 5-deoxy-5-fluorouridine (5dFU). It is a systemic prodrug which is designed to mimic a constant rate infusion and generate FU preferentially in tumours [87, 108, 109]. It is prominent in the treatment of breast cancer, as monotherapy or in combination with other chemotherapeutics [109], metastatic colorectal cancer [108], nasopharyngeal carcinomas [47] and in the preoperative treatment of locally advanced rectal cancer [87]. Thymidine phosphorylase (TP) activates 5dFU intracellularly to its active cytotoxic metabolite 5FU and inactive metabolite 5-fluorouridine [108, 109]. This activation requires the drug to be absorbed through the gastrointestinal tract [5], which means administration must be *per os*. A percentage of the total dose is excreted through the faecal route thereby passing the colon and a good therapeutic treatment of colon cancers [108]. Possible synergy was seen with coadministration of tamoxifen and capecitabine in preclinical

studies on oestrogen receptor positive cells of mice xenograft models. This is seen by the elevation of TP and in turn enhancement of capecitabine activity due to increased conversion to its cytotoxic metabolite [109]. Improvement of pathological tumour regression was seen when capecitabine was administered with statin, as a preoperative chemotherapy in locally advanced rectal cancer cases. From this study, we see that the toxicity of the combination was tolerable. This is supported by the lack of mortality related to treatment [87]. Another favourable combination is that of gemcitabine and capecitabine. They show synergistic effects and positive interaction in preclinical studies with pancreatic adenocarcinoma, biliary tract adenocarcinoma and thymic epithelial tumours [47].

Six adult female mixed breed dogs were used to determine the ocular toxicity of capecitabine in renal transplant model [110]. All dogs in the study were administered with increasing doses (50 mg/m^2 to 200 mg/m^2, twice a day) of capecitabine from the day of renal transplant till the end of study. The incidence of blepharospasm and conjunctival hyperaemia in 2 dogs (henceforth named as dog 1 and dog 2) in the 6 to 8 weeks after operation prompted the ophthalmic examination of all dogs. Ophthalmic examinations in dogs 1 and 2 were performed weekly after onset of clinical signs whereas the remaining dogs were examined after roughly 12 weeks of treatment. Both dogs above developed superficial keratitis, which was depicted by multifocal epithelial erosions and rapid, superficial pigmentation of corneal epithelium. Unilateral neovascularization of cornea with the associated oedema was seen in these dogs in the following weeks. There was a slight decrease in tear production in dog 1 over a 6-week period, followed by the development of unilateral, axial, superficial crystalline corneal deposit in one eye at 8 weeks. Treatment was discontinued in both dogs for 7-14 days, resulting in improvement of clinical signs however these signs (conjunctival hyperaemia) recurred within one week after reinstitution. Ophthalmic finding in the remaining four dogs was slightly decreased tear break up times. Euthanasia and necropsy of all animals was performed 14 weeks after surgery, making sure that dogs 1 and 2 had at least one week of readministration of capecitabine before euthanasia. At necropsy, the eye globes were removed, fixed in paraffin, HE and periodic acid Schiff (PAS) stained and analysed. Histopathology and axial epithelial thickness of cornea of 5 normal beagle dogs was examined for comparison. Ocular histopathological abnormalities were limited to corneal epithelium and anterior corneal stroma. Sections of sparse pigmentation (dog 1), epithelial thinning, abnormal morphology of basal cells and epithelial disorganisation (83%) were the epithelial abnormalities seen. Anterior stromal abnormalities were only seen in dogs 1 and 2 and included neovascularization, fibroblast accumulation and mild lymphoplasmacytic

inflammatory infiltrate. The histopathological evidence of crystalline deposit seen in dog 1 was reactive fibroblast clusters within superficial corneal stroma [110].

30 Wistar rats were given a dose of 359 mg/kg of capecitabine through oral gavage one week before intestinal anastomosis surgery and continued for the rest of the study, to study the effects of the drug on the healing of anastomosis [111]. This group was further divided in 3 groups, sacrificed at postoperative day 3 (inflammatory stage), day 7 (fibroblast proliferation, collagen synthesis) and day 14 (remodelling stage). All rats were weighed prior to administration of drug, operation, and sacrifice. A significant and progressive increase of body weight was seen in treated animals whereas control animals lost weight in the seven days post operation. The increase in body weight of study animals was thought to be due to oedema, protein, or fat gain however there is no typical evidence of this. Animals of the study group killed on post operative day (POD) 3, showed lower degree of adhesions as compared to control group at same time point, which showed higher degree. This trend continued where sacrificed animals on POD 7 showed first degree adhesions in the study group (4 rats) and second degree in the control group (all rats), and on POD 14, control group showed second or higher degree of adhesions whereas study group did not show any adhesions. The bursting pressure of anastomosis is a measure of its mechanical strength, and it was found to be higher in the study group as compared to the controls at all time points, with the difference in values getting lower from day 3-14 post operation. Biochemical tests did not yield any significant differences between control and study group, except in the levels of cytokines. Control group animals were seen to have higher levels of IL-6 at POD 7 and TNF-alpha at POD 14. Macroscopic inspection of anastomosis revealed a thickening of the bowel wall for study group animals sacrificed at POD 3, with preservation of multilayered structure but minor necrotic signs [111].

6 male BDF1 mice were treated with a dose of either 1.1 mmol/kg/day (low) or 2.2 mmol/kg/day (high) for a period of 36 days, after which the blood was collected, and organs removed for examination [112]. Nuclear degeneration of crypt cells (duodenum, jejunum, ileum) was seen at both doses, with mild effects seen in 33% of mice at low dose, 50% of mice at high dose with moderate effects in the remainder of high dose mice.

3 male Cynomolgus monkeys were treated with a dose of either 0.5 mmol/kg/day (low) or 1 mmol/kg/day (high) for a period of 27 days and similarly, blood and organs were collected for examination. Severe degeneration of crypt cells was seen in both small and large intestine of monkeys treated at high dose [112].

A retrospective study of client owned dogs with carcinoma of any site that had received at least two cycles of capecitabine therapy (2018-2022) was performed [113]. Oral capecitabine was administered for two weeks at a dose of 750 mg/m^2, followed by a one-week rest period. This three-week cycle was continued until disease progression or toxicity was observed. All dogs were evaluated by physical examination, blood count and chemistry and urinalysis after two cycles and every 2-3 cycles thereafter. Out of a total of 25 dogs examined in the study, the adverse side effects were in the form of gastrointestinal (68%), neurological (12%, afinalistic vocalisation, isolated epileptic seizures), haematological (4%, neutropenia), ocular (8%) and dermatological (8%) toxicities [113].

Healthy male albino rats were given an oral dose of 16 mg/kg twice daily for 14 consecutive days [114]. At the end of treatment, all rats were sacrificed for blood and histopathological examination of the liver. Clinical signs observed included huddling, conjunctivitis, mild tremor, piloerection, and diarrhoea. The body weight and liver weight showed a marked decrease when compared to the control group.

Another group of rats was given a dose of 3 mg/kg twice daily, which caused abnormal function of liver enzymes in all treated rats. The serum levels of liver enzymes, such as alkaline phosphatase (ALKP), were increased significantly. Histopathological examination displayed an abnormal structure of hepatic lobules and characteristic cord-like arrangement of liver cells was lost. In addition, degenerated hepatocytes with vacuolated cytoplasm, ruptured sinusoidal endothelia, areas of necrosis and inflammatory cell infiltration was also observed [114].

9 male albino rats were given a dose of 40 mg/kg by oral gavage once daily for a period of one month to assess the ocular toxicity of capecitabine [115]. This dose is similar to the effective human therapeutic dose. Animals were anaesthetised at the end of treatment period and eyes were enucleated. The cornea was collected for histological and immunohistochemical analysis. No treatment related deaths were seen during the experimental period. Haematoxylin-eosin (HE) stained sections showed multifocal structural alterations with the epithelium being the most affected. Focal areas of epithelial separation, desquamation and in some cases pale vacuolated cytoplasm and deeply stained nuclei were seen. Some focal areas also showed decreased epithelial thickness as well as disruption and separation of stromal fibres. Neovascularization and inflammatory cellular infiltration were seen close to the surface epithelium in the anterior region of the stroma. Corneal epithelial cells, stromal keratocytes and endothelial cells showed strongly positive cytoplasmic and/or nuclear p53 immunoreactions. Electron microscopy confirmed the above-mentioned

structural changes along with evidence of widening and focal separation of intracellular spaces with partial loss of desmosomal junctions. In addition to cytoplasmic vacuolation, swollen mitochondria with disrupted cristae were affected in most epithelial cell layers and some nuclei were seen to have irregular outlines, were shrunken and hyperchromatic. Disorganised collagen fibres in stroma, cytoplasmic vacuolation and dilated rough endoplasmic reticulum in keratocytes and variable sized cytoplasmic vacuoles in endothelial cells [115].

4.3. Raltitrexed

Development of new compounds focusing on creating one that was more water soluble but still had specificity and strong inhibition for TS [83]. A previous compound CB3717 showed good activity in breast and ovarian cancers but also nephrotoxicity and hence was not further developed. By substituting the NH_2 moiety with methyl group, we achieved a compound with better solubility and cytotoxicity [76]. And so, raltitrexed (RTX), a hydrophilic quinazoline compound was created [61]. This drug is used in patients with advanced colorectal cancer, especially those whose cancer has become resistant or intolerant to 5-fluorouracil [61, 76]. A phase III trial conducted discovered that when RTX was administered in combination with cisplatin, an alkylating platinum derivative, the overall survival of patients with malignant pleural mesothelioma was increased [61, 76]. It is mainly transported into the cell with the RFC and FR [61]. Upon entry it is polyglutamylated, by the action of FPGS, which enhances its potency and intracellular retention. Only polyglutamated metabolites have been found in plasma in animal studies, suggesting that no other metabolites are produced [76]. The polyglutamylated form has an increased specificity for TS as compared to the parent compound, however it does not show any compelling inhibitory activity against GARFT or DHFR, other folate dependent enzymes [76]. Protein binding of raltitrexed is nearly 100% as seen in rat plasma. Tested rodents, rats, and mice, show rapid elimination through the biliary system [76]. However, in humans, half of total clearance is done through the renal system, with the remaining through faecal elimination and tissue sequestration [76]. Raltitrexed has good activity with FPGS and immediately undergoes extensive polyglutamylation once entering the cell. The resulting polyglutamate metabolites significantly elevates its TS inhibition and maintains the drug in the intracellular space, both leading to a prolonged effect [61, 63, 83]. As compared to humans and other tested animal species, rodents tolerate higher

doses of raltitrexed due to the higher levels of circulating thymidine in their bodies. However, with repeated doses, cytotoxicity can be induced. Body weight loss, myelosuppression and epithelial damage in the small intestine were seen in BALB/c mice [76].

Pregnant C57BL/6 mice were divided into 6 groups of 10 mice each and treated with different doses of raltitrexed intraperitoneally on gestational day 7.5 [116]. This day was chosen as it is a critical point for the development of neural tubes in murine species. One control group was administered 0.9% NaCl, and five groups were given a dose of 5 mg/kg, 10 mg/kg, 11.5 mg/kg, 13.5 mg/kg, and 15 mg/kg. All mice were sacrificed 4 days after treatment, on gestational day 11.5, and embryos were examined. A dose dependent increase in the rate of resorbed foetuses and growth retardation were seen. At the highest dose (15 mg/kg), the majority of embryos were resorbed and as a result no neural tube defects (NTD) were seen. Embryos with NTDs were seen from the doses of 10-13.5 mg/kg, the rate of which increased with the dose. The highest rate of embryos with NTDs and the lowest resorption rate was seen at a dose of 11.5 mg/kg, therefore this dose was considered the optimal dose to establish murine NTDs. The main defects seen included exencephalia (lack of neural tube in hindbrain), craniofacial malformations and growth retardation. Embryonic growth retardation at 11.5 mg/kg was seen as shorter crown-rump lengths as compared to control. Histological examination of HE stained samples demonstrated the infused nature of the neuroepithelial wall of hind brain, the dorsal edges of the hindbrain were open and neural tube cells had no structure as compared to control [116].

Single intraperitoneal dose of 50 mg/kg in male BALB/c and DBA2 mice did not show any signs of toxicity or body weight loss [117]. To evaluate the toxic effects of raltitrexed on small and large intestine, mice were injected with an intraperitoneal dose of 10 mg/kg (low), 100 mg/kg (mid) or 500 mg/kg (high) for up to 5 days. Five mice from each dose group were sacrificed 24 hours after each dose, with some mice in the low dose group sacrificed 48 and 72 hours after the final dose. Mice were monitored daily for signs of distress, and if they could not freely access food/water or lost >30% of their body weight, they were sacrificed. Only small reduction in body weight was seen in both strains of mice in the low dose group in the first 48 hours, however in the next 24 hours (day 3) BALB/c mice displayed a significant amount of weight loss, around 93% of starting weight, reducing further to 79% (day 6) and 74% (day 8). By this time however, DBA2 mice remained at 95% of their original weight. The maximum tolerated dose for 5 days in BALB/c mice was determined to be 5-10 mg/kg/day, with adverse effects such as weight loss and diarrhoea. When DBA2 mice were given a dose of 10 mg/kg/day for 5 days, they demonstrated less weight loss and

no diarrhoea leading to their MTD being >500 mg/kg/day. Changes in small intestine crypt framework, villus atrophy and changes in the colon were seen to a greater extent in BALB/c mice given a dose of 10 mg/kg/day. A dose of 100 mg/kg/day given to DBA2 mice for 5 days showed similar changes to gastrointestinal tract as mentioned above in BALB/c mice at 10 mg/kg/day but showed later onset and recovery. Haematological effects were studied in groups of five mice receiving *intraperitoneal* injections of raltitrexed. BALB/c mice received 5 mg/kg and DBA2 mice received 5 mg/kg and 100 mg/kg/day for a period of 5 days. Mice was similarly monitored, and blood was collected for full blood count and differential white blood cell analysis. DBA mice displayed severe neutropenia on day 8 after treatment with 100 mg/kg/day [117].

4.4. Plevitrexed

Development of a new drug was needed, one that retains water solubility, specificity for TS enzyme, is transported via RFC and cannot be acted upon by FPGS, due to its altered glutamate moiety, to be converted to its polyglutamated form [76]. Such a compound was chemically synthesised with a C7 methyl group and gamma-carboxyl tetrazole ring in the glutamate chain, allowing for a higher level of TS inhibition and absence of polyglutamylation. This compound came to be known as plevitrexed or ZD9331 and demonstrated better TS inhibitory activity than its parent compound, raltitrexed [83]. A broad spectrum of tumour types, such as ovarian, colon and gastric, were tested *in vitro* on human xenografts. Plevitrexed showed promising activity [83]. Preclinical pharmacokinetic studies were performed in canines. These found a substantial level of distribution due to 98% binding with serum albumin, and a short half-life [83]. It is excreted predominantly through the renal system and this process is decelerated due to tubular reabsorption, that is dose dependent [76]. It displayed clearance in 3 phases, and its terminal half-life was longer as compared to predictions from canine studies, where it was short (approximately 10 hours) [83]. This may be due to the strong tissue binding and the fact that it is not retained intracellularly due to lack of polyglutamation [83]. *In vivo* testing was done in rodents with tumour cell lines deficient in thymidine kinase. Rodents have increased levels of thymidine circulating in the body which allows them to bypass TS inhibition through the thymidine kinase salvage pathway [83]. It showed increased levels of radioactivity in proliferating tissues such as gastrointestinal tract [85]. These studies also showed significant nephrotoxicity, but this

effect was not seen in clinical trials. Another preclinical study in dogs showed diarrhoea as the most prominent toxicity [83].

Adult female DBA-2 mice were given a plevitrexed dose of either 50 mg/kg (low), 150 mg/kg (mid) or 200 mg/kg (high) through intraperitoneal or intravenous administration [118]. Animals were then sacrificed, blood collected, liver and kidneys removed and stained for histological examination. Renal function was also assessed by measuring the glomerular filtration rate (GFR) after 4 and 24 hours and compared to the controls. Low dose group showed slight effects on renal function 4 hours after administration. Mid dose group demonstrated a slight increase in GFR following intravenous administration, but not IP administration. The increase in GFR was significantly higher when high dose was given intravenously, but as seen above did not show any increase with IP route. At 24 hours, the trends of GFR seen at 4 hours with the highest dose were maintained but the increase in GFR was not as intense. Histological examination 4 hours after high intravenous dose, showed evidence of dilated tubules with fine granular casts. The same dose given IP, produced tubular dilation but no cast formation, and these effects were also seen in tissue samples from other doses and time points [118].

5. GARFT INHIBITORS

Glycinamide ribonucleotide formyltransferase (GARFT) is an important part of the *de novo* pathway of DNA synthesis. Although healthy tissues tend to favour the salvage pathway, tumour cells seem to have increased activity of the *de novo* pathway, and it could possibly be the primary source of DNA for these cells. This reliance provides us with specific targets so that we may more effectively treat malignancies [119, 120].

5.1. AG2034

AG2034, is a second generation GARFT inhibitor. It was designed with the help of the crystal structure of the *E. coli* enzyme and human enzyme domain of GARFT [119, 120]. Models tested *in vitro* and *in vivo* demonstrated a wide range of anticancer activity and an increased potency as compared to predecessor, lometrexol [120]. AG2034 is transported into

the cell with the help of the reduced folate carrier (RFC) and membrane folate binding protein (MFBP) [119, 120]. Preclinical studies focused on the inhibition of GARFT showed strong effect and increased substrate binding to FPGS. Similar results were seen with lometrexol, but in contrast AG2034 is seen to have higher affinity for MFBP [119, 120]. It was also observed that tumour cells with the mutant p53 were "preferentially targeted" and experienced a more severe cytotoxic effect. Levels of hypoxanthine, FPGS enzyme activity, RFC and MFBP expression can also influence the cytotoxicity of AG2034 [120]. Mice and dogs were used to test the preclinical toxicology of AG2034, with dogs observed to be more sensitive to its effects. Toxicities were primarily seen in the gastrointestinal tract and bone marrow. These adverse effects were seen to be more severe in animals with dietary folate deficiency [119, 120]. Normal tissues relying on the p53-mediated apoptosis pathway could be the reason behind the observed systemic toxicities [120].

Intravenous administration of AG2034 daily over a period of five days in mice, demonstrated the MTD to be 40 mg/kg/day on a normal diet. However, when administered with a folate deficient diet, the MTD reduced to 0.2 mg/kg/day [119]. The same effect of dietary folate absence was seen when C3H/He female mice were administered AG2034 daily for nine days [121]. The MTD was 7.5 mg/kg/day on a normal diet, however when mice were given a low folate diet the MTD decreased to 0.05 mg/kg/day [121]. Toxicity studies performed in mice and dogs demonstrated that the latter species is more sensitive to effects of AG2034, which primarily affect the gastrointestinal and haematopoietic systems [122]. The highest dose that showed no effect level (NOEL) in dogs when administered daily for a period of 5 days was 0.2 mg/kg/day [119], or 4 mg/m^2/day [122]. Whereas the NOEL for mice administered AG2034 on the same schedule was 60 mg/m^2/day [119].

DISCUSSION AND CONCLUSIONS

Purine antagonists

The drugs reviewed in this study displayed common toxicities on the bone marrow, liver, gastrointestinal tract (reducing body weight and food intake) as well as haematological toxicities. The severity of these effects was directly related to the dose administered. There is a species toxic effect of mercaptopurine seen only in rats upon administration with oral or intraperitoneal route. This effect is manifested in the form of pulmonary and cardiac lesions in addition to the other toxicities mentioned above. In addition to the above-mentioned toxicities, lesions are also seen on the thymus, bile ducts, lymph nodes, in thoracic and abdominal spaces. This multi-organ toxicity makes mercaptopurine the most toxic out of the purine antagonists. For reduce the overall toxicities of mercaptopurine, a study combined the drug with chitosan nanoparticles (MCP-CN) in Wistar rats [12]. The LD_{50} value of the MCP-CN (1000 mg/kg) was double that of MCP alone (500 mg/kg). MCP-CN displayed not only an absence of mortalities, but also decreased severity of hepatic and renal damage as compared to MCP given alone. The findings of this study show the advantageous effect of drug delivery systems, such as nanoparticles, at reducing the toxicity and improving the therapeutic index. Fludarabine toxicities studied are seen to be those of the group itself. It does not produce renal toxicity such as that seen in mercaptopurine. And unlike the next two drugs, it does not demonstrate neurological toxicity either. However, neurotoxicity is a common toxicity in human trials, which indicates that animal models might not be appropriate to evaluate this toxicity [16, 22]. Nelarabine toxicity is a special one in this group, as its effects were studied primarily on monkeys and rabbits as compared to the mice, rats and dogs used for the other members of this group. Nelarabine shares the toxicities of the group, except that of gastrointestinal toxicity as this is not seen in the animal studies. However, it does demonstrate significant neurological and reproductive (maternal and foetal) toxicities. Due to the close relation between monkeys and humans, it suggests that the administration of nelarabine would cause a similar neurological effect in humans, so trials and treatment of humans with nelarabine should be done with care. Nelarabine should also be avoided in treatment of pregnant animals/humans. The above mentioned reproductive and dose limiting neurological toxicities were seen in human trials using nelarabine for the treatment of paediatric and adult T-cell acute lymphocytic leukaemia/lymphoblastic leukaemia [26], demonstrating that monkey and rabbit species are good models for the

identification of these toxicities. Thioguanine demonstrates a mixture of the toxicities mentioned for this group. It displays pulmonary toxicity similar to that of mercaptopurine, however, it does not demonstrate the species-specific toxicity seen in rats with MCP. Unlike the rest of the group, toxic effects were not seen in the stomach or kidneys. Instead, it is similar to nelarabine in that it shows neurological toxicity such as ataxia and weakness. When HGPRT deficient mice were administered doses of thioguanine, there was minimal toxicity seen [31], demonstrating the important role of this enzyme in producing toxicity. A multiple dose study performed with prodrugs of TG, trans AVTG and cis AVTP showed decreased toxicity as compared to administration of equal doses of TG [34]. Trans AVTG showed a lower effect on peripheral blood counts and bone marrow, while cis AVTP showed no effect on the above parameters. This study did not examine the possible delayed toxicities that could be seen with these prodrugs, but the absence of severe toxicities is a good starting point and further examination of these prodrugs should be performed.

Pyrimidine antagonists

The common toxicities seen with this group of drugs is similar to that of purine antagonists due to their mechanisms of action being alike. The shared toxicities of the drugs in this review are that of bone marrow, gastrointestinal system, liver, kidney and haematological. In addition to the group toxicities, cytarabine treatment exhibited adverse effects in the brain (neurotoxicity), eyes (ocular toxicity) and in foetuses, which makes it the most toxic drug in the pyrimidine antagonist group. Neurotoxicological effects, which are a common adverse effect seen in humans [38], were only seen in rats suggesting that this species is more accurate at predicting this toxicity in humans. The specific toxicities of gemcitabine were related to the route of administration of drug, that is severe bladder toxicity seen with intravesical administration and severe lung toxicity seen in isolated lung perfusion study. Toxicity of azacytidine was demonstrated on adrenal gland and spleen in addition to the effects of the group. No renal or hepatic toxicity was seen in the studies performed with decitabine, however adverse effects were seen in thymus and a delayed effect seen in testis.

Antifolates

The common toxicities seen in this group are also bone marrow, haematological, hepatic, spleen, kidney, testes, epididymis gastrointestinal (causing reduction of body weight and food intake). Methotrexate is by far the most toxic antifolate drug examined in this review. In addition to multiple toxicities attributed to this group, it also affects lungs, heart, eyes

(chromodacryorrhea, blepharospasm) and skeletal growth in young animals. Administration of folinic acid following treatment with MTX demonstrated heights and lengths of femur, tibia, and growth plates as well as levels of plasma MTX and erythrocyte folate comparable to those of untreated controls, seemingly reversing the growth inhibition [72]. Piritrexim, although being a lipid analogue of MTX, does not display as severe toxicities as its parent drug. In the absence of liver, kidney, and testes toxicity, however, it demonstrates adverse effects in thymus and gallbladder. Pemetrexed is the second most toxic antifolate in this study, demonstrating additional neurological (hyperactivity, abnormal movement), dermal (redness, flaky skin), ocular (iritis, conjunctivitis), reproductive (maternal and foetal) and thymic toxicities. Pralatrexate similarly also displayed dermal and reproductive (only foetal) toxicities.

Thymidylate synthase inhibitors

There are fewer common toxicities seen amongst the members of this group and they are gastrointestinal and haematological. Fluorouracil is the most toxic TS inhibitor, displaying toxicities in multiple organs (lung, brain, liver, lung, kidney, spleen, ovary) as well as dermal and reproductive toxicity (only maternal). The renal toxicity of FU can be reduced with concurrent administration of vitamin C [104]. It also displayed a negative effect on intestinal anastomosis, causing high mortality due to leakage and subsequent peritonitis. Despite of these toxicities, fluorouracil does not cause ototoxicity when administered by intratympanic administration in rats [91]. Capecitabine toxicity was seen in many other organs (brain, eyes, liver, skin), however, the severity of adverse effects were not as high as those seen with its prodrug/predecessor. This is seen by the significant and progressive healing of intestinal anastomosis when treated with capecitabine. Raltitrexed did not show as widespread toxicities as seen above, however, it did hinder the development of neural tube resulting in foetal toxicity (resorption, growth retardation) in mice. Plevitrexed demonstrated dose-dependent nephrotoxicity without any significant effects in mice.

GARFT inhibitors

The main toxicities associated with administration of these agents is seen in gastrointestinal tract and bone marrow, and the effect is exacerbated when animals have low dietary folate intake. This is evidenced by the reduction of MTD in mice by 200 and 150 times for daily AG2034 dosing over a period of five and nine days, respectively. The toxicities seen in

humans are stomatitis, diarrhoea, mucositis (gastrointestinal), anaemia, neutropenia, and thrombocytopenia (hematopoietic) [119, 122].

From the above collection of results, the drugs with the most severe toxicities include nelarabine, decitabine, and methotrexate. We should seek to replace the use of these drugs with safer analogues or alternatives. The drugs showing moderate toxicities include mercaptopurine, fludarabine, azacytidine, pralatrexate, and raltitrexed. The side effects of these drugs are tolerable and can be managed with concomitant therapy with protective agents. Drugs such as thioguanine, cytarabine, piritrexim and AG2034 show good clinical activity without excessive toxicity. This final group of drugs shows us the future of chemotherapy, where the side effects are not as detrimental due to their specific tumour targeting mechanisms.

Summary

This literary review summarises the kinetic profile, transport systems, mechanisms of action and toxic effects of the most common antimetabolite drugs used in clinical cancer therapy today,

The purpose of this review was to collate published data regarding the toxic effects of these drugs *in vitro* or *in vivo*, in different animal species, and correlate them to the toxicities seen during chemotherapy regimens. Data was also collected on the alleviation of toxic effects by supplementation of different molecules or drugs.

The 5 categories of antimetabolite drugs are purine antagonists, pyrimidine antagonists, antifolates, thymidylate synthase inhibitors and lastly GARFT inhibitors. The mechanism of antitumour activity of these categories is due to their effect on a part of the cell cycle. Purine and pyrimidine antagonists compete with their respective analogues in the body for uptake into the cell for synthesis of DNA and/or RNA. Antifolate drugs compete with the body's supply of folates for attachment to the folate receptors found in cancer cells. Thymidylate synthase and GARFT inhibitors inhibit these key enzymes, which are an essential part of the *de novo* pathway of DNA synthesis and repair.

Performing a literature review allows us to consolidate the information on the existing drugs being studied and in use. This allows us to explore future areas in targeting alternative pathways in the tumour cell cycle. This is especially relevant considering increasing resistance, prevalent adverse effects and dosing concerns observed in the aforementioned drugs.

By highlighting the mechanism of toxicity of these drugs, we can better understand how to create better and safer alternatives.

A strength of this review would be the large number of published works that were reviewed, analysed, and consolidated for this study. Some limitations faced during collection of data, was that certain articles reviewed were dated in the late 90s to early 2000s with very preclinical studies being performed in recent times despite these being the common drugs associated with antitumour activity.

To sum up, this review of the literature has offered a thorough examination of the present status of research on the preclinical toxicology of antimetabolites. This review advances knowledge about the toxicities in the various drugs assessed by clarifying important themes, patterns, and knowledge gaps in the body of existing research and by highlighting areas that warrant more research. To improve our understanding of the toxic effects of antimetabolite

drugs and provide guidance for future research, and practice in this area, it will be crucial to fill in the gaps found in this study and explore new lines of investigation.

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