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Andrews University

College of Arts and Sciences

TEMOZOLOMIDE-NSAID MIXTURES AND NOVEL DICLOFENAC-PURINE HYBRIDS AS POTENTIAL ANTIGLIOBLASTOMA AGENTS

A Thesis

Presented in Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

Joshua Kiprono Rotich

2022

ABSTRACT

TEMOZOLOMIDE-NSAID MIXTURES AND NOVEL DICLOFENAC-PURINE HYBRIDS AS POTENTIAL ANTIGLIOBLASTOMA AGENTS

By

JOSHUA ROTICH

Co-chairs: Denise L. Smith and Desmond H. Murray

ABSTRACT TO GRADUATE STUDENT RESEARCH

Thesis

Andrews University

College of Arts and Sciences

Title: TEMOZOLOMIDE-NSAID MIXTURES AND NOVEL DICLOFENAC-PURINE HYBRIDS AS POTENTIAL ANTIGLIOBLASTOMA AGENTS

Name of researcher: Joshua Rotich

Name and degree of faculty co-chairs: Denise L. Smith, Ph.D. and Desmond H. Murray Ph.D.

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Glioblastoma (GBM) is the most common, lethal and aggressive brain tumor in adults. Standard treatment involves surgery, radiation therapy and temozolomide (TMZ) chemotherapy. However, GBM recurs and the average survival rate is between 12 to 18 months with 25% 1-year survival rate and 9% 5 years survival rate. Treatment options and advancement is limited by the blood brain barrier (BBB) which restricts drug entry into the brain and the immense heterogeneity of the tumor which limit adequate control of the entire tumor using one drug. In this research, we explored whether a combination mixture of TMZ and non-steroidal anti-inflammatory drugs (NSAIDs) which have shown anticancer properties (diclofenac, aspirin, ibuprofen, ketoprofen, naproxen, oxaproxin) may have synergistic or additive effects on U87MG cell line. All the combination mixtures in the ratio 1:1 had a lower LC_{50} value compared to

individual compounds indicating that combination mixtures could have a synergistic or additive effect against GBM. We also examined whether novel hybrid of diclofenac (which had the lowest LC_{50} value, cell motility changes and morphological changes) and purines (which have shown to be able to enhance TMZ antitumor efficacy) could have a higher efficacy compared to individual compounds. All the novel hybrids of diclofenac and purines had lower LC_{50} compared to individual compounds. Therefore, hybrids and mixtures could have a higher efficacy and a better promise to GBM patients.

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Marlene Murray, Ph.D. Date approved

For Faith, Emmanuel, Ruth, Abi, Dad &Mum

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CHAPTER 1

INTRODUCTION

Research goal: To investigate the efficacy of mixtures and hybrids of temozolomide (TMZ) and non-steroidal anti-inflammatory drugs (NSAIDs), and amino purines (AP) against glioblastoma multiforme (GBM).

Cancer

Cancer refers to a group of more than 100 diseases that develop over time, are characterized by uncontrolled cell division, growth and metastatic properties. Though cancer can develop in any part of the body's tissues, and each type of cancer disease is unique, the basic processes that lead to development of cancer are similar[1].

Cancer develops from a cell that stops responding to normal cell division restraints then begins to proliferate continuously [1]. Daughter cells from this parent cancerous cell also show abnormal and inappropriate proliferation. Continued cell division leads to formation of a mass of cells called a tumor. Though tumors begin with a single cell, at the time of clinical diagnosis, majority of the tumors show startling heterogeneity in morphological and physiological features such as angiogenic and proliferative potential[2]. Inter tumor heterogeneity is characterized by distinct genetic alterations which occur in individual tumors that originate from the same organ while intratumor heterogeneity is characterized by genetic alterations within individual tumors[3]. Heterogeneity is a hallmark is some cancers like glioblastoma multiforme (GBM) which have pronounced heterogeneity on histological, genetic and gene expression levels[4]. This is why single drug therapies do not show significant benefit on GBM patients[5].

Some tumors remain within the tissue it originated. These are called benign tumors. Tumors which develop metastatic properties, attributed by down-regulation of cell adhesion receptors and up-regulation of receptors enhancing cell motility, are said to be malignant. The abnormal tumor growth becomes life threatening when they disturb the normal tissue and organ functions[1, 6, 7].

There are a number of differences between cancer cells and normal cells. Below is a table with a list of notable differences.

Table 1:Comparison between normal cells and cancer cells

Normal cells	Cancer cells
Controlled by external growth signals	Do not depend on external growth signals.
Most do not move around the body	Can move around the body
They stop growing when they encounter other cells	Invade and spread to other areas of the body
Can be eliminated by the immune system when damaged or abnormal	Hide from the immune system
Do not have multiple changes in chromosomes	Accumulates multiple changes in chromosomes
Its growth is controlled	Growth rate is uncontrolled
Cell shape is uniform	Cell shape irregular
Has single nucleus	Has multiple nucleus
Has spheroid nucleus	Nucleus take multiple shapes

Has fine distributed chromatin	Have coarse and aggregated chromatin
Always mature into very specialized cells	Remain immature
They favor aerobic respiration	Favor anaerobic respiration

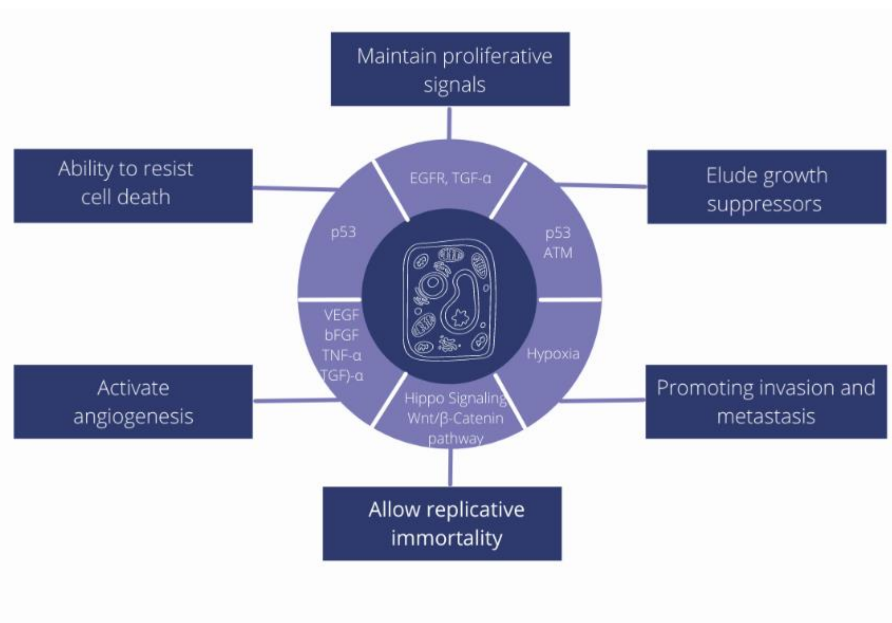


Figure 1:Cancer hallmarks. Cancer characteristics common in all cancer cells

In 2000, two cancer researchers named Douglas Hanahan, founding member of Whitehead Institute and Robert Weinberg, Director of the Swiss Institute for Experimental Research, proposed six cancer hallmarks. Together, these six cancer hallmarks (figure 1) constituted an organizing principle that provided a logical framework for understanding the highly diverse types of cancer diseases. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis[8]. Benign and malignant tumors share all the hallmarks except invasion and metastasis[9]. Behind all these hallmarks are genomic instabilities in the cancer cells which are responsible for the genetic diversities[10]. In 2011, a decade later, Douglas Hanahan and Robert A. Weinberg introduced two enabling characteristics into the

cancer hallmark canon. These characteristics include genome instability and mutation[11-13], and tumor promoting inflammation[14-17].

In this research, we investigate the efficacy of drug mixtures and hybrids to address cell proliferation and inflammation associated with GBM. The specific questions we address are: a) could physical mixtures of temozolomide (a drug targeting cell proliferation) and non-steroidal anti-inflammatory drugs (NSAIDs) which target inflammation have a higher efficacy? b) TMZ is a current first line chemotherapeutic drug given to (GBM) patients. Could addition of NSAIDs drugs which act against an enabling factor (tumor promoting inflammation), be a better and promising strategy in GBM treatment?

Glioblastoma multiforme (GBM)

Glioblastoma multiforme (GBM) is the most common, most devastating, the most fatal primary brain tumor[18]. It is highly aggressive, progressive and invasive, often invading the brain parenchyma then progressing through corpus callosum into other parts of cerebral hemispheres [19]. Its highly invasive nature currently represents the most challenging hurdle to surgical resection[20].

GBM is an astrocytoma. Astrocytoma is a brain tumor that develop from glial cells called astrocytes. They are classified into four grades. Grade I astrocytoma is a localized tumor that can be removed surgically, and they can be cured. They have a high prognosis and are considered low-grade neoplasms. An example is pilocytic astrocytoma[21]. Grade II astrocytoma are malignant and invasive but not to the extent of higher grades. An example is fibrillary astrocytoma[22]. Grade III astrocytoma is a highly invasive and malignant. Moreover, they have a poor prognosis. An example of grade III astrocytoma is anaplastic astrocytoma. Glioblastoma is a grade IV astrocytoma. Very resistant to therapies, the most aggressive and malignant[23, 24].

There are two different classifications of GBM. Primary, which arise without a known precursor and secondary where low grade tumor transforms into GBM. Primary GBM are more common, occur in older aged people and have poorer prognosis compared to secondary GBM[25].

In 2020, American Society for Clinical Oncology (ASCO) estimated that 23,890 adults and 3,540 children would be diagnosed with primary brain tumors in America. Glioblastoma multiforme (GBM) represents 15-20% of primary brain tumors, 48 % of primary malignant tumors of the central nervous system and 57 % of all gliomas. Moreover, the highest number of people diagnosed with GBM are aged between 75-80 years and the median age at diagnosis is 64[26, 27].

GBM is initially difficult to detect since the symptoms vary among patients and depend on the tumor's size and location in the brain [18]. Some of the more common general symptoms include headaches, changes in mood and personality, and seizures. Patients who show an increase in intercranial pressure as a result of the rapid growth of the tumor typically will experience nausea and vomiting as well as headaches and seizures [28]. As the tumor grows, there is also indication of neurological damage by aphasia, sensory loss, and hemianopsia [29]. When the neurological symptoms become more apparent, such as the seizures and personality changes, the patient will most likely have to undergo a head CT or MRI scan [28]. At this point, the GBM will be at stage four.

There are many challenges when it comes to treatment of GBM e.g. rapid tumor progression, intra and inter-tumor heterogeneity, tumor location that is hard to reach, the blood brain barrier that limits drug efficacy and tumor relapse. This is why mortality rates remain high. Over the past decade, there have been improvements in surgical approaches, chemotherapy and radiotherapy. These improvements have led to slight improvements in survival rates as well as

quality of life for GBM patients. However, prognosis is still a major issue [30] Currently, management of GBM patients is more inclusive, in that, therapeutic management is offered alongside effective supportive care. Therapeutic management involves administration of anti-tumor drugs while supportive care involves the management of signs and symptoms. These signs and symptoms include seizures, cognitive impairment, edema, osteoporosis, venous thromboembolism, mood disorders as well as gastrointestinal tract disturbances among others[31]. Supportive care is aimed at improving the quality of life. Some of the drugs administered as supportive care medicine include corticosteroids and Dexamethasone for symptomatic relief and Levitiracetam for seizures[32]. Therapeutic management involves surgery followed by radiation therapy and chemotherapy.

Surgery remains the principal part of standard care for GBM patients[33]. Surgery can be used to reduce the tumor burden, control seizures, reverse neurological deficits and introduce local therapeutic agents. There are limitations on the extent to which resections can be done. These limitations are dependent on the site of the tumor and eloquence of the brain area. Sites like basal ganglia, brain stem and cortex have very poor prognosis and may not be amenable to surgical resections. Unfortunately, patients inevitably face recurrence often 2-3 cm from the margin of the previous lesion[34, 35]. This is because GBM is highly invasive making it almost impossible to eliminate through surgical resection. GBM cells interact with many different cells in the brain including endothelial cells, astrocytes, neurons and extracellular matrix (ECM). Interestingly, it has been shown that it can shrink its cell body to infiltrate into narrow spaces of the brain, thereby invading many different regions of the brain and escaping from surgery[36]. The main process involved not only in invasion but also in metastasis is epithelial to mesenchymal transition (EMT), whereby cells lose cell to cell adhesion, polarity and the

expression of cell surface and cytoskeletal proteins, thus enabling cells to acquire migratory properties[37].

GBM response to radiation varies from patient to patient. In many cases, it induces a phase of remission seen in the reduction of the tumor size. However, this phase of remission is often short since the tumor recurs [38]. Research has shown that combination of surgery, radiation therapy and chemotherapy prolong the survival rates of GBM, that is, when compared with surgery alone. Radiotherapy and surgery increase the survival rates from 4 months to 12 month[39]. Radiation dose of up to 4500 cGy in GBM patients results in median survival rate of 13 weeks while a radiation dose of 6000 cGy administered 5 days every week (1.8 -2 cGy per day) results in a median survival of 42 week[40].

In this research, we are investigating chemotherapeutic mode of treatment using TMZ which is a first line drug given to GBM patients in combination with NSAIDs.

Temozolomide

Temozolomide (TMZ) was first synthesized in the late 1970s but was FDA approved for medical use in 1999 to treat refractory anaplastic astrocytoma[41, 42] and glioblastoma multiforme (GBM) in 2005 [43]. TMZ is an imidazotetrazine lipophilic prodrug which can cross through the blood brain barrier hence it can be administered orally. It is stable in acidic pH but at pH higher than 7, TMZ is spontaneously

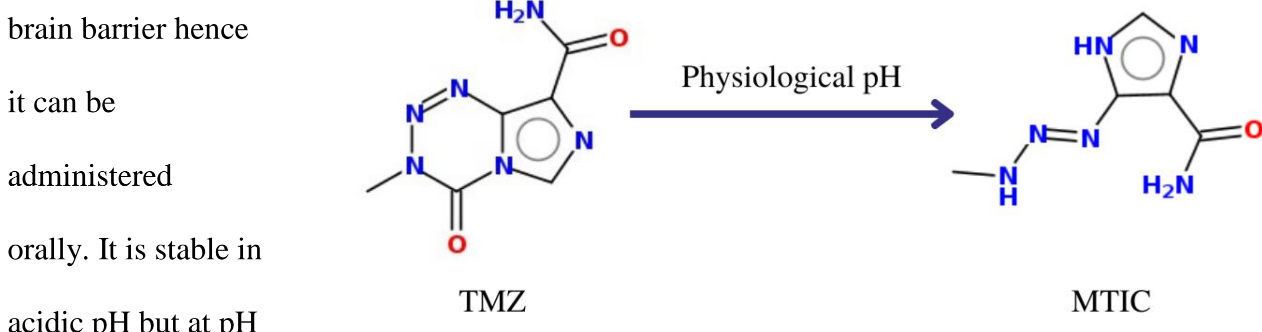


Figure 2: Conversion of temozolomide (TMZ) to 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide (MTIC)

hydrolyzed into active form 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide (MTIC). Thereafter, MTIC is hydrolyzed to methyldiazonium ions (figure 2). Methyl group from methyldiazonium ions are transferred to either guanine at N^7 (60-80%) or O^6 (5%) as well as adenine at N3 (10-20%) site on negatively charged DNA which acts as a nucleophile[44, 45].

This process is called alkylation and it leads to substitution of thymine for cytosine during DNA replication which results in multiple DNA adducts that have a high opportunity for mismatched base pairing. Mismatched base pairs triggers mismatch base repair pathways which aim to correct the damage but results in G2/M cell cycle arrest and eventually apoptosis[46, 47].

Research on O^6-methylguanine-DNA methyltransferase (MGMT) deficient cells showed that alkylation product O^6-methylguanine (O^6MeG) is the most powerful trigger for apoptosis compared to N^7-methylguanine and N^3-methyladenine products[48]. Moreover, O^6-methylguanine lesions mediates antitumor activity of TMZ which requires functional DNA mismatch repair (MMR) of tumor cell[49]. O^6-methylguanine is mispairing with a thymine. MMR machinery recognizes this. The mis-paired thymine is excised and replaces with another thymine upon repair synthesis. This leads to an energy consuming cycle of DNA repair[50]. The methylated guanine on the opposite strand cannot be repaired by MMR, hence, it persists leading to replication fork arrest and eventual apoptosis[51, 52].

Research done in Maastricht University Medical Center between January 2005 and January 2008 on effectiveness of temozolomide showed that temozolomide was an effective chemotherapeutic drug for glioblastoma. The survival rate for groups with radiotherapy alone was 8 months while the medial survival rate for patients who received TMZ and radiotherapy was 12 months. The two-year survival rate was 4% for patients who used radiotherapy alone and 18% for patients who used TMZ and radiotherapy[53].

TMZ efficacy is mainly limited by O6-methylguanine-DNA methyltransferase (MGMT). DNA alkylation at O^6 position of guanine leads to formation of crosslinks between adjacent strands of DNA. Cross linking of adjacent double stranded DNA by alkylating agents is inhibited by O^6-methylguanine-DNA methyltransferase (MGMT) which rapidly reverse alkylation at O^6 position of guanine. MGMT therefore plays a role in resistance to alkylating drugs[54, 55] [56]. TMZ use may also lead to side effects such as severe nausea and vomiting, constipation, diarrhea and loss of appetite[57].

Use of multi-target drugs such as combination drugs and hybrid drugs have shown to be more advantageous. Hybrid drugs for instance target many points of the signaling network, reduce the possibility of drug interaction occurrence, diminish the number of side effects and adverse reactions as well as toxicity levels. In this research, we investigated if a hybrid containing TMZ and nonsteroidal anti-inflammatory drugs (NSAIDs) target different signaling networks and have a higher efficacy compared to TMZ and NSAIDs compounds?

NSAIDs

Tumor promoting inflammation is a cancer enabling factor. Often, tumors grow on sites of chronic inflammation[58-60]. Virchow first linked cancer and inflammation in 19th century[60]. However, the link was out of favor for a long time. There has been a growing interest, a line of evidences[60-62] that substantiate the link and its now generally accepted that cancer is linked to inflammation. This is why it has been added as an enabling factor in the cancer hallmarks[10]. Noteworthy, inflammation mediators like chemokines, cytokines, tumor growth factor, vascular endothelial growth factor (VEGF) and cyclooxygenase (COX) are also upregulated in several cancer cases[63].

Nonsteroidal anti-inflammatory drugs (NSAIDs) refers to drugs approved by Food and Drug Administration (FDA) federal agency for use as anti-inflammatory, antipyretic and analgesic agents[64]. Therefore, NSAIDs are used to reduce inflammation, treat muscle pain, trauma cases, migraines etc [65, 66]. Research also shows that some NSAIDs are potential anticancer agents.

The following is a list of NSAIDs that have shown anticancer properties; aspirin[67-69], diclofenac[70-73], ibuprofen[74-76], ketoprofen, naproxen and oxaprozin.

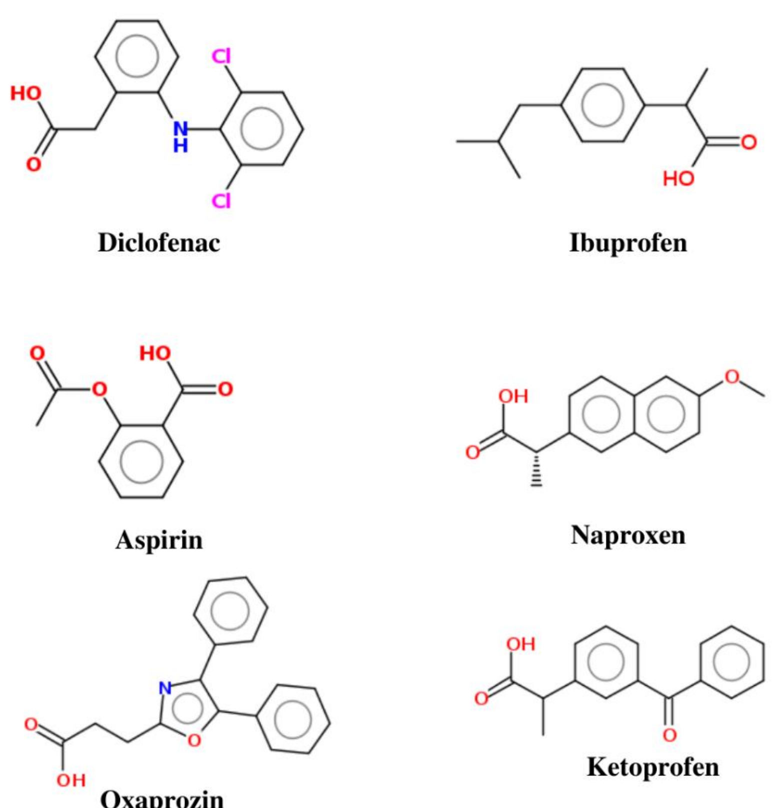


Figure 3: Structires of NSAIDs used.

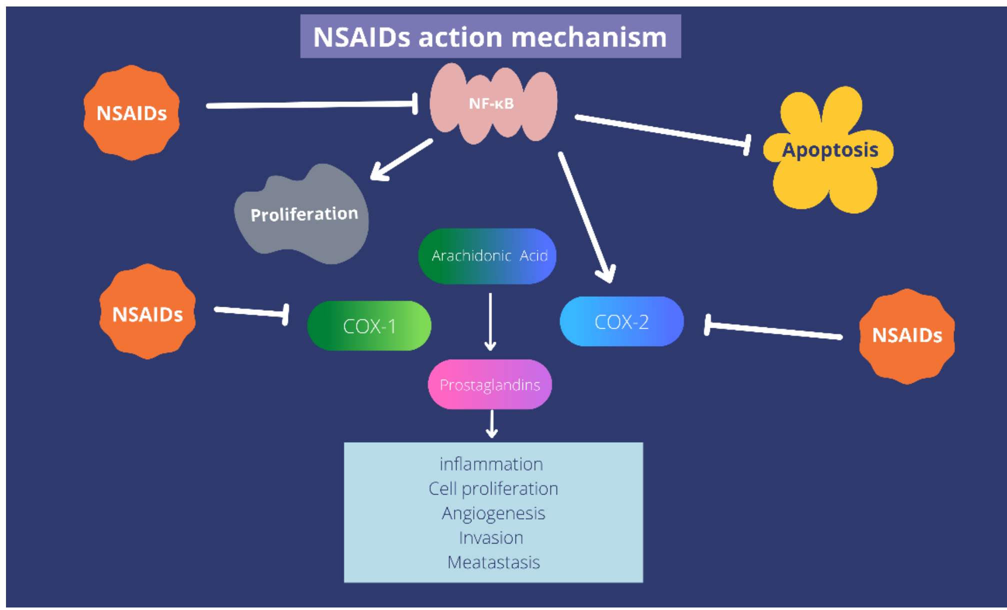


Figure 4: Mechanism of action by non-steroidal anti-inflammatory drugs (NSAIDs). They inhibit COX-1, COX-2 and NF-κB which are involved in activation of arachidonic acid. Arachidonic acid is converted to prostaglandins and prostaglandins are responsible for inflammation, cell proliferation, angiogenesis, invasion and metastasis. NF-Kb inhibits apoptosis but enhances cell proliferation.

NSAIDs exert their anti-inflammatory effects by inhibiting an enzyme called cyclooxygenase (COX). There are two COX isoforms, COX-1 and COX-2. COX-1 is common in mammalian tissues but COX-2 is expressed quickly in response to pro-inflammatory stimuli[77]. COX-2 is also often upregulated in cancers[77-79]. COX is an enzyme responsible for converting arachidonic acid into prostaglandins (PGs). Prostaglandins are normally produced in response to injury or infection and they cause inflammation which is associated with fever, pain and swelling symptoms. PGs influence cancer angiogenesis, metastasis, apoptosis, and invasion [80, 81]. Moreover, they are often highly expressed in cancer cells[82].

Experimental evidence has been mounting recently about influence of nuclear factor-kappa B (NF-κB) on initiation, promotion and progression of cancer[83]. NF-κB is a transcription factor involved in inflammatory, proliferation cell survival and immune responses. It has 5 proteins RelA(p65), c-Rel, Rel B, p50, and p52 which dimerize and are held by NF-κB

inhibitors (IκBs) in the cytoplasm[83]. Cytokine-responsive IκB kinase -α (IKK-α) and IκB kinase -β (IKK-β), are protein kinases whose function is needed for NF-κB activation by pro-inflammatory stimuli[84]. Research shows that NF-κB is activated in GBM/ astrocytes and suppression of its activity can lead to up 90% suppression of tumor proliferation[85]. NSAIDs have shown to inhibit/ suppress expression of NF-κB[86].

Interestingly, NF-κB is linked to COX in that the promoter region of the COX-2 gene contains binding sites for NF-κB, which acts as a transcription factor to regulate the induction of COX-2[87]. Since cancer has been linked to inflammation and inflammation is associated with COX and NF-κB, NSAIDs use could be advantageous to cancer patients. This is why we ventured into an examination of TMZ-NSAIDs imide hybrids as potential antiglioblastoma agents. TMZ-NSAID compounds are imides. Research has shown that imides can act as immunomodulatory anticancer drugs. Thalidomide analogues, for example, has been used to treat several neoplasms[88]. There is a possibility that our imide hybrids may have immunomodulatory properties in addition to antiglioblastoma properties of TMZ.

Anticancer Imides

Imides are organic compounds containing two acyl groups (figure 6) that are bound to a nitrogen. According to the drug bank, there are more than 22 drugs that are imides. Among those listed to have anticancer include lenalidomide used to treat multiple myeloma[89, 90], thalidomide (figure 5) used to treat newly diagnosed multiple myeloma[91-93], amonafide (figure 5) which is currently under investigation for treatment of breast, ovarian and prostate cancer[94, 95], pomalidomide used to treat patients with multiple myeloma[96], LY-2090314 used to treat pancreatic cancer and finally[97], glucoraphanin (figure 5) which is under investigation as an anti-cancer[98,99].

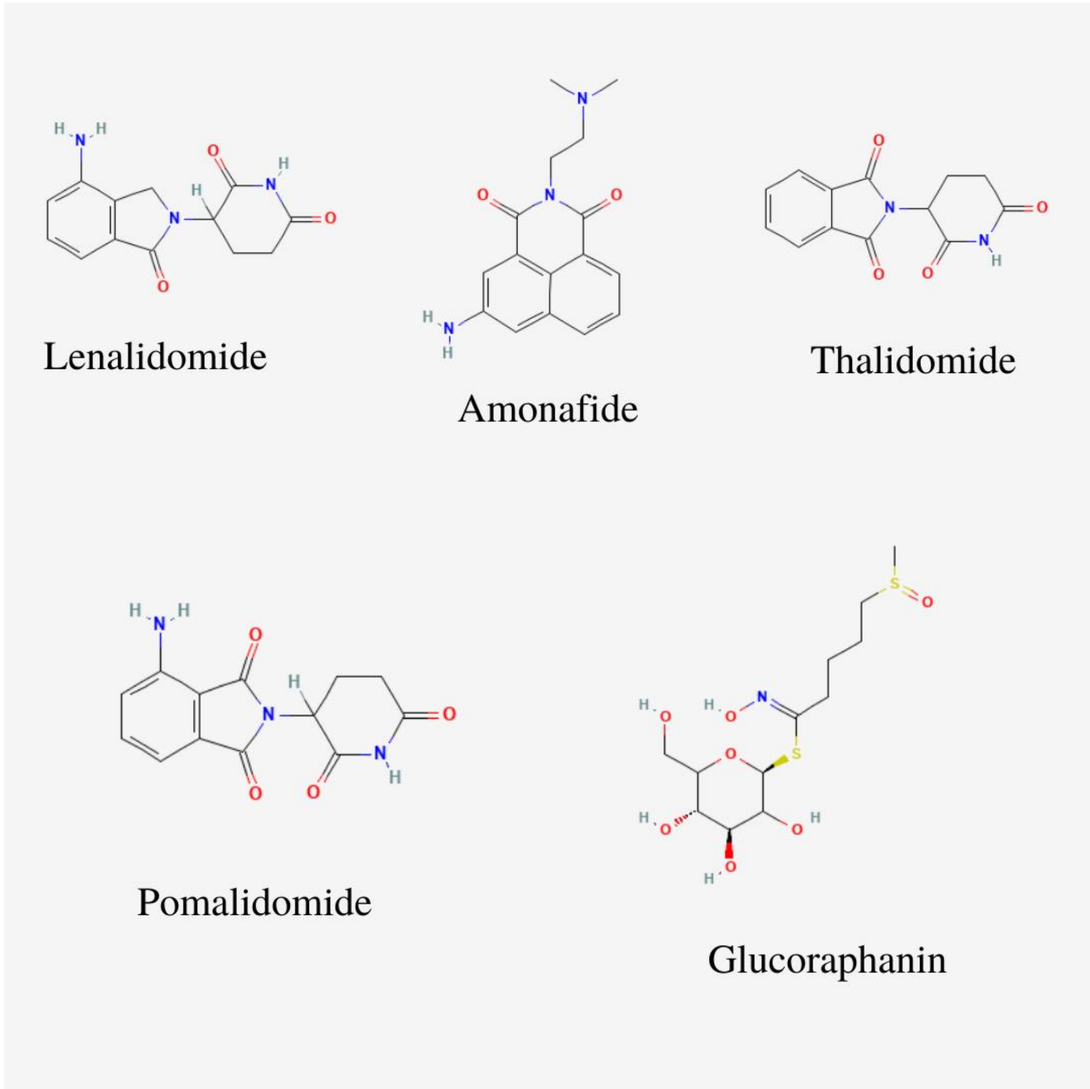


Figure 5: Structures of imides that have shown anticancer properties.

Imides seem to have different mechanism through which they inhibit progression of tumors. Pthalimides have been studied and researched for their varied pharmacological effects. They have shown anti-inflammatory, antimycobacterial, analgesic as well as anticonvulsant properties. Actually, some of its derivatives like thalidomide have been used against multiple myeloma, oral squamous cell carcinoma and prostate cancer[88]. Pthalimide-based curcumin derivatives have shown anti-proliferative activity and anti-migrating activities. It inhibited

anchorage-dependent activities as well as independent growth of prostate cancer cells. It was found that it worked by altering expression of genes that are involved in cell proliferation [100]. Since TMZ-NSAID hybrid are imides, there are chances that it could have anticancer properties associated with imides for example anti-proliferation or anti-inflammatory and anti-angiogenesis. To synthesize TMZ-NSAIDs hybrids, we considered the following synthetic methods:

Acylation of amides with activated forms of carboxylic acids

This is the most frequently used method to prepare imides. Acylation refers to the process of adding an acyl group to a compound. Acylating agent is the compound that provides the acyl group. An acyl group has a double bonded oxygen atom attached to an alkyl group. Usually, acyl group is derived from carboxylic acids. An acyl group (figure 6) can be used to form carboxylic acid, acid halides, acid anhydride, esters, amides, thioesters and acyl phosphates. Activated forms of carboxylic acids like acid chlorides, methylarenes, potassium acyltrifluoroborates, esters and anhydrides can be used in acylation of amides to form imides[101].

Figure 6: Acyl bonded to halogen

Figure 7:Acylation of amides with activated forms of carboxylic acids

Mumm rearrangement

Mumm rearrangement was first discovered in 1910 and stands for intramolecular acyl transfer [62]. It is an electrochemical four component reaction cascade and the last step is called Mumm rearrangement. The rearrangement is an O - N-acyl shift. It involves interactions between an amine, an isonitrile, a carbonyl compound and a carboxylic acid[102]. Mumm rearrangement process is very exothermic and can drive the entire process thermodynamically. Bronsted acids mediates the acyl transfer[102].

Figure 8: Mumm rearrangement

Carbonylative cross-coupling of aryl halides

Carbonylation is a process that involves incorporation of CO into an organic molecule like an alkene, alkenes, alcohol or existing C-X (X=I, H, Cl, Br) bond to form a carbonyl group. This process is very important because it allows construction of new bonds as well as an introduction of a carbonyl group.

X=Br,I,H

Figure 9:Carbonylative cross-coupling of aryl halides

Carbonylative transformation takes place in presence of a metal catalyst. Some of the transition metal catalysts which have been studied include palladium, ruthenium, rhodium and

manganese. Carbonylative synthesis of alkyl iodides and amides using manganese as a catalyst have been used in synthesis of imides [102].

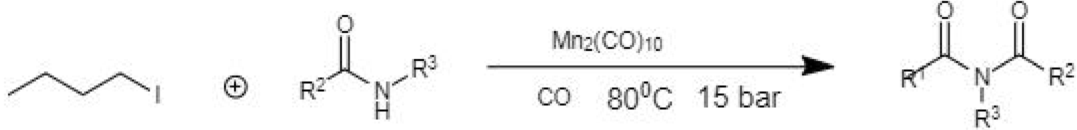


Figure 10:Manganese-catalyzed carbonylative synthesis of imides from alkyl iodides

Oxidative decarboxylation of amino acids

Decarboxylation refers to a chemical reaction which removes a carboxyl group and releases CO_2. Oxidative decarboxylation is therefore a chemical reaction that leads to production of carbon (iv) oxide and removal of a carbon group as a result of oxidation reaction.

N-Acyl amino acids can be converted to imides through oxidative decarboxylation. This conversion takes place at room temperature, and it is induced by $Ag^{+}/Cu^{2+}/S_2O_8{}^{2-}$. The imide yield through this process is pretty good, can be as high as 89%[102].

Figure 11:Oxidative decarboxylation of amino acids

Oxygenation of amides

Amides are primarily inert towards electrophilic oxidants. Manganese oxide has been used in catalytic oxidation of amides to imides. Manganese oxide is thermally stable and it is readily available in a number of oxidation states. Moreover, it is highly abundant. It has been used in catalytic oxidation of quite a good number of reactions such as oxidation of alcohols to aldehydes, alcohols to amides, hydrocarbons to alcohol, amines to imines and styrene to styrene

oxide. It can also be used in oxidation of amides into imides [102]. Copper (1) bromide and Selectfluor combination have a very strong oxidation ability. It has been used in oxidizing amides into imides. The oxidation process takes place at room temperature in acetonitrile and is complete in less than an hour [102].

Figure 12:Copper mediated oxidation of amides to imides by selectflour *(https://www.sciencedirect.com/science/article/abs/pii/S0040403911002826)*

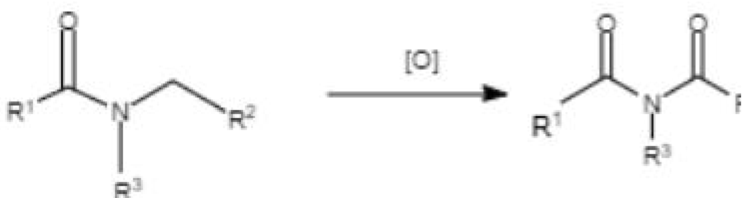


Figure 13:Oxidation of amides to imides

In this research, we attempted synthesis of TMZ-NSAIDs imide hybrids using "acylation of amides with carboxylic acid derivatives" method to synthesize TMZ-NSAID compounds. This is because NSAIDs have a carboxylic acid functional group and temozolomide has an amide group. However, we did not successfully synthesize the hybrid even after many trials. This prompted us to synthesize hybrids of diclofenac and purines using acylation method. Our choice of diclofenac was influenced by the results observed while testing LC_{50} values of selected NSAIDs. Diclofenac changed U87 MG cell motility and had the lowest LC_{50} value. We chose purines because research shows they can enhance TMZ antitumor efficacy. Fortunately, we were successful in synthesizing novel hybrids of diclofenac and purines.

Purines

Purine is a six-membered and a five-membered nitrogen-containing ring (figure 14). It is used by cells to make DNA and RNA building blocks[103]. Examples of purines are Guanine and Adenine. Rapidly proliferating cells demand purines, which are basic biochemical compounds, in large quantities for nucleotide synthesis as well as DNA replication. Moreover, purines are known to be sources of energy that drive cellular biological processes, are involved in host-tumor interaction and modulate immune cell responses. This is why they are upregulated in tumor microenvironment[104, 105].

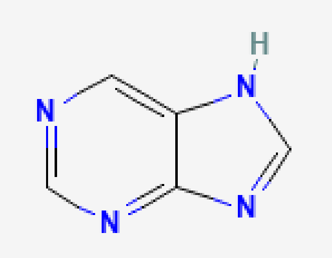


Figure 14: Purine structure

One of the characteristics of tumors is, they proliferate rapidly[106]. Targeting this characteristic has been exploited in the past in development of anticancer drugs. These drugs either alter or inhibit DNA synthesis. Generally, a mature human being has few cells replicating. However, some cells are continually replicating e.g cells in the bone marrow, hair follicles and gastrointestinal lining. This means that targeting DNA replication may result in high levels of toxicity thus limiting the amount of drug that can be tolerated by a patient. Despite the toxicity levels, effective drugs targeting DNA replication have been developed in the past and they actually increased survival rates and even cured patients [107].

Interestingly, purine analogues have been used since 1953 against cancer. Purine analogues refers to compounds which have a structure similar to that of purines but they have a different mechanism of action, metabolism, pharmacokinetics and adverse conditions. They are considered as antimetabolites, that is, they compete or interfere with nucleoside triphosphates in the synthesis of DNA and RNA. These compounds have been used against leukemia and lymphomas[108, 109]. 6-mercaptopurine was approved by FDA in 1953 for treatment of leukemia in children. It is curative and currently considered the standard treatment for the cancer. Thioguanine was then approved in 1966 and has been used in treatment of chronic myeloid leukemia, acute myeloid leukemia and acute lymphocytic leukemia[110]. Other purine analogues that have been approved since then include fludarabine, cladribine, pentostatin, azathioprine, clofarabine, nelarabine, thioguanine and pentostatin [107, 111]. In this research, we have investigated aminopurines (figure 16) including guanine, adenine, 2-chloro-7H-purine-

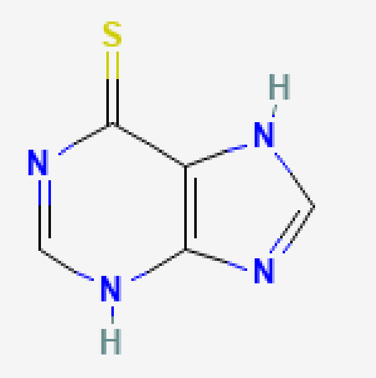


Figure 15: Structure of 6-mercaptopurine

6-amine, 2-fluoro-7H-purine-6-amine, 2-amino-6-chloropurine amines and p-anisidine.

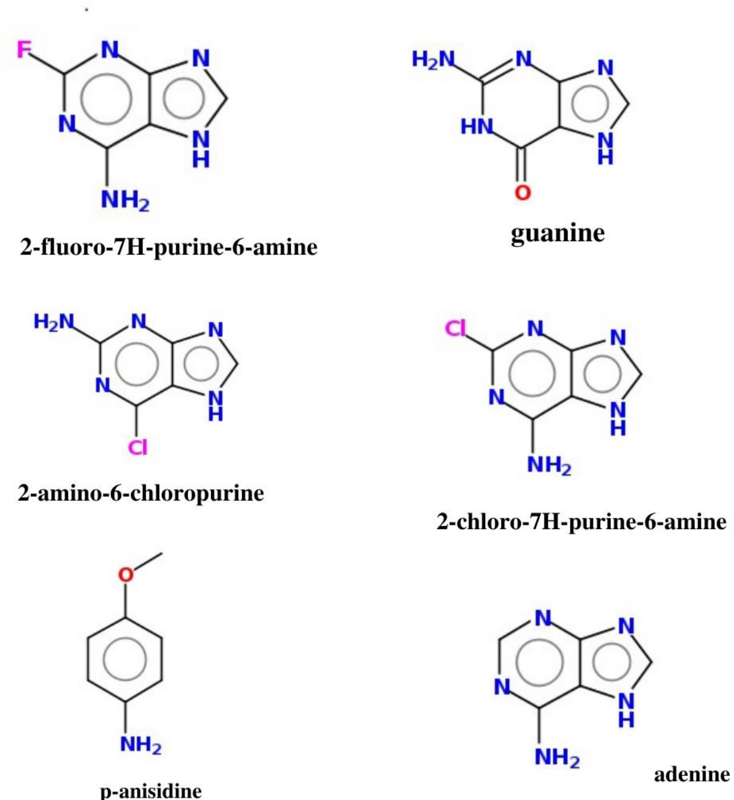


Figure 16: Structures of purines used

Mechanism of action

Nucleoside analogues diffuse into cells with the aid of membrane transporter. Once in the cell, they are converted into analogues of cellular nucleotides by enzymes[112]. Polymerases then incorporate the analogues into the DNA during normal DNA synthesis. They can also be incorporated into DNA during DNA excision repair synthesis. Thereafter, these analogs compromise the integrity of DNA structure or inhibit one or more enzymes that are critical for DNA synthesis. This leads to stalling of replication forks as well as chain termination. Molecular mechanisms can sense when there is a stalling in the replication process and activate cell cycle checkpoints as well as DNA repair processes. Other DNA damage sensors include ataxia

telangiectasia and Rad3-related protein (ATR) ataxia-telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK). DNA repair processes are to a large extent responsible for drug resistance. Apoptosis is initiated when DNA repair processes fail to repair the DNA or when the stalled forks are not stabilized[107, 113, 114].

Purine association with GBM

Research shows that purine nucleotides increases the toxicity of TMZ when combined[115]. Additionally, purine synthesis has been linked to the highly aggressive nature of GBM[116]. Also, it has been observed that there is an elevated expression in purine synthetic enzymes whenever there is a poor prognosis in GBM patients[117]. Moreover, high rates of purine synthesis in tumorigenic cells increase their ability to repair DNA damaged cells hence leading to tumor recurrence. Even DNA damage caused during GBM radiation therapy is repaired when purine synthesis is upregulated. This leads to tumor recurrence and therapy resistance[117-119].

Noteworthy, de novo purine synthesis can generate adenosine triphosphate (ATP) and guanosine triphosphate (GTP). ATP is a nucleoside triphosphate that is made up of three components; adenine, sugar ribose and a triphosphate. On the other hand, GTP is made up of; guanine, sugar ribose and triphosphate. ATP and GTP act as sources of energy or activators of substrates in metabolic reactions. They are also molecular precursors of DNA & RNA. In GBM, de novo GTP synthesis is upregulated and de novo ATP synthesis is active in both normal brain tissues and GBM but not upregulated. Upregulation of GTP synthesis enhances GBM proliferation. Inhibiting de novo GTP synthesis overcomes resistance to GBM radiation therapy[118] [120].

Blood Brain Barrier

Glioblastoma is a brain tumor. All orally administered anti-glioblastoma drugs have to pass through blood brain barrier (BBB). BBB is a membrane barrier found between the blood vessels of the brain and the rest of the cellular brain tissue. It is selectively permeable in that it is responsible for regulating the passage of molecules into the brain neuronal environment[121]. It was discovered by Paul Ehrlich a German physician in the later part of the 19th century[122]. The BBB function is enabled by its various components. The key structure to the function of the BBB is the endothelial cells that line the inner part of the brain blood vessels. The endothelial cells are closely adhering to each other thus forming the functional tight junctions which thus allows specific molecules to pass through to the brain tissue [123]. Some of these molecules are mainly fat soluble or smaller molecules. Other large molecules can also pass through with the aid of protein transporters. These include glucose, amino acids, nucleotides and ions[124].

Astrocytes are another group of cells that make up the blood brain barrier. These star shaped cells form feet like projections that surround the endothelial cells of BBB[125]. Their role is mainly to provide support to the endothelial cells and providing nutrients to the tissues. Astrocytes also facilitate repair and fluid regulation via the aquaporin 4 water channel[126].

Neurons also form the BBB. They are closer to the blood vessels and the astrocytes, and this closeness makes them sensitive to the changes in the ion balance. Neurons are also responsible for blood flow regulation and controlling permeability.

Pericytes are another group of cells found on the endothelial cells surface and are attached to it by gap junctions. They have a major role in stabilizing the endothelial cells by developing tight junctions and depositing the extracellular matrix[127].

The BBB action is mainly to offer protection to the brain from external pathogens that are restricted from getting to the brain tissue. Antibodies are large to penetrate the BBB to offer immunity to the brain tissues and thus this is the main protective mechanism of the brain. Majority of the drugs cannot pass through the BBB due to the selective permeability thus making it difficult to treat many neurological diseases. The BBB becomes permeable during inflammation after infections such as Toxoplasmosis and meningitis and this allows bacteria and viruses to gain access to the BBB[128]. Apart from protecting the brain from pathogens, the BBB also allows passage of essential nutrients to the brain such as glucose through the various transporter proteins[128].

Indeed, it is paramount to find out if a drug can pass through the BBB to reach its target site. In this research, I used molinspiration software to predict the permeability of hybrid compounds, TMZ and NSAIDs and determined whether they can pass through the BBB.

Lipophilicity

Another important factor worth predicting before drug synthesis is lipophilicity. Lipophilicity is a Greek word which means “fat friendly”. It is used to refer to the solubility of compounds in fats, lipids, oils and non-polar compounds e.g toluene and hexane. As a physiochemical parameter, lipophilicity plays a role in absorption, distribution, metabolism, excretion and toxicity (ADMET) of a drug. This therefore means lipophilicity of a drug influence its potency and selectivity. When lipophilicity is too high, then the drug will lead to toxicity and metabolic clearance hence low potency. When it is too low, it leads to low potency too[129].

Lipophilicity of a compound is measured as a partition coefficient. Partition coefficient refers to the ratio of the sum total of all compounds in two phases. The most common method of measuring lipophilicity is called “shake flask method”. In this method, a sample or a compound

is dissolved in a mixture of water and octanol then the mixture is shaken and agitated until an equilibrium is reached. The two phases, octanol and water, are then separated. Octanol-water partition coefficient (Log P) can then be calculated. Optimal range of lipophilicity is between 0 and 4[129, 130].

Oral Bioavailability

Oral bioavailability is also a physiochemical parameter that's important to consider before synthesizing drugs. Oral bioavailability refers to the ratio between the amount of drug administered orally and the amount that reaches systemic circulation. Unlike drugs administered intravenously that are fully available in the bloodstream upon injection, orally administered drugs must pass through many barriers before reaching the target point of action.

High levels of oral bioavailability reduce the quantity of drug administered. Lower quantities of drug lead to reduced toxicity and relatively reduced side effects. On the other hand, low oral availability levels lower drug efficacy[131].

In 2005, Lepinski's rule of 5 was formulated in order to set guidelines for measuring oral bioavailability of drugs. According to this rule, poor oral bioavailability is likely when there are more than 5 hydrogen bond donors, 10 hydrogen bond acceptors, the molecular weight (MW) is greater than 500, and the calculated Log P (CLog P) is greater than 5. Lipinski also stated that this rule would only hold for compounds that were not substrates for active transporters[132, 133].

CHAPTER 2

MATERIALS AND METHODS

Drawing of Chemical Structures

To draw structures of different compounds and hybrids, Chemdraw online software, (https://chemdrawdirect.perkinelmer.cloud/js/sample/index.html), Reaxys structure editor (https://www.reaxys.com/#/structure-editor) and Marvin Sketch online software was used. Figures were illustrated using BioRender software, canva software and Adobe software.

Calculation of Molecular Properties

To determine if the temozolomide, NSAIDs and hybrids could pass through the blood brain barrier, we used Molinspiration software to calculate molecular properties.

Chemistry

Reagents and solvents used to synthesize and analyze the products were bought from Sigma-Aldrich company. All the experiments were done in a conventional fume chamber. Flasks, beakers, magnetic stirrer, separating funnel, stir bar and rotavapor were supplied by the Andrews University Chemistry department and Dr Desmond Murray’s lab. IR spectra was obtained using Thermo Scientific Nicolet iS50 ATR Infrared Spectroscopy.

Experimental procedures for imide and amide synthesis

4-methoxybenzamide Aspirin Imide

Figure 17:Chemical equation for imide synthesis from 4-methoxybenzamide and aspirin

Below is a general procedure representing the trials done using aspirin as the carboxylic acid and 4-methoxybenzamide and TMZ as the amides. The procedures were done in different variations e.g different times of refluxing or only stirring the mixtures.

An oven dried 50 ml round bottom flask containing a stir bar was clamped on top of a magnetic stirrer. 25 ml of dry dichloromethane was then added into the flask before attaching a drying tube and stirring gently. Thereafter, the following reagents were added; (1) 0.002 mol carboxylic acid, (2) 0.0025 mol oxalyl chloride and (3) 10 drops of dry dimethylformamide (DMF). The drying tube was then removed and a water-cooled condenser was attached. The mixture was refluxed with continued stirring for 45 minutes to produce acyl chloride. After 45 minutes of reflux, the condenser was replaced with a drying tube then the mixture was dried in an ice-bath for 5 minutes. 0.002 mol potassium carbonate was then added into the cooled mixture and stirred for 10 minutes. The following reagents were then added into the mixture; add (a) 0.002 mol of amide and (b) 0.002 mol diisopropylethylamine. The mixture was then stirred using a magnet stirrer at room temperature for 24 hours. Thereafter, the reaction mixture was poured into a 250 ml beaker containing ice and 30 ml 1M HCl. The mixture was then stirred for 15

minutes. The mixture was then extracted three times with ethyl acetate. The combined organic layers were then dried using anhydrous sodium sulphate then filtered into a round bottom flask and rotovated. The product was weighed and analyzed using IR and NMR. IR and NMR spectra of the starting compounds and product was then compared.

Below is an amide synthesis procedure that was used to synthesize novel hybrids of diclofenac and purines.

An oven-dried 50 ml round bottom flask containing a stir bar was clamped on top of a magnetic stirrer. 25 ml of dry dichloroethane was then added into the flask before attaching a drying tube and stirring gently. The following reagents were then measured out and added into the flask: (1) 0.002 mol diclofenac, (2) 0.0025 mol oxalyl chloride and (3) 10 drops of dry dimethylformamide (DMF). Drying tube was then removed and a water-cooled condenser was attached to the flask. The mixture was then refluxed with continued stirring for 1 hour to produce the acyl chloride. The condenser was replaced with a drying tube then the mixture was allowed to cool in an ice-bath for 5 minutes. 0.002 mol potassium carbonate was then added to the mixture and stirred for 10 minutes. The following reagents were measured out and added into the flask (a) 0.002 mol purine and (b) 0.002 mol diisopropylethylamine. Thereafter, the mixture was stirred for 4 hours. Slowly, the reaction mixture was poured into a 250 ml beaker containing ice and 10 ml 1M HCl and 20ml saturated NH_4Cl. Stirring continued for 15 minutes before extracting the mixture three times with ethyl acetate. Combined organic layers were dried with anhydrous sodium sulphate then filtered into a round bottom flask. A rotavapor was used to gently remove the solvent. IR of the product was then taken and compared with IR of the organic starting materials. Finally, the actual yield and % yield of the product was determined.

p-Anisidine + Diclofenac → JRP1

2-chloro-7H-purine-6-amine + Diclofenac → JRP2

Adenine + Diclofenac → JRP3

Guanine + Diclofenac → JRP4

2-Amino-6-chloropurine + Diclofenac → JRP5

2-fluoro-7H-purine-6-amine + Diclofenac → JRP6

Figure 18: Chemical equations for the amides synthesized. JRP1, JRP2, JRP3, JRP4, JRP5, JRP6 represent hybrid compounds synthesized.

Biology

Once novel hybrids had been synthesized, I tested their LC_{50} and observed changes in morphology. These tests were done on U87MG cell line in Dr. Denise's lab at the Biology department, Andrews University. Below are procedures, activities and methods done in the lab.

Maintaining of U87MG Cells

U87MG Glioblastoma cells were grown in standard tissue 100 mm or 60 mm culture dishes. They were then allowed to grow in 8 ml or 3 ml respectively of MEM (Minimal Essential Media; Invitrogen) that was supplemented with 100U/ml penicillin, streptomycin and 10% fetal bovine serum (FBS). The cells were then kept in a humidified incubator with Carbon dioxide concentrations of 5 % and a temperature of 37 °C.

The media was changed three times every week. Whenever the cells covered more than 80% of the tissue culture dishes, the media was removed followed by addition of 2ml for 100 mm dishes 1ml for 60 mm dishes of 1X TE (trypsin EDTA; Invitrogen). Trypsin was used to detach the cells from the dish. Half of the cells floating in trypsin were then removed. 8ml or 3ml, respectively of new media was then added before incubation of the cells.

Determination of LC_{50} lethal concentration

Determination of LC_{50} was a process that took 72 hours. The first step was transferring 10,000 U87 MG cells into each well in the 12 well plate followed by treating the cells with drug, fixing, staining, and counting.

Transferring 10,000 U87 MG cells

90 µl of trypan blue was transferred into a microcentrifuge tube. Old media was then removed from the dish before adding 2 ml or 1 ml respectively of trypsin. Trypsin was used to wash the cells off the dish. Thereafter, 10 µl of cells suspended were transferred into the microcentrifuge tube containing 90 µl of trypan blue. Mixing of the cells and trypan blue was done using a pipette. Immediately after, 10µl of the mixture was introduced into the hemocytometer via capillary action. The number of cells was determined then diluted to create 10,000 cells for each well of a 12-well plate. 1ml of media containing 10,000 cells was introduced into each well of the 12-well plate. The cells were then incubated in a humidified incubator with Carbon dioxide concentrations of 5 % and a temperature of 37 °C for 24 hours to allow them to attach to the plate.

Treating U87 MG cells with drugs

0.02 g of compound was first dissolved in 1 mL of dimethyl sulfoxide (DMSO) to create a stock solution. As long as we kept the DMSO concentration below 1%, cells behaved and grew normally with no additional cell death. Eleven serial dilutions of half concentration in MEM media were created starting at 2mg/ml. The final well contained media only. After treating the cells with the drug, they were incubated again in a humidified incubator with Carbon dioxide concentrations of 5 % and a temperature of 37 °C for another 24 hours.

Cell viability count

First, the media was removed then 1 mL of methanol was added into each well and incubated for 5 minutes to fix the cells. Methanol was then removed before adding 1 mL of crystal violet stain. Incubation for another 5 minutes then followed. Thereafter, crystal violet was

carefully removed using a pipette. Excess stain was removed with repeated washing in water. The wells were then allowed to dry for another 24 hours.

Using a sharpie, three dots were randomly marked under the well. An inverted light microscope at 400X and Clay Adams Laboratory Counter was used to count the number of cells in the field of view for the three dots. An average was then calculated before determining the total number of cells in each well.

Compounds screened

The following are the compounds and mixtures screened; TMZ, aspirin, diclofenac, ibuprofen, naproxen, oxaprozin and ketoprufen. 1:1 and 2:1 ratio of TMZ and aspirin, TMZ and diclofenac, TMZ and oxaprozin as well as TMZ and ketoprufen were also screened. The following purines and their hybrids with diclofenac were also screened: guanine, adenine, 2-chloro-7H-purine-6-amine, 2-fluoro-7H-purine-6-amine, 2-amino-6-chloropurine p-anisidine.

CHAPTER 3

RESULTS

Computational predictions for TMZ, NSAIDs, purines and novel hybrid compounds.

Molinspiration online software was used to calculate molecular properties of TMZ, NSAIDs, purines and novel hybrid compounds (table 2). Drug potency depends on chemical and physical properties of the drug. Drugs must be transported and distributed in a complex multicompartment biological system to site of action and should be complementary to a structurally unknown receptor. Lipophilicity plays a role in this process. The term lipophilicity which means 'friendly' in Greek, refers to the ability of a compound to dissolve in oils, fats, lipids as well as non-polar compounds[129, 134]. Therefore, it contributes to absorption, distribution, metabolism, elimination and toxicity (ADMET) properties of a drug. Most of the drugs synthesized and tested have lipophilicity (LogP) value within the optimal range of 1-5. TMZ had the lowest LogP value of -1.9 while a novel hybrid of p-anisidine and diclofenac had the highest LogP value of 6.18. Diclofenac has a LogP value of 4.57. All the hybrids with diclofenac compound had a LogP value higher than 4.57 except hybrid of diclofenac and TMZ which has a LogP of 2.80 and hybrid of diclofenac and guanine which has a LogP value of 4.20.

Lipinski rule of 5 states that poor oral bioavailability is likely when there are more than 5 hydrogen bond donors, 10 hydrogen bond acceptors, the molecular weight (MW) is greater than 500, and the calculated Log P (CLog P) is greater than 5. Only TMZ, guanine and hybrid of TMZ and aspirin have a negative LogP value. On the other hand, only a hybrid of p-anisidine

and diclofenac had a LogP value greater than 5. All the other compounds had a value within or very close to the range of 1-5 LogP value. All the compounds tested had less than 5 hydrogen bond donors(nOHNH), 10 or less than 10 hydrogen bond acceptors and molecular weight of less than 500 (table 2).

Table 2: Predicted blood-brain barrier permeability and Bioavailability of TMZ, NSAIDs, purines and novel hybrid compounds. LogP – Octanol-water partition coefficient, TPSA – Topical polar surface area, nAtoms – Number of atoms, nON – Number of hydrogen-bond accepted, nOHNH – Number of hydrogen bond donors, MW – molecular weight, nRotB – Number of rotatable bonds

Compound	LogP	TPSA	nAtoms	nON	nOHNH	MW	nRotB	Volume	Lepinski rule
TMZ	-1.9	108.19	14	8	2	194.15	1	152.12	pass
Aspirin	1.43	63.60	13	4	1	180.16	3	155.57	pass
Diclofenac	4.57	49.33	19	3	2	296.15	4	238.73	pass
Ibuprofen	3.46	37.30	15	2	1	206.28	4	211.19	pass
Naproxen	3.38	46.53	17	3	1	230.26	3	213.97	pass

Oxaprozin	3.75	63.33	22	4	1	264.88	5	264.88	pass
Ketoprofen	3.59	54.37	19	3	1	254.28	4	234.83	pass
p-anisidine	1.07	35.26	9	2	2	123.16	1	120.88	Pass
Guanine	-0.93	100.46	11	6	4	151.13	0	119.97	Pass
Adenine	0.23	80.49	10	5	3	135.13	0	111.84	Pass
2-Chloro-7H-purine-6-amine	1.23	80.49	11	5	3	169.57	0	125.38	Pass
2-Fluoro-7H-purine-6-amine	0.72	80.49	11	5	3	153.12	0	116.77	Pass
2-amino-6-chloropurine	0.43	80.49	11	5	3	169.57	0	125.38	Pass
p-anisidine + diclofenac	6.18	50.36	27	4	2	401.29	6	340.07	

Guanine + diclofenac	4.20	115.56	29	8	4	429.27	5	339.16	Pass
Adenine + diclofenac	4.94	95.59	28	7	3	413.03	5	331.03	Pass
2-Chloro-7H-purine-6-amine + diclofenac	5.94	95.59	29	7	3	447.71	3	344.57	Pass
2-Fluoro-7H-purine-6-amine + diclofenac	5.42	95.59	29	7	3	431.26	5	335.96	Pass
2-amino-6-chloropurine + diclofenac	5.55	95.59	29	7	3	447.71	5	344.57	Pass

IR Spectroscopy Analysis

Novel hybrid compounds were synthesized in the Chemistry department. IR spectroscopy was done to confirm that the desired compounds were synthesized. The product was also weighed to find out the percentage yield of the product.

Imide N-H stretch, which falls between 3150 and 3250 was not present in all products. This means that imides were not successfully synthesized (table 3). O-H carboxylic acid band at 2800-3500 cm^{-1} was expected in aspirin's IR spectra. This peak was observed at 2830 cm^{-1}. The N-H amide peak expected between 3100 - 3500 cm^{-1} was observed at 3158 cm^{-1}. TMZ doublet N-H peaks were also observed at 3328 and 3421 cm^{-1}. Diclofenac had a peak at 3321 cm^{-1}. We expected two peaks for primary amines (N-H) within the range of 3000 and 3300 cm^{-1} range. All the primary amines had these two peaks. All the products synthesized had an amide (C=O) peak within the expected range (table 4).

Table 3: Diagnostic IR peaks for TMZ and 4 methoxybenzamide (amides), aspirin (carboxylic acid) and diagnostic peaks for hybrids of aspirin and 4-methoxybenzamide and hybrid of aspirin and TMZ are missing

Compound	Diagnostic Peaks	Results
TMZ	3328 (amide) 3421 (amide)	NA (Not Applicable)
4-methoxybenzamide	3158 (amide)	NA
Aspirin	2830 (Carboxylic acid)	NA
4-methoxybenzamide + aspirin		Imide peak not observed
TMZ + aspirin		Imide peak not observed

Table 4: Diagnostic IR peaks for carboxylic acid(diclofenac), amino purines (guanine, adenine, 2-Chloro-7H-purine-6-amine, 2-Fluoro-7H-purine-6-amine, 2-amino-6-chloropurine) amines (p-anisidine) and amides (hybrid of 2-amino-6-chloropurine + Diclofenac, 2-Chloro-7H-purine-6-amine + Diclofenac, Adenine + Diclofenac, Guanine + Diclofenac, P-Anisidine + Diclofenac and 2-Fluoro-7H-purin-6-amine + Diclofenac) analyzed

Compound	Diagnostic peaks
Diclofenac	3321 (carboxylic acid)
2-Chloro-7H-purine-6-amine	3274 (amine) 3125 (amine)
2-Fluoro-7H-purine-6-amine	3289 (amine) 3117 (amine)
2-Amino-6-chloropurine	3297 (amine) 3127 (amine)
p-anisidine	3219 (amine) 3006 (amine)
Guanine	3314 (amine) 3110 (amine)
Adenine	3286 (amine) 3103 (amine)
2-amino-6-chloropurine + Diclofenac	1675 (amide)
2-Chloro-7H-purine-6-amine + Diclofenac	1673 (amide)
Adenine + Diclofenac	1672 (amide)
Guanine + Diclofenac	1663 (amide)
p-Anisidine + Diclofenac	1664 (amide)
2-Fluoro-7H-purin-6-amine + Diclofenac	1663 (amide)

Table 5 below shows detailed structures and their product yield. Product yield ranged between 54% and 133%. Hybrid of 2-amino-6-chloropurine and diclofenac had the lowest yield of 54% followed by hybrid of 2-Fluoro-7H-purine-6-amine and diclofenac with a 61% yield. Hybrid of adenine and diclofenac had a % yield of 68 while a hybrid of guanine and diclofenac had a % yield of 70. Percentage yield of novel hybrid of p-anisidine and diclofenac was relatively high at 86% and product yield of novel hybrid of 2-chloro-7H-purine-6-amine and diclofenac was the highest at 133%. Novel hybrid of 2-chloro-7H-purine-6-amine and diclofenac most likely had impurities from solvents or catalysts hence the product yield higher than 100%.

Table 5: Reactants and products structures

Carboxylic acid	Amine	Amide	Product yield %
Diclofenac	p-Anisidine	Hybrid of diclofenac and p-Anisidine	86
	Guanine	Hybrid of diclofenac and guanine	70
	Adenine	Hybrid of diclofenac and adenine	68
	2-Chloro-7H-purine-6-amine	Hybrid of diclofenac and 2-Chloro-7H-purine-6-amine	133
	2-Fluoro-7H-purine-6-amine	Hybrid of diclofenac and 2-Fluoro-7H-purine-6-amine	61
	2-Amino-6-chloropurine	Hybrid of diclofenac and 2-Amino-6-chloropurine	54

Biology

LC_{50} Values

LC_{50} values ranged from 0.0056 to 0.2021. Two NSAIDs had an LC_{50} lower than that of TMZ. These compounds include diclofenac which had the lowest LC_{50} of 0.0139 and oxaproxin with 0.0455. TMZ had LC_{50} of 0.059. All 1:1 mixture of TMZ and NSAIDs had a lower LC_{50} compared to LC_{50} of the individual compounds except in case of mixture between ketoprofen and TMZ (figure 19).

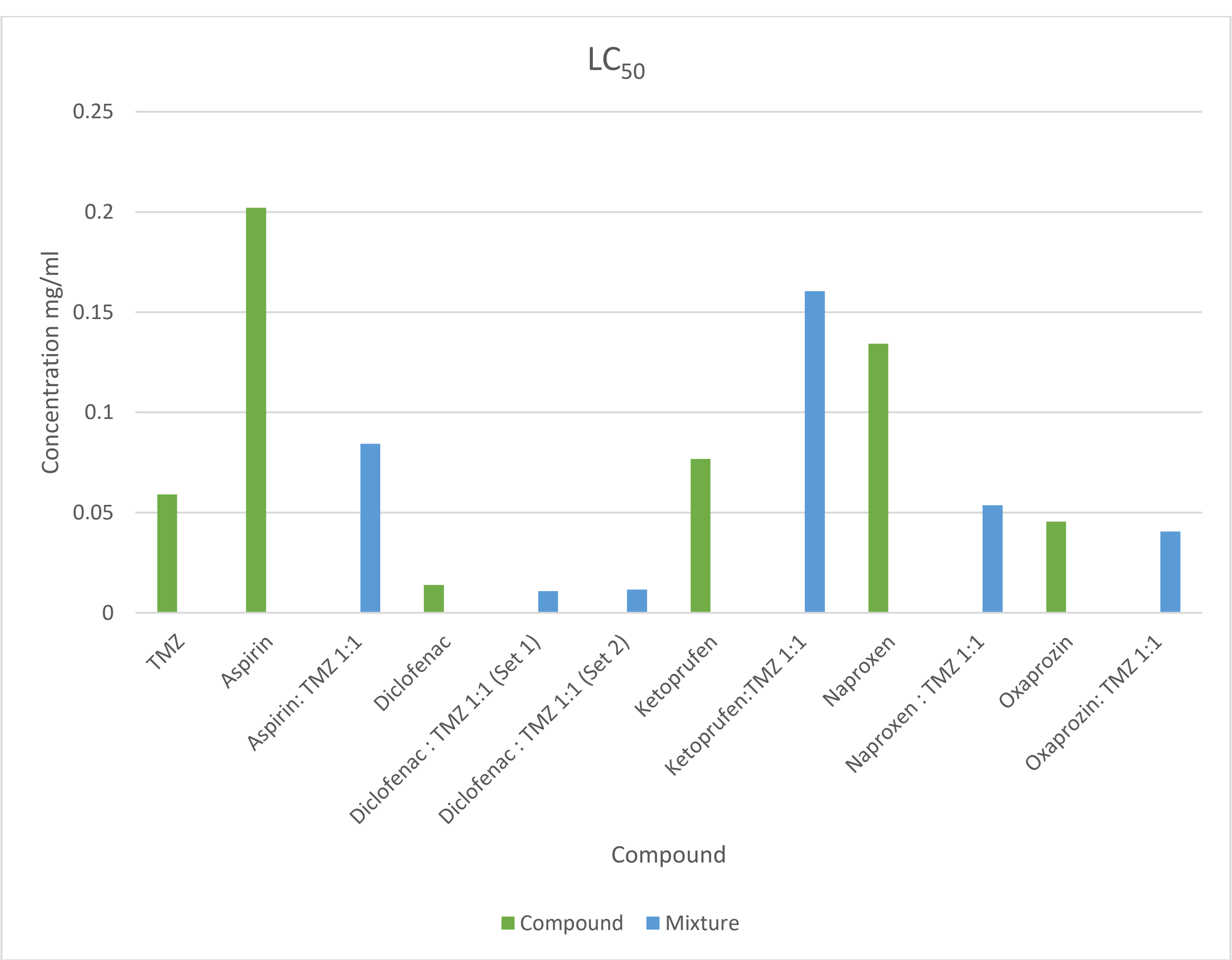


Figure 19:Comparison between LC_{50} of individual compounds and LC_{50} of 1:1 mixtures of the compound and TMZ

In the case of TMZ: NSAIDs 2:1 mixture, LC_{50} values changed to a value closer to that of TMZ's LC_{50}. The table below illustrates these findings (figure 20).

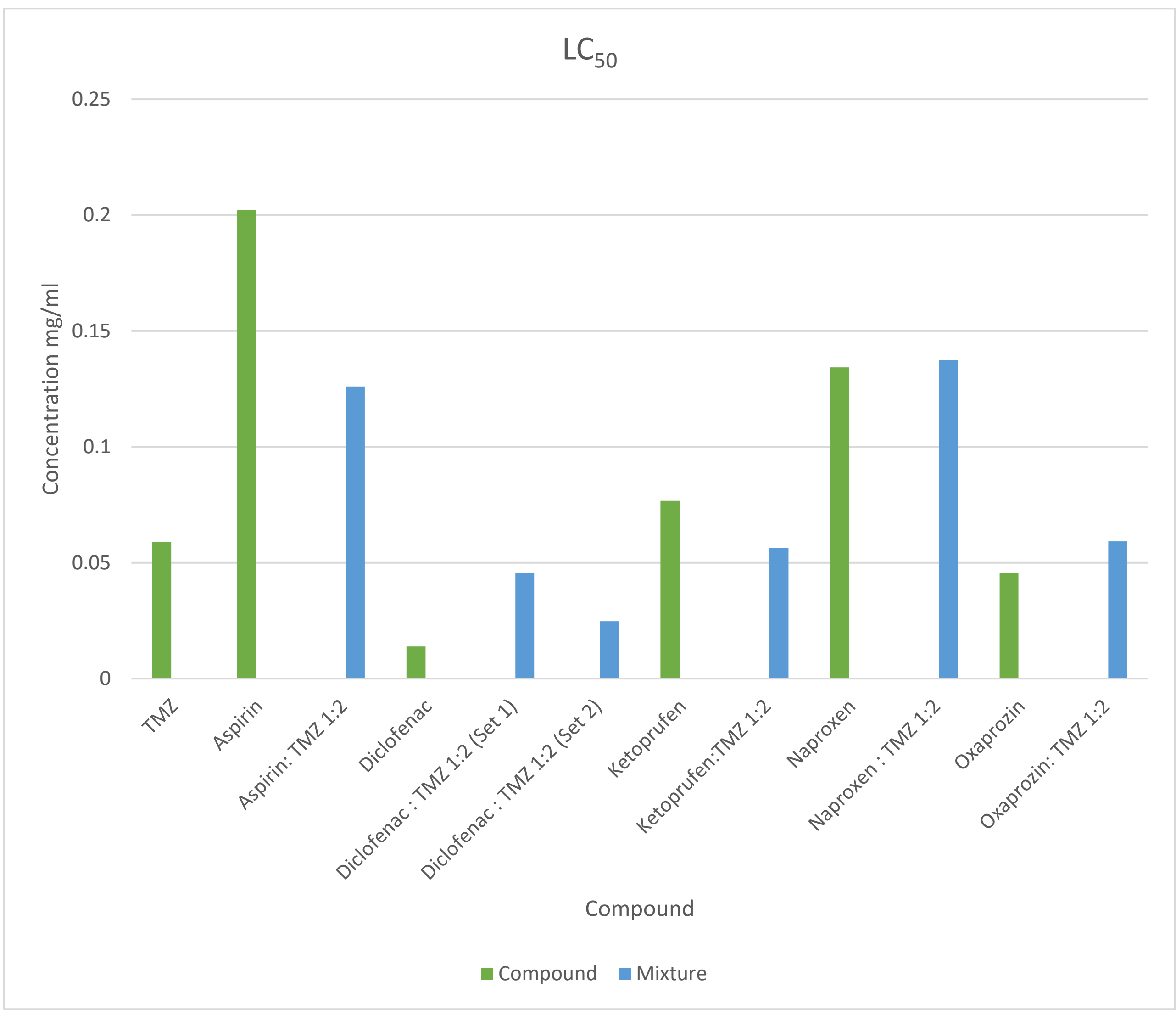


Figure 20: Comparison between LC_{50} of individual compounds and LC_{50} of 1:2 mixture of the compound and TMZ respectively.

Novel hybrid compounds had LC_{50} values ranging from 0.0056 and 0.0247. Only novel hybrid of p-anisidine and diclofenac had LC_{50} value higher than that of diclofenac. Generally, the hybrids had lower LC_{50} values compared to LC_{50} values of individual compounds (figure 21).

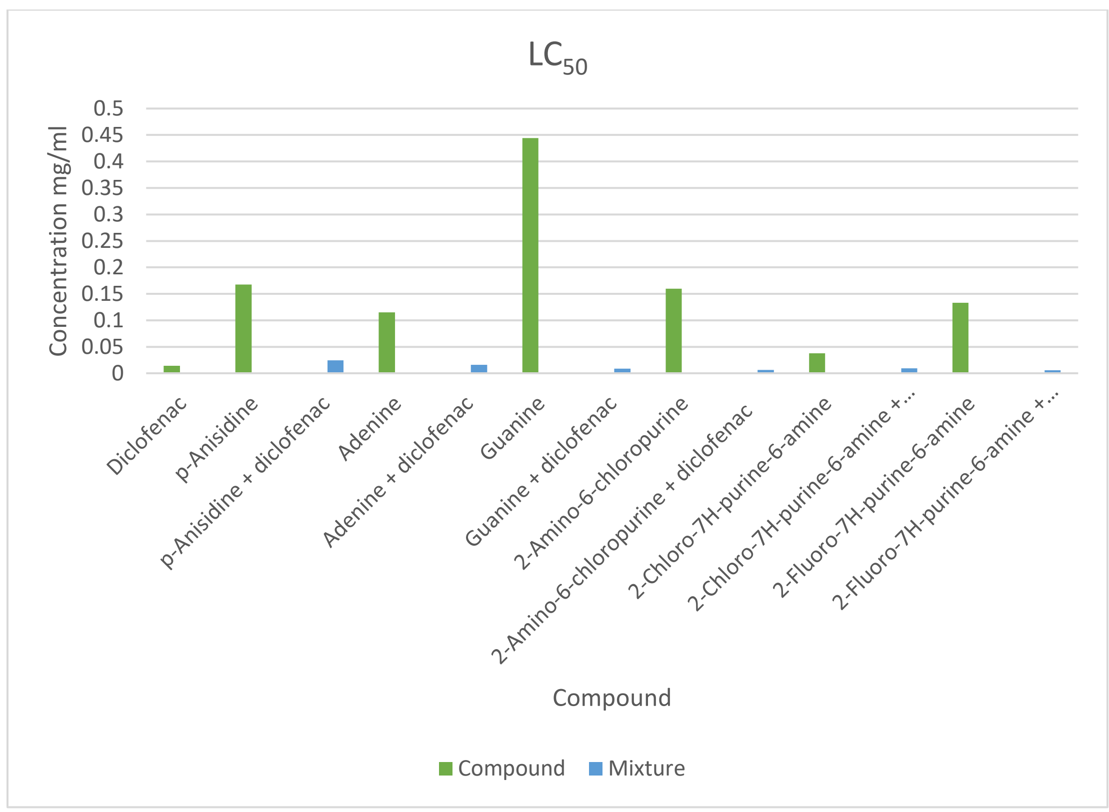
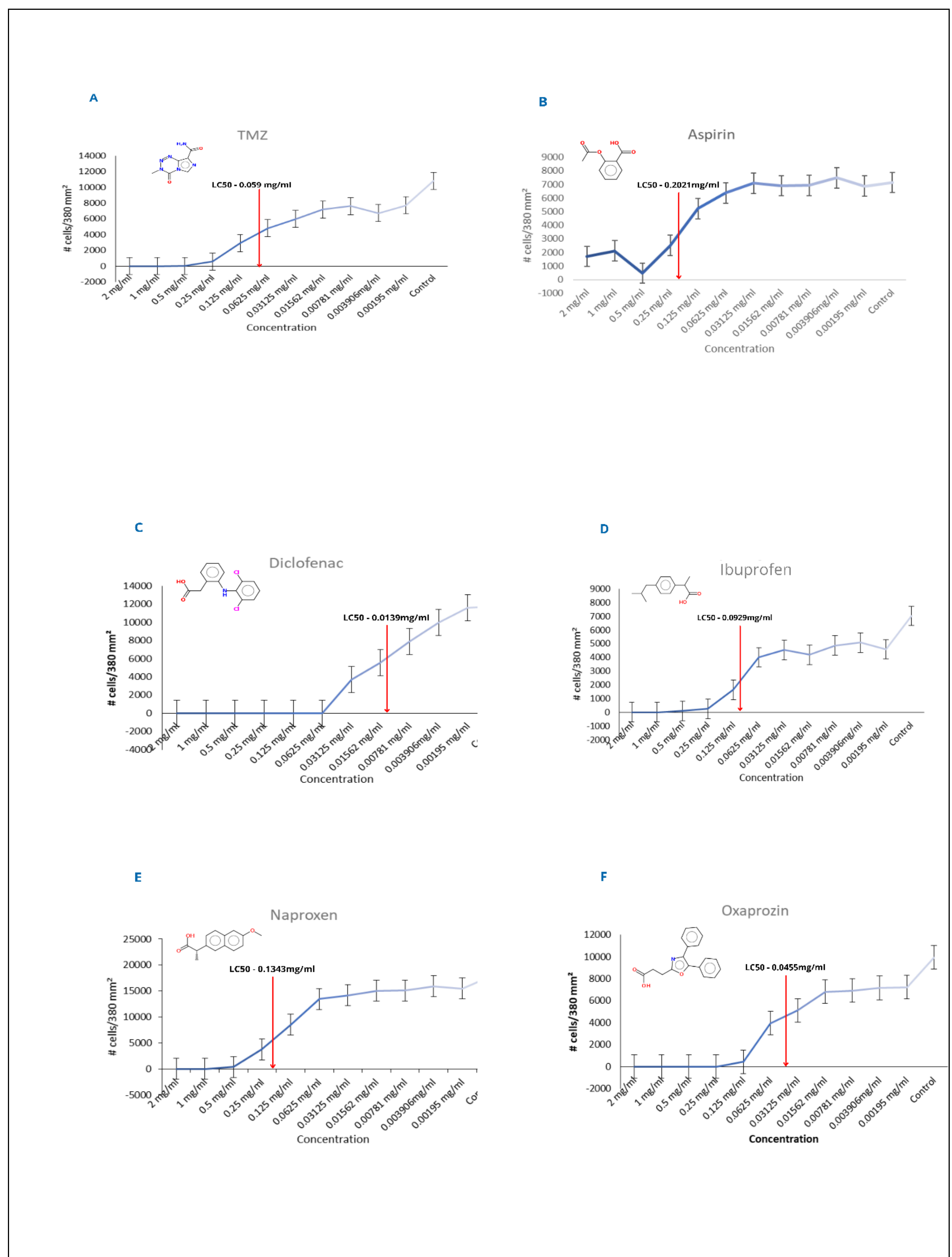
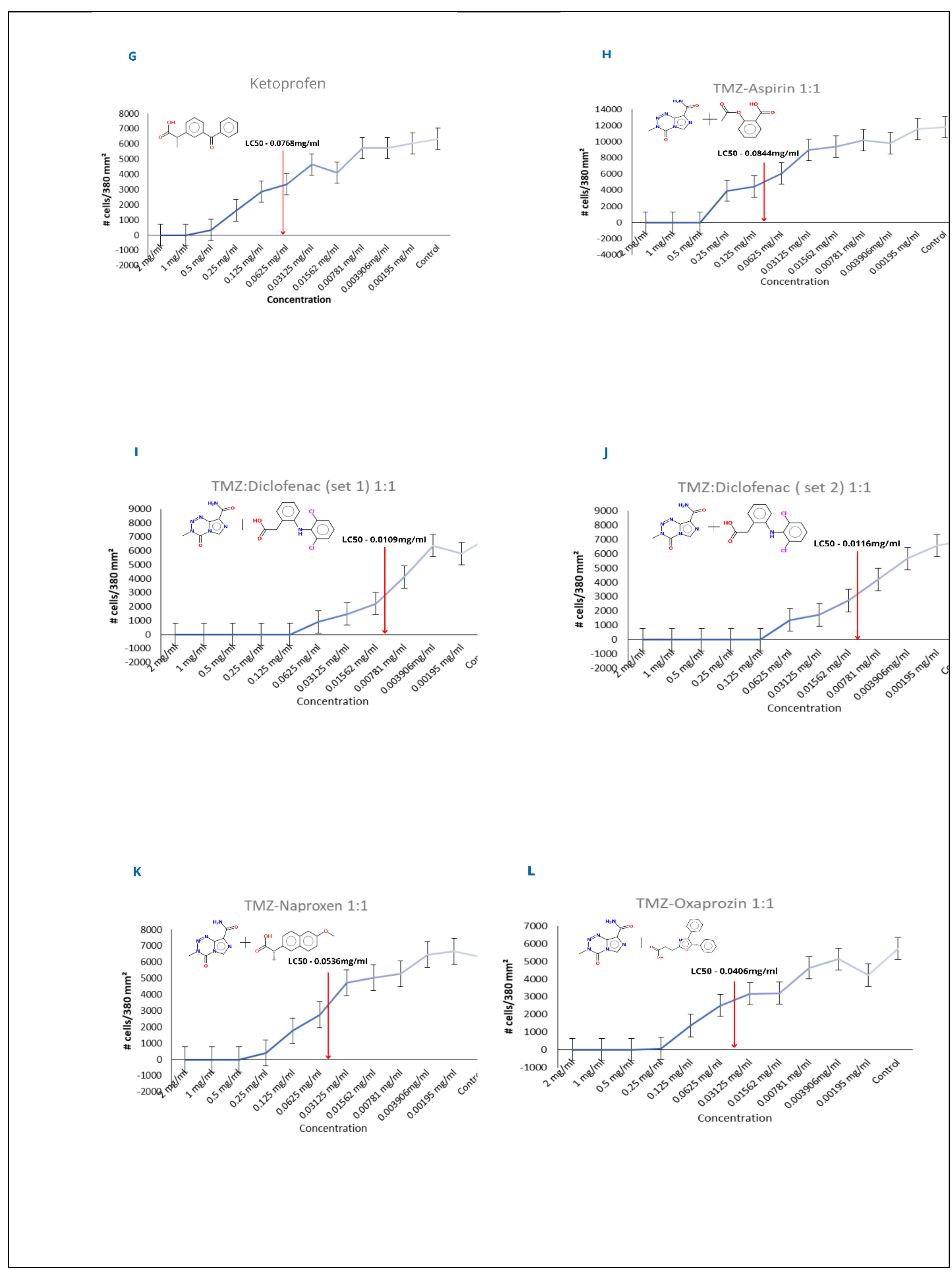
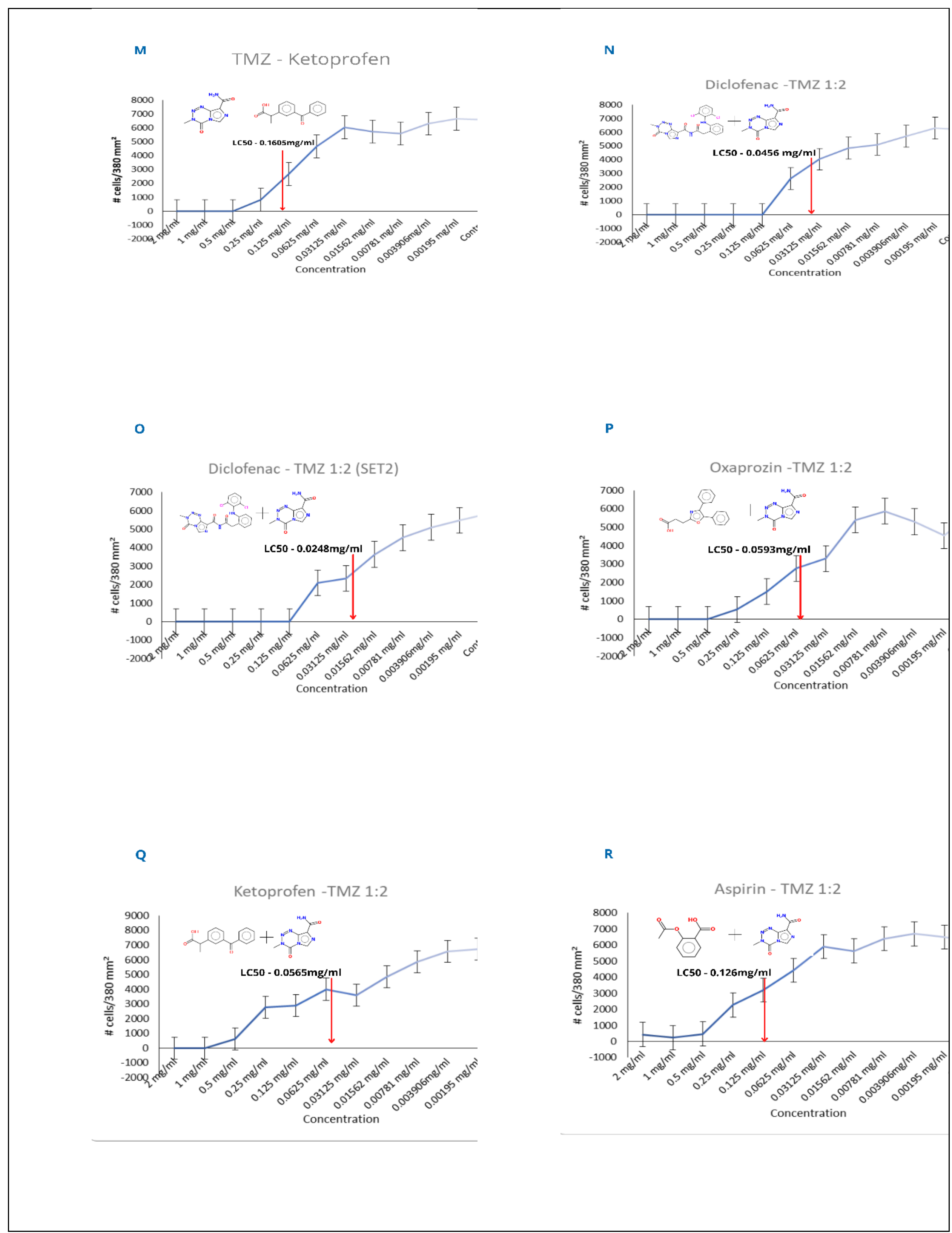


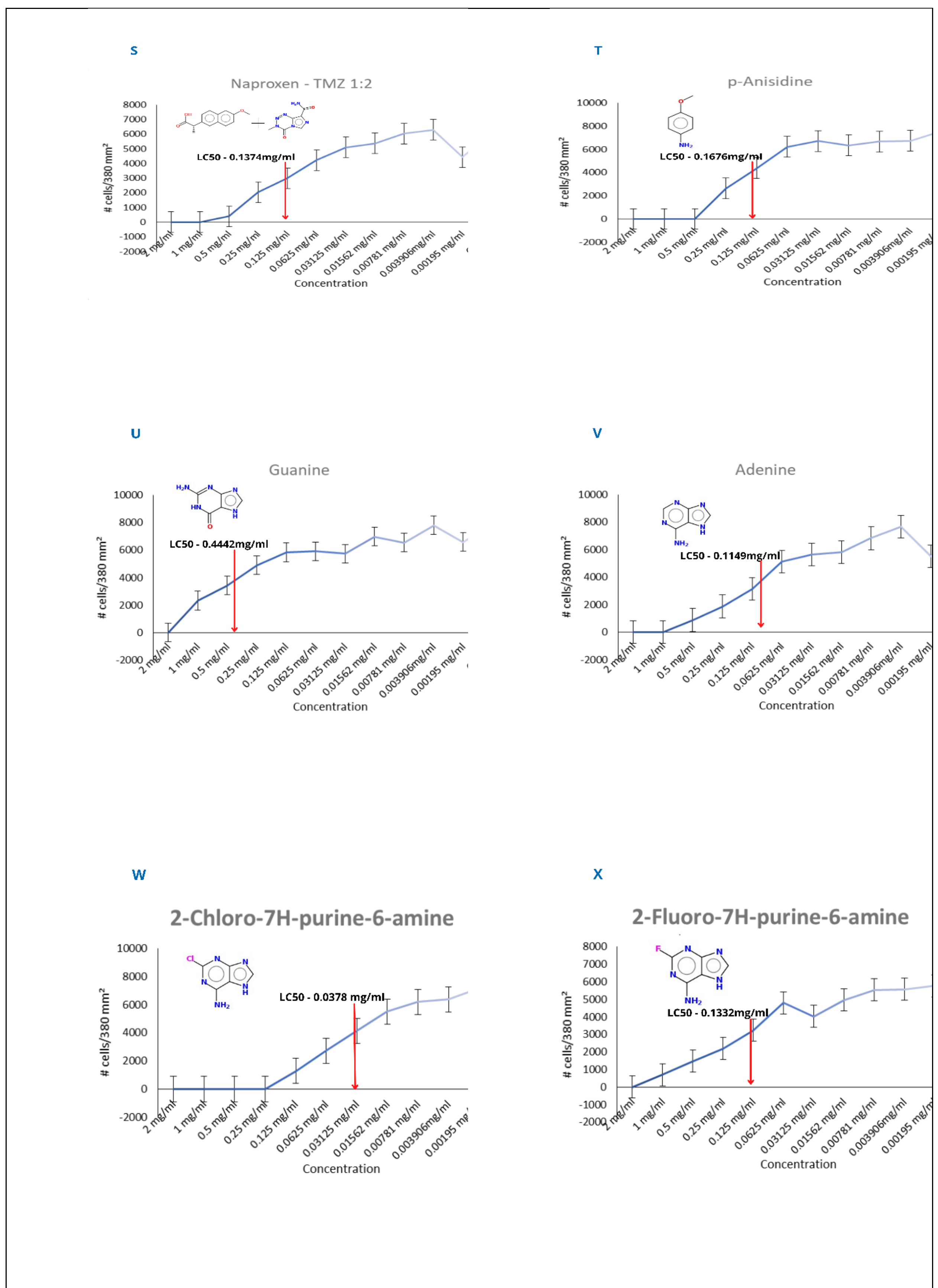
Figure 21: Comparison between LC_{50} of individual compounds and LC_{50} of novel hybrids of purines and diclofenac

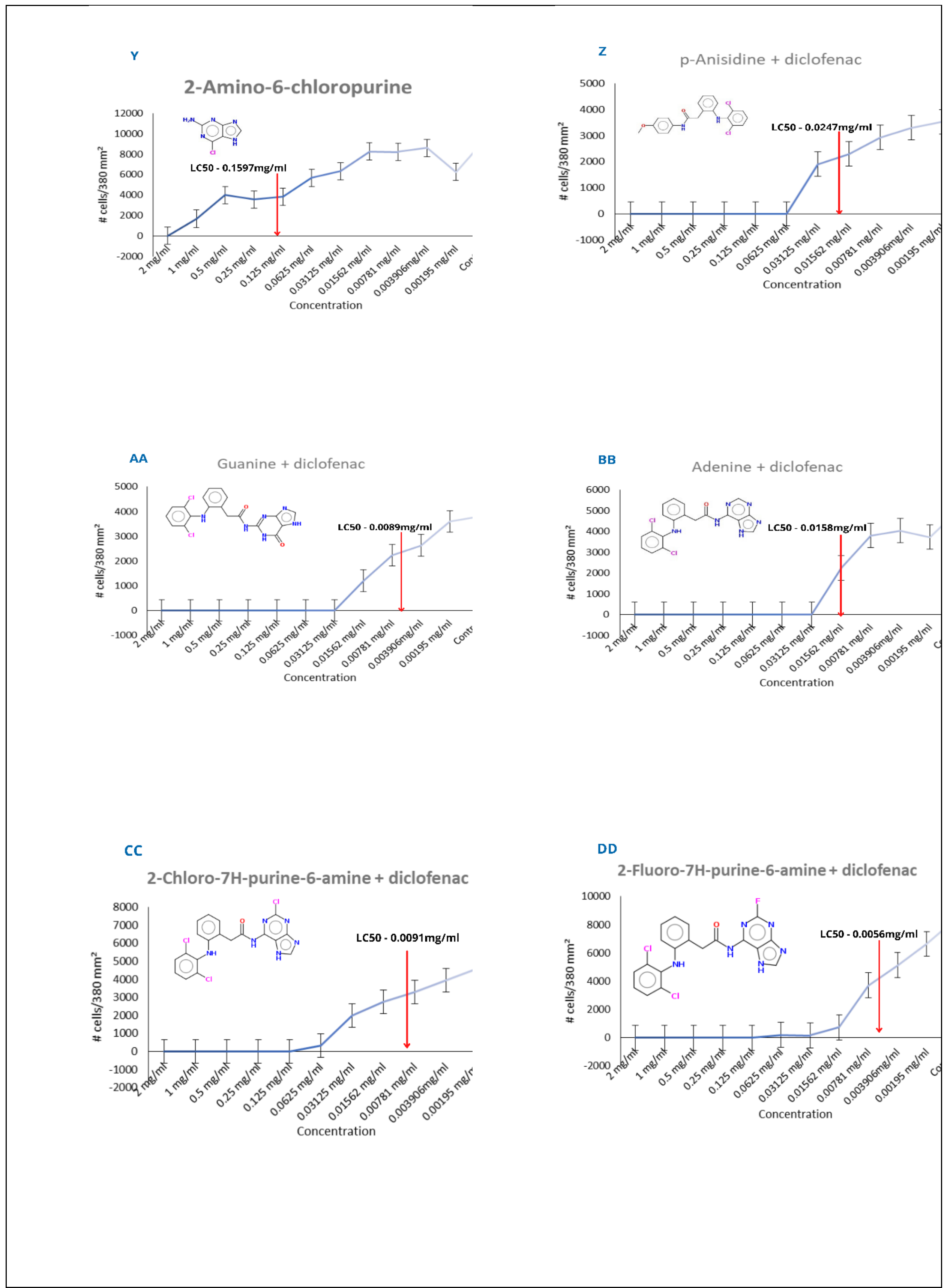
Figure 22 below shows effect of TMZ, NSAIDs, amino purines, p-anisidine and novel hybrids of diclofenac and purines on U-87MG glioblastoma cell viability compared to untreated U-87MG cells. Red arrows indicate the LC_{50} values of each compound. Error bars indicate standard error, n = 3 independent experiments in triplicate.


A
TMZ
LC50 - 0.059 mg/ml
cells/380 mm²
Concentration
B
Aspirin
LC50 - 0.2021mg/ml
cells/380 mm²
Concentration
C
Diclofenac
LC50 - 0.0139mg/ml
cells/380 mm²
Concentration
D
Ibuprofen
LC50 - 0.0929mg/ml
cells/380 mm²
Concentration
E
Naproxen
LC50 - 0.1343mg/ml
cells/380 mm²
Concentration
F
Oxaprozin
LC50 - 0.0455mg/ml
cells/380 mm²
Concentration


G
Ketoprofen
LC50 - 0.0768mg/ml
cells/380 mm²
Concentration
H
TMZ-Aspirin 1:1
LC50 - 0.0844mg/ml
cells/380 mm²
Concentration
I
TMZ:Diclofenac (set 1) 1:1
LC50 - 0.0109mg/ml
cells/380 mm²
Concentration
J
TMZ:Diclofenac (set 2) 1:1
LC50 - 0.0116mg/ml
cells/380 mm²
Concentration
K
TMZ-Naproxen 1:1
LC50 - 0.0536mg/ml
cells/380 mm²
Concentration
L
TMZ-Oxaprozin 1:1
LC50 - 0.0406mg/ml
cells/380 mm²
Concentration


M
TMZ - Ketoprofen
LC50 - 0.1605mg/ml
cells/380 mm²
Concentration
N
Diclofenac -TMZ 1:2
LC50 - 0.0456 mg/ml
cells/380 mm²
Concentration
O
Diclofenac - TMZ 1:2 (SET2)
LC50 - 0.0248mg/ml
cells/380 mm²
Concentration
P
Oxaprozin -TMZ 1:2
LC50 - 0.0593mg/ml
cells/380 mm²
Concentration
Q
Ketoprofen -TMZ 1:2
LC50 - 0.0565mg/ml
cells/380 mm²
Concentration
R
Aspirin - TMZ 1:2
LC50 - 0.126mg/ml
cells/380 mm²
Concentration


S
Naproxen - TMZ 1:2
LC50 - 0.1374mg/ml
cells/380 mm²
Concentration
T
p-Anisidine
LC50 - 0.1676mg/ml
cells/380 mm²
Concentration
U
Guanine
LC50 - 0.4442mg/ml
cells/380 mm²
Concentration
V
Adenine
LC50 - 0.1149mg/ml
cells/380 mm²
Concentration
W
2-Chloro-7H-purine-6-amine
LC50 - 0.0378 mg/ml
cells/380 mm²
Concentration
X
2-Fluoro-7H-purine-6-amine
LC50 - 0.1332mg/ml
cells/380 mm²
Concentration


Y
2-Amino-6-chloropurine
LC50 - 0.1597mg/ml
cells/380 mm²
Concentration
Z
p-Anisidine + diclofenac
LC50 - 0.0247mg/ml
cells/380 mm²
Concentration
AA
Guanine + diclofenac
LC50 - 0.0089mg/ml
cells/380 mm²
Concentration
BB
Adenine + diclofenac
LC50 - 0.0158mg/ml
cells/380 mm²
Concentration
CC
2-Chloro-7H-purine-6-amine + diclofenac
LC50 - 0.0091mg/ml
cells/380 mm²
Concentration
DD
2-Fluoro-7H-purine-6-amine + diclofenac
LC50 - 0.0056mg/ml
cells/380 mm²
Concentration

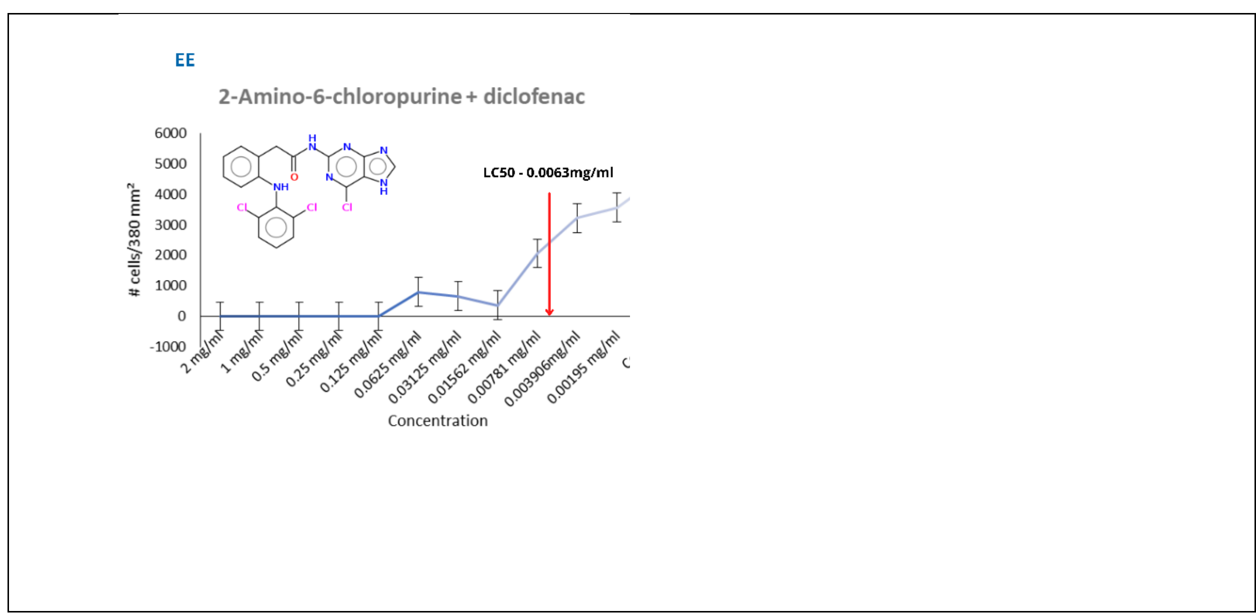
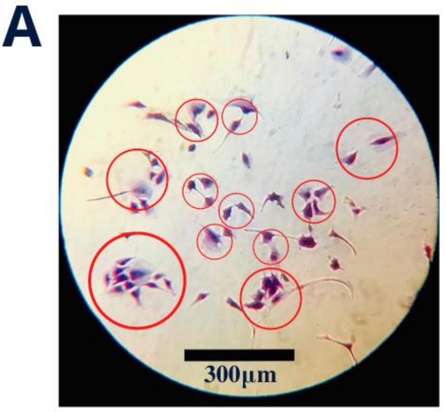
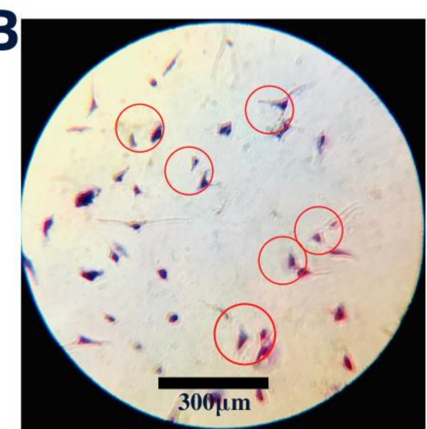


Figure 22: The effects of TMZ. NSAIDs, purines and novel hybridsl on U-87MG glioblastoma cells compared to untreated U-87MG cells. Red arrow indicates the LC_{50} value. Error bars indicate standard error, n=3 independent experiments in triplicate.

Interesting morphological observations were observed in cells treated with diclofenac, adenine and 2-amino-6-chloropurine. In the case of diclofenac, it was observed that at high concentrations, cells divided and remained localized or close to parent cell. This suggests that diclofenac influences cell motility. Moreover, it was observed that the number of cell clusters increased with decreasing concentration and eventually there were no clusters at low concentrations. Cell shape was observed to be more of an irregular circle and not star shaped as in the case of normal U-87MG glioblastoma cells.





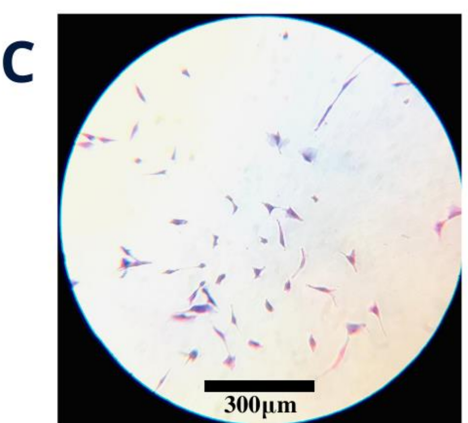


*Figure 23:Morphological observations on diclofenac. **A**. A picture showing field of view on a Leica DMIL type 090-135.001 inverted microscope. Red cycles show cluster of U87MG GBM cells treated for 24 hours with 0.03125 mg/ml of diclofenac and stained with crystal violet. **B.** picture showing field of view on a Leica DMIL type 090-135.001 inverted microscope. Red cycles show cluster of U87MG GBM cells treated for 24 hours with 0.01562 mg/ml of diclofenac and stained with crystal violet. **C.** A picture showing field of view on a Leica DMIL type 090-135.001 inverted microscope. On the image are U87MG GBM cells not treated with diclofenac and stained with crystal violet (control).*

CHAPTER 4

DISCUSSION

Lipophilicity

Lipophilicity is a Greek word that means "fat friendly" or "fat loving". It is often referred to as LogP. LogP represents the ratio at equilibrium of the concentration of a compound between two phases, an oil and a liquid phase. It is a physiochemical parameter that predicts how soluble a compound is in fats, lipids, oils and non-polar compounds. Therefore, it plays a role in absorption, distribution, metabolism, excretion and toxicity of a drug [88][134]. When lipophilicity is high, i.e. greater than 5, it results in high metabolic turn over, poor oral absorption and low solubility. There is also a risk of increased toxicity associated with high lipophilicity. Low lipophilicity, i.e. below 1, leads to low efficacy of the drug since it negatively impacts permeability and drug potency. Study shows that optimal logP is between 1 and 4[135]. The compounds synthesized have LogP values within the range of 1-5 or close except the novel hybrid compound of diclofenac and p-anisidine which has a logP value of 6.18. The high logP values may be attributed to the high logP value of diclofenac which is 4.57. A hybrid of diclofenac and other compounds with optimal logP values generally have higher logP values. Temozolomide has a low logP value of -1.9. This low lipophilicity may not be a problem since TMZ is a prodrug, that is, it does not require metabolic activation and it is converted to active compound spontaneously at blood pH[52, 136]. Prior research shows that TMZ has100% bioavailability when taken orally and can pass through the BBB. This is attributed to its small size and lipophilic properties [137].

Oral Bioavailability

As stated earlier, oral bioavailability refers to the ratio between the amount of drug administered orally and the amount that reached the systemic circulation. Oral drugs pass through many barriers before reaching target point of action. Drugs with high level of oral bioavailability are given in low quantities, which leads to reduced toxicity as well as side effects. On the other hand, drugs with low oral bioavailability have lower drug efficacy[131]. Lipinski's rule formulated in 2005 sets the guidelines for measuring oral bioavailability. According to this rule, poor oral bioavailability is likely when there are more than 5 hydrogen bond donors, 10 hydrogen bond acceptors, the molecular weight (MW) is greater than 500, and the calculated Log P (CLog P) is greater than 5. All the compounds tested had less than 5 hydrogen bond donors(nOHNH), 10 or less than 10 hydrogen bond acceptors and molecular weight of less than 500. Only a novel hybrid of p-anisidine and diclofenac had Log P value of 6. Therefore, all the compounds except the hybrid of p-anisidine and diclofenac passed the Lipinski's rule.

Blood Brain Barrier

Drugs pass the blood brain barrier mainly through transmembrane diffusion. There are factors that influence the ability of substances to cross the blood brain barrier. Lipophilicity and molecular weight are two of the main factors. Generally, molecules with low molecular weight of not more than 500 and high lipophilicity can cross the blood brain barrier. When lipophilicity is too high for instance more than LogP value of 5, it results in low solubility, high toxicity, poor oral absorption and high metabolic turnover. Low lipophilicity leads to reduced efficacy and poor bioavailability. Drugs that pass the Lipinski's rule of 5 are more likely to pass the blood brain barrier as well. Therefore, all the compounds tested except the novel hybrid of diclofenac

and p-anisidine may pass the blood brain barrier. This is in congruence with prior research which showed that TMZ, aspirin, diclofenac, ibuprofen, naproxen, ketoprofen can pass through BBB [138-142]. Purines can pass through BBB via carrier proteins[143, 144].

Chemistry

Synthesis of TMZ-NSAIDs imides

There are various synthetic methods for synthesizing imides. They include acylation of amides with carboxylic acid derivatives, Mumm rearrangement of isoimides, oxidative decarboxylation of amino acids, oxygenation of amides and carbonylative coupling of aryl halides[102]. Based on the nature of our compounds, acylation of amides with carboxylic acid derivatives was the most feasible synthetic method. Acylation refers to the process of adding an acyl group to a compound. NSAIDs are carboxylic acids. Acyl group is therefore derived from the NSAIDs. The first step in our procedure involved a reaction between carboxylic acid (NSAIDs) and oxalyl chloride in presence of dimethylformamide (DMF). DMF acted as a catalyst and oxalyl chloride or thionyl chloride reacted with carboxylic acid to form acyl chloride.

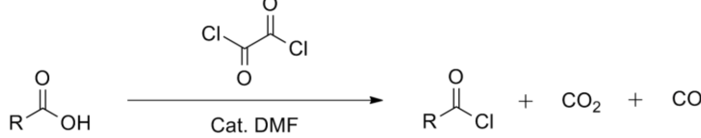


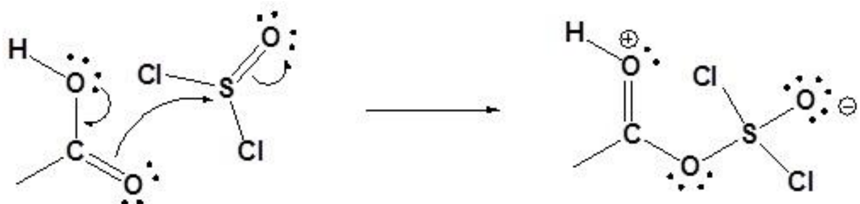
Figure 24: Conversion of a carboxylic acid to acyl chloride using oxalyl chloride and a DMF catalyst

$RCOOH \xrightarrow{SOCl_2} RCOCl + HCl + SO_2$

Figure 25: Conversion of a carboxylic acid to acyl chloride using thionyl chloride

Below is a mechanism for conversion of carboxylic acids to acid chlorides using thionyl chloride.

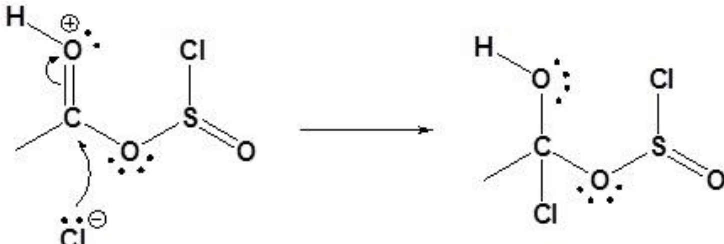
1. Nucleophilic attack on Thionyl Chloride



2. Removal of Cl leaving group

A Chlorosulfite

3. Nucleophilic attack on carbonyl



4. Removal of leaving group

5. Deprotonation

Figure 26: mechanism for conversion of carboxylic acids to acid chlorides using thionyl chloride.

Activated forms of carboxylic acids, like acid chlorides in our case, can be used in acylation of amides to form imides. Therefore, the second part of the reaction involved a reaction between acid chloride and TMZ (amide).

Figure 27:Acylation of amides with acid halides to form imides.

Ethyl acetate was used to extract the product (imide). Unfortunately, NMR and IR result showed that the imide was not successfully synthesized. More trials were done with different variations but none has been absolutely successful. Noteworthy, the first step that involved conversion of carboxylic acid to acid chloride was successful.

Synthesis of diclofenac-purine hybrids

Six amides were synthesized through a reaction between diclofenac and each of six amines which included p-anisidine, guanine, adenine, 2-chloro-7H-purine-6-amine, 2-fluoro-7H-purine-6-amine and 2-amino-6-chloropurine. The first step of this process was the conversion of diclofenac which is a carboxylic acid to acid chloride. DMF was used as a catalyst in the process.

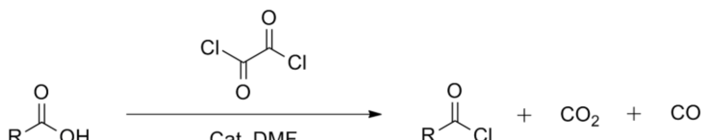


Figure 28: Conversion of a carboxylic acid to acyl chloride using oxalyl chloride and a DMF catalyst

The resultant acyl chloride was reacted with an amine to form the amide product.

Acid Chloride Amine Amide

Figure 29: Acyl chloride reaction with amine to form amide

Below is a reaction mechanism for this step.

1. Nucleophilic attack by the amine

2. Removal of leaving group

3. Deprotonation

Figure 30: Reaction mechanism for reaction of acyl chloride with amine

IR spectroscopy

Infrared spectroscopy (IR) is a measure of interactions of infrared radiation and matter. It is often used in analysis of chemical compounds to identify functional groups. In this research, amide and carboxylic acids were reacted with a goal to have a hybrid which is an imide. Amides used included 4-methoxybenzamide and TMZ. Aspirin was used as carboxylic acid. Imide N-H stretch, which falls between 3150 and 3250 was not present in all products. O-H carboxylic acid band at 3000–2500 cm^{-1} was expected in aspirin's IR spectra. This peak was observed at 2830 cm^{-1}. The N-H amide peak expected between 3100 - 3500 cm^{-1} was observed at 3158 cm^{-1}. TMZ doublet N-H peaks were also observed at 3328 and 3421 cm^{-1}.

In the second part of this research, diclofenac, a carboxylic acid, was reacted with purines which are primary amines to form novel hybrids which are amides. Carboxylic (O-H) peak was expected between 2800-3500 cm^{-1} range. Diclofenac had a peak at 3321 cm^{-1}. We expected two peaks for primary amines (N-H) within the range of 3000 and 3300 cm^{-1} range. All the primary amines had these two peaks. The product would therefore not have either the carboxylic acid peak or the primary amine peaks but have amide peak which was expected at 1630-1690 cm^{-1} range. All the products synthesized had an amide (C=O) peak within the expected range.

Biology

As mentioned earlier, GBM is the most common, most lethal and most aggressive primary malignant brain tumor[145]. It has a very low median survival rate of 15 months only and often poor prognosis[146]. The current standard care for GBM patients involves surgical resections which is followed by radiation and chemotherapy. TMZ is the first line chemotherapeutic drug. However, GBM develops resistance to TMZ hence limiting effectiveness

of the drug[147]. There are a number of mechanisms responsible for resistance observed. These mechanisms include DNA repair systems, O6-methylguanine-DNA methyltransferase repair mechanism, extracellular vesicle production, epigenetic modification mechanisms and autophagy. Coupled with these mechanisms is the fact that GBM is highly heterogeneous[148, 149]. Our main aim in this research was to find out if hybrids of TMZ and NSAIDs would overcome resistance and have a higher efficacy, i.e. more active than TMZ alone. Unfortunately, we have not yet successfully synthesized the hybrids as explained before. Instead of the hybrids, we used physical mixtures of TMZ and NSAIDS in the ratio of 1:1 and 1:2 respectively.

It was interesting to find out that diclofenac and oxaprozin were more active than TMZ. TMZ had an LC_{50} of 0.059 while diclofenac and oxaprozin had an LC_{50} of 0.0139 and 0.045 respectively. Research had been previously done to find out if diclofenac could be a potential drug against glioblastoma[150, 151]. In some cases, a combination of diclofenac and other compounds like metformin which is the most prescribed drug in treatment of type 2 diabetes was used[152]. Other research works focused on NSAIDs as anti-glioma agents[153]. Our findings are in congruence with these prior research works. Diclofenac specifically, could play a role as anti-glioblastoma agent.

It was also exciting to find out that LC_{50} values decreased in all the TMZ: NSAIDs mixtures of ratio 1:1. For example, LC_{50} for aspirin changed from 0.2021 to 0.0844. This could imply that hybrids with both TMZ and NSAIDs constituents could have higher efficacy compared to individual compounds.

In the case of TMZ: NSAIDs mixtures in the ratio 2:1 respectively, LC_{50} values changed towards the LC_{50} value of TMZ. LC_{50} value of diclofenac and Oxaprozin mixtures with TMZ increased while the LC_{50} value of the other NSAIDs mixtures with TMZ decreased. This was expected since the quantity of TMZ was double the quantity of the NSAIDs.

CHAPTER 5

CONCLUSION

In this research, we successfully synthesized novel hybrids of diclofenac and purine amines. This was after finding out that diclofenac had more interesting and exciting characteristics. There were observed changes in cell morphology and motility in cells treated with diclofenac and not in cells treated with either TMZ or the other NSAIDs. Moreover, diclofenac had the lowest LC_{50} value compared to all the other NSAIDs tested and TMZ. Though we were unsuccessful in synthesizing and confirming that we synthesized novel hybrids of TMZ and NSAIDs, we worked with TMZ and NSAIDs physical mixtures. We found out that TMZ: NSAIDs mixtures in the ratio 1:1 respectively, had lower LC_{50} values compared to LC_{50} values of individual compounds. In the case of TMZ: NSAIDs mixtures in the ratio 2:1 respectively, we found out that LC_{50} values changed towards the LC_{50} value of TMZ. This was expected, considering that TMZ was double the quantity of NSAIDs. Novel hybrid compounds of diclofenac and purines had LC_{50} values significantly lower than LC_{50} values of individual compounds. This suggests that hybrid compounds are more effective than individual compounds. Future work includes confirming LC_{50} values, synthesizing the novel hybrids of TMZ and NSAIDs and finding out their LC_{50} values. Two main research questions brought up by this research include: what is the mechanism responsible for change in motility of cells observed in

diclofenac and are there any proteins upregulated or downregulated? Would GBM develop resistance against hybrids?

CHAPTER 6

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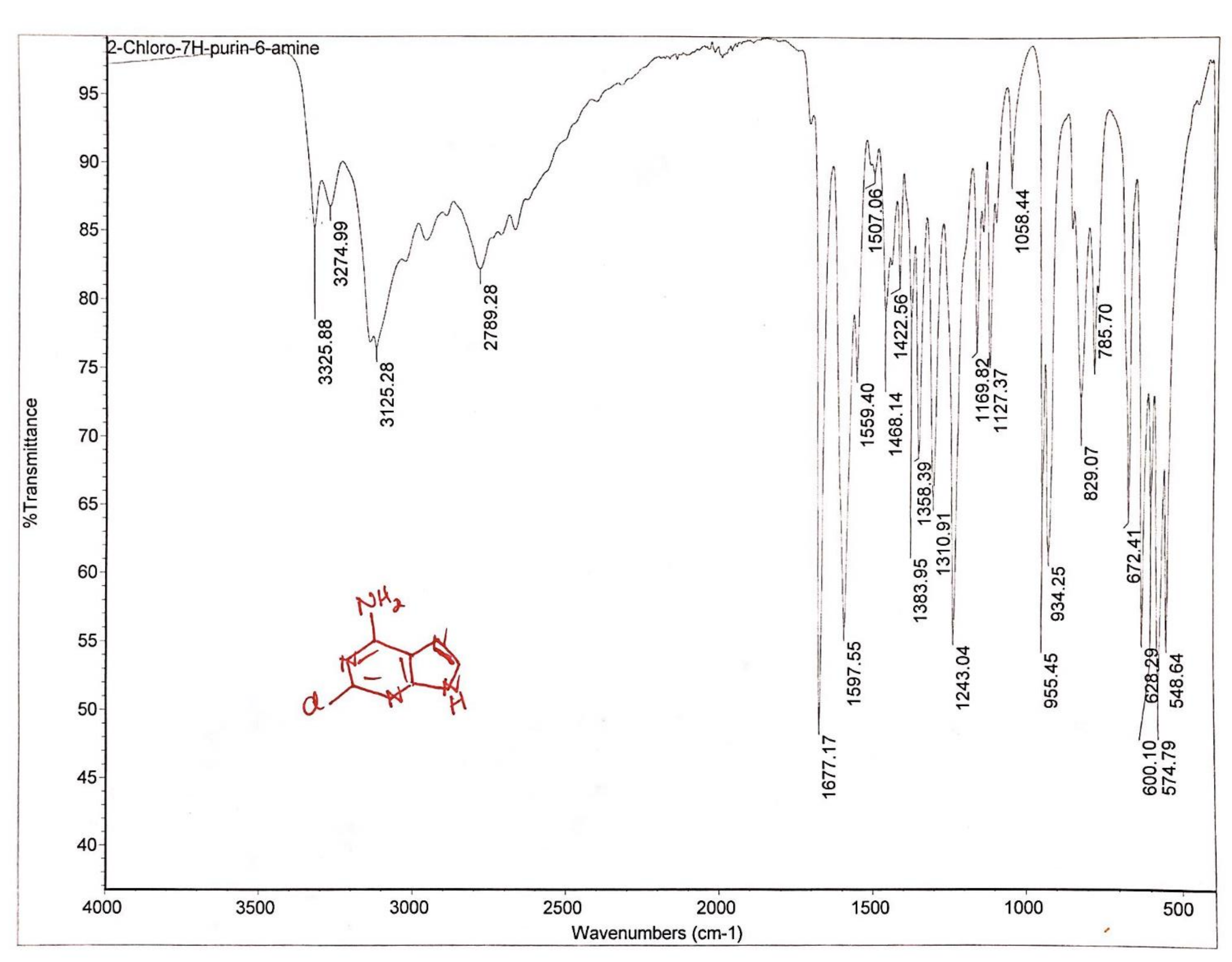
APPENDIX

IR SPECTRA



2-Fluoro-7H-purin-6-amine

%Transmittance

Wavenumbers (cm-1)

3289.38
3117.84
2988.33
2785.90
2701.61
1691.47
1606.68
1525.67
1485.35
1430.24
1367.62
1278.27
1244.27
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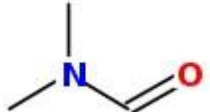

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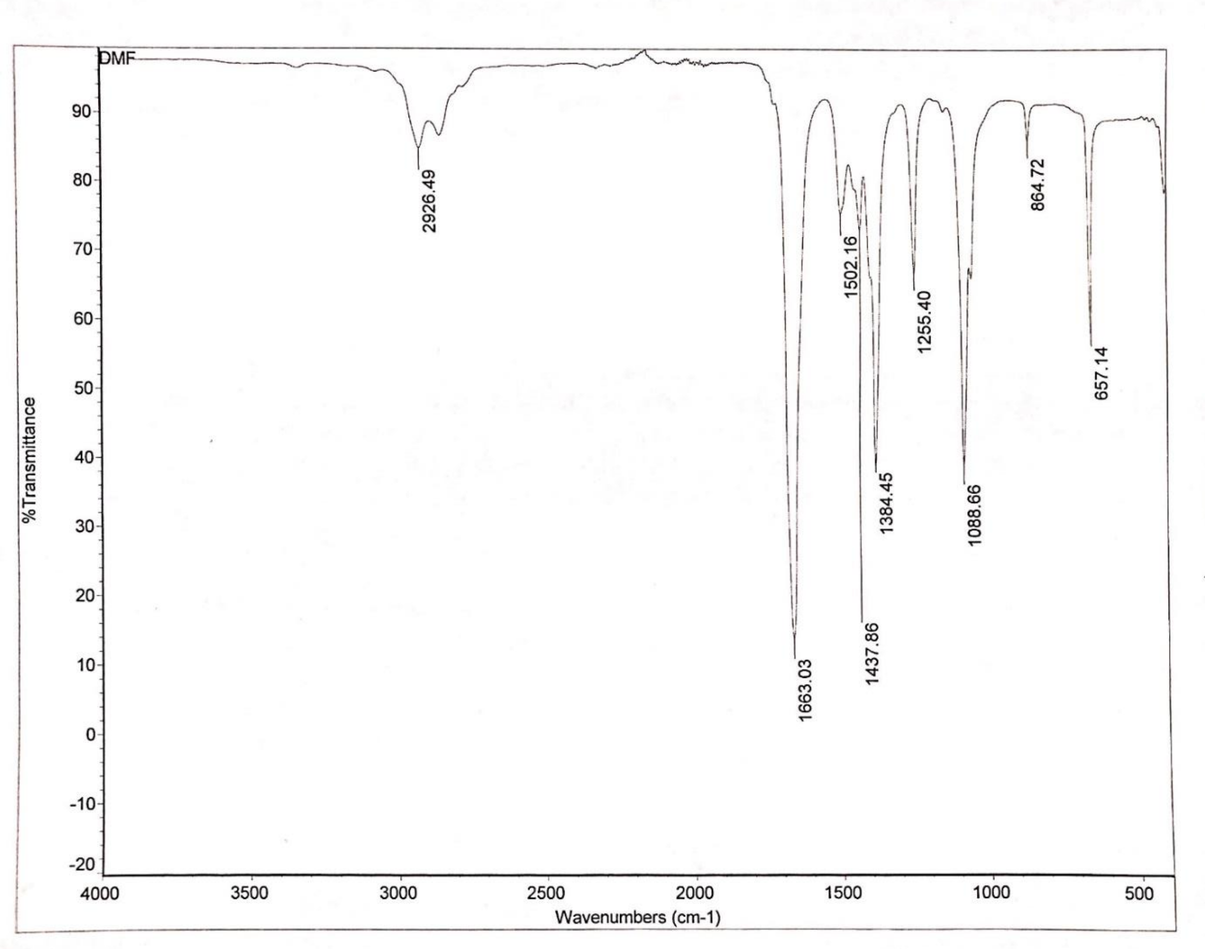

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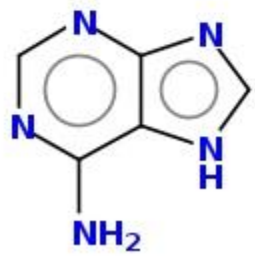

Adenine - JR

%Transmittance

95
90
85
80
75
70
65
60
55
50
45
40
35
30

3286.81
3103.58
2973.11
2689.37
1670.69
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1450.19
1417.29
1366.62
1332.55
1306.31
1250.27
1156.43
1123.92
1023.05
937.78
910.45
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721.16
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617.43
541.25

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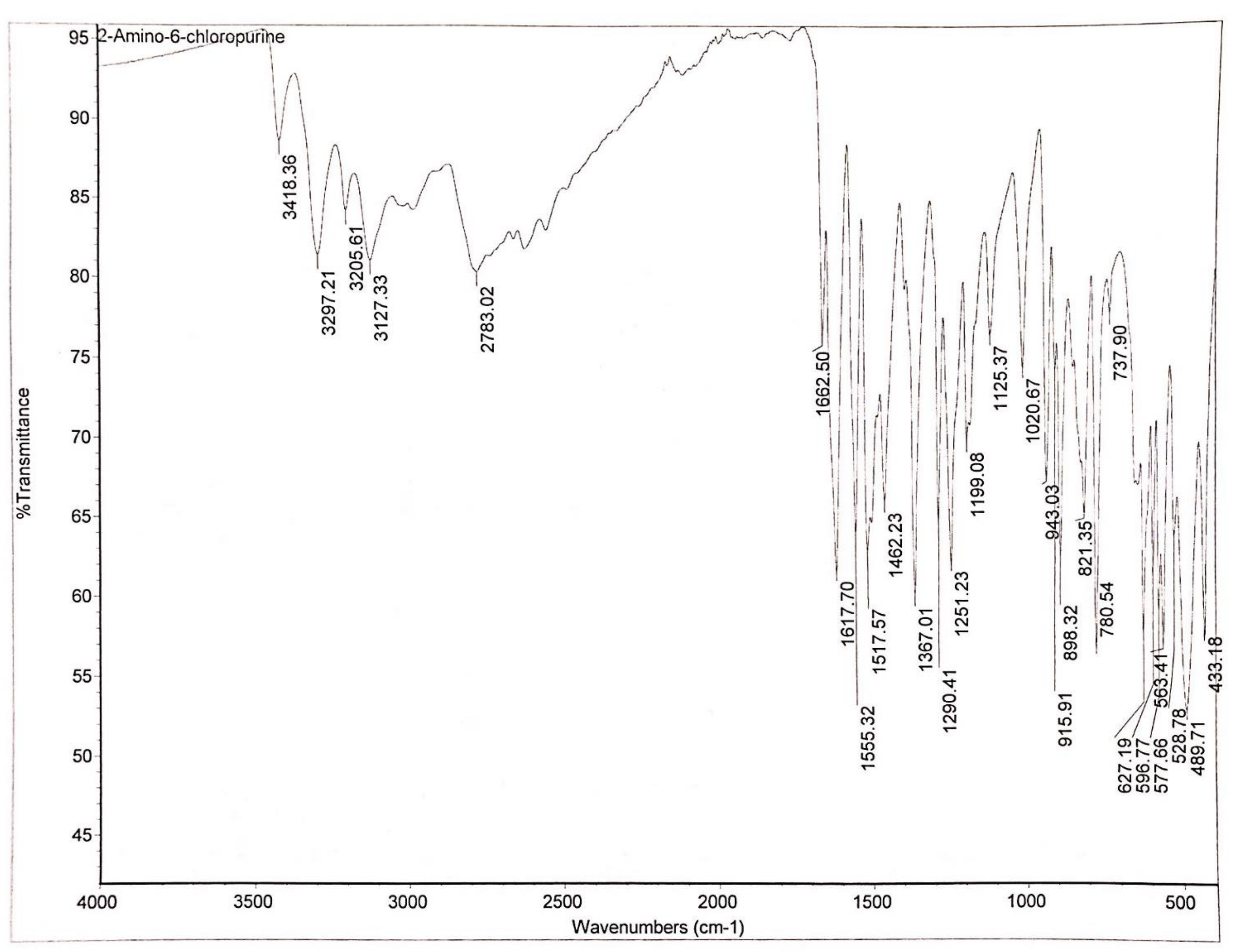
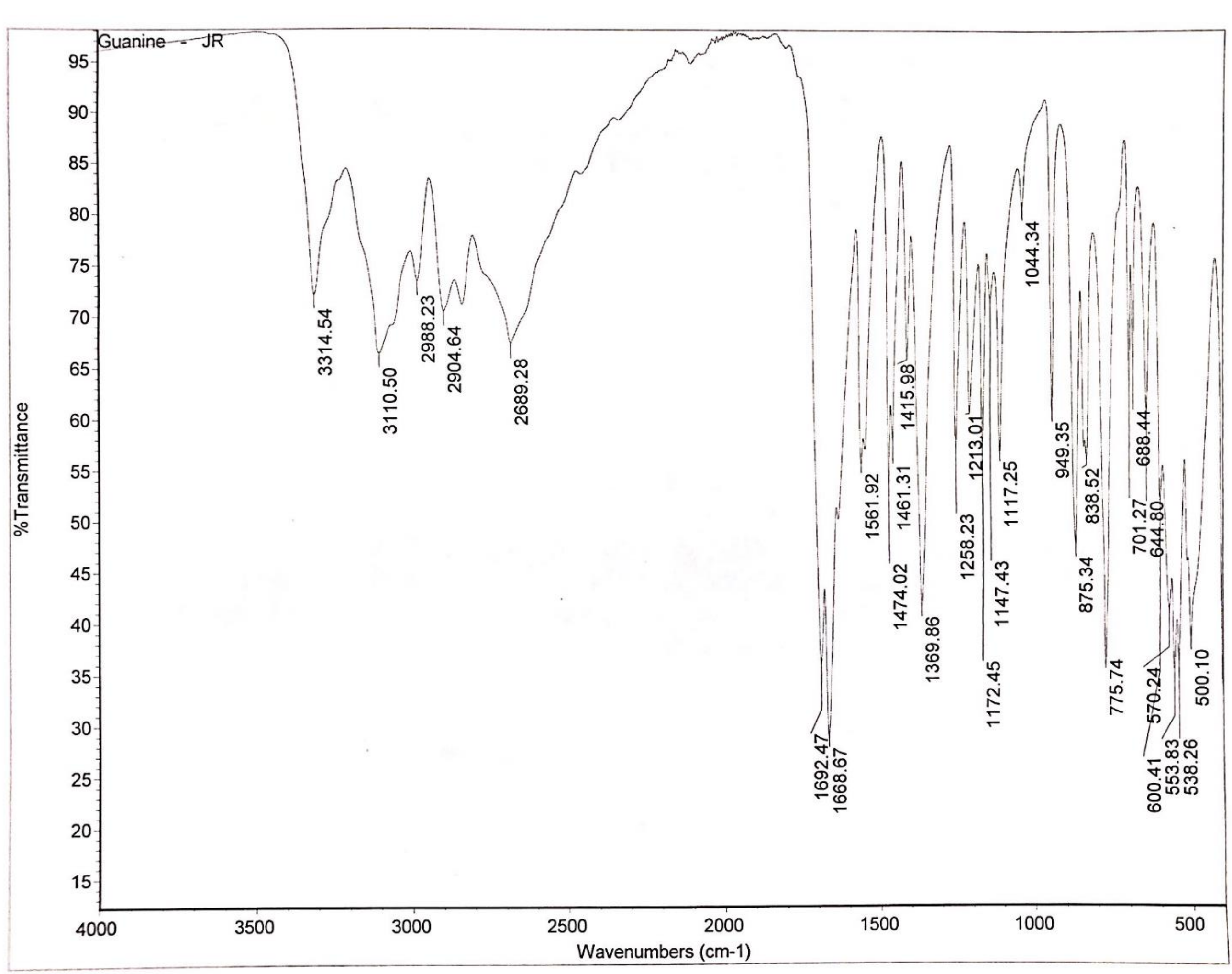
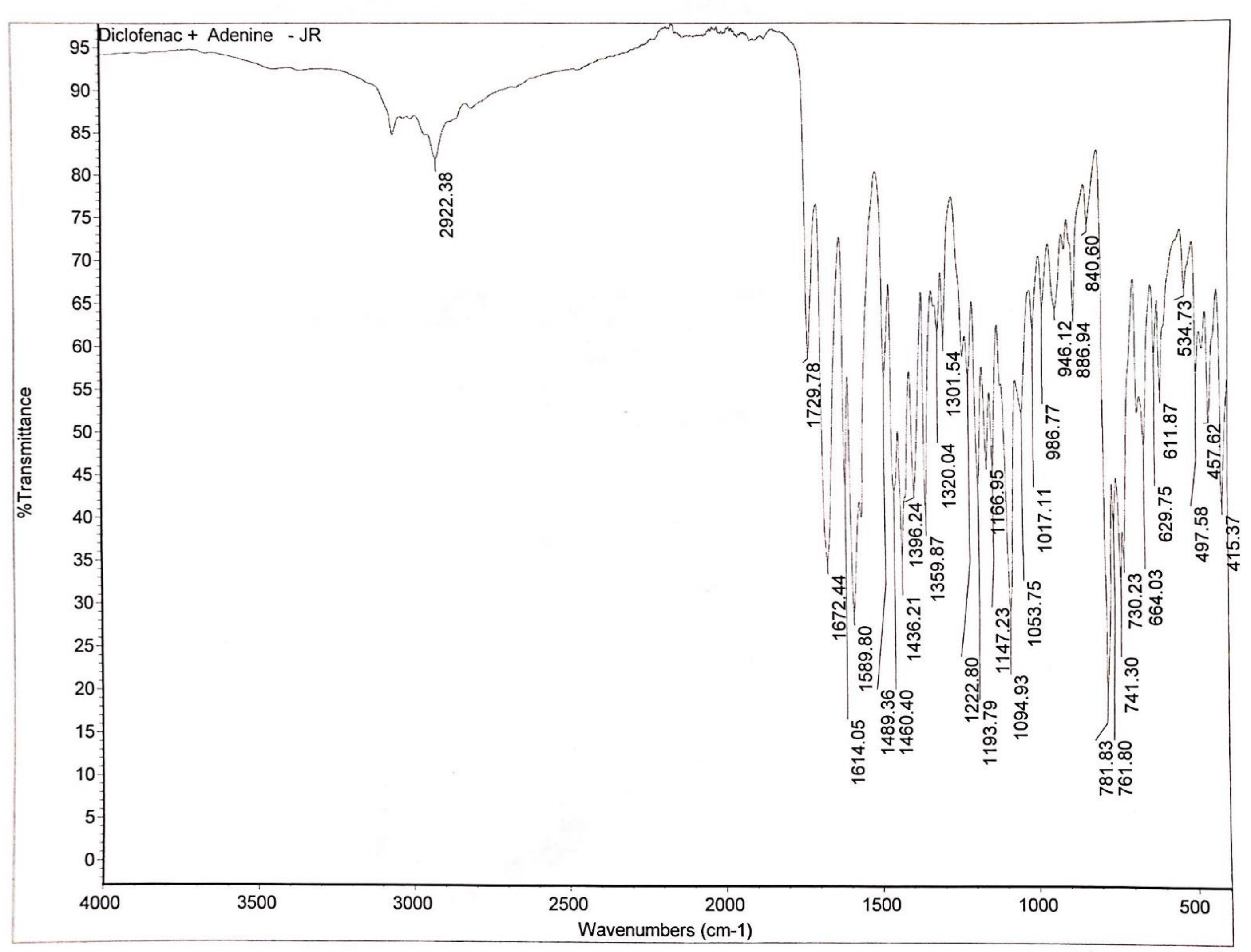
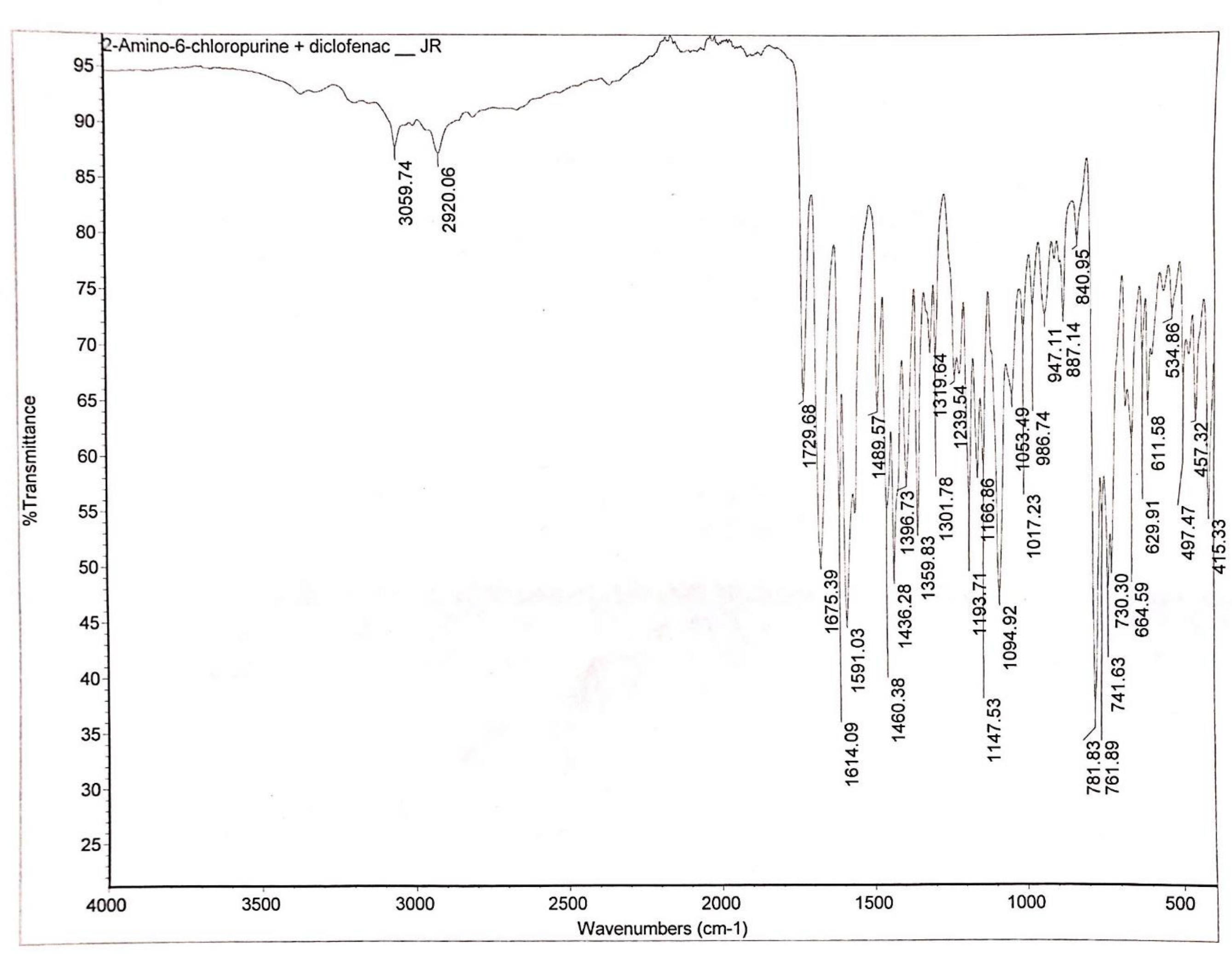
Wavenumbers (cm-1)


2-Amino-6-chloropurine
%Transmittance
Wavenumbers (cm-1)
3418.36
3297.21
3205.61
3127.33
2783.02
1662.50
1617.70
1555.32
1517.57
1462.23
1367.01
1290.41
1251.23
1199.08
1125.37
1020.67
943.03
915.91
898.32
821.35
780.54
737.90
627.19
596.77
577.66
563.41
528.78
489.71
433.18


Guanine - JR
%Transmittance
Wavenumbers (cm-1)
3314.54
3110.50
2988.23
2904.64
2689.28
1692.47
1668.67
1561.92
1474.02
1461.31
1415.98
1369.86
1258.23
1213.01
1172.45
1147.43
1117.25
1044.34
949.35
875.34
838.52
775.74
701.27
688.44
644.80
600.41
570.24
553.83
538.26
500.10



Diclofenac + Adenine - JR
%Transmittance
Wavenumbers (cm-1)
2922.38
1729.78
1672.44
1614.05
1589.80
1489.36
1460.40
1436.21
1396.24
1359.87
1320.04
1301.54
1222.80
1193.79
1166.95
1147.23
1094.93
1053.75
1017.11
986.77
946.12
886.94
840.60
781.83
761.80
741.30
730.23
664.03
629.75
611.87
534.73
497.58
457.62
415.37


2-Amino-6-chloropurine + diclofenac __ JR
%Transmittance
Wavenumbers (cm-1)
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
4000
3500
3000
2500
2000
1500
1000
500
3059.74
2920.06
1729.68
1675.39
1614.09
1591.03
1489.57
1460.38
1436.28
1396.73
1359.83
1319.64
1301.78
1239.54
1193.71
1166.86
1147.53
1094.92
1053.49
1017.23
986.74
947.11
887.14
840.95
781.83
761.89
741.63
730.30
664.59
629.91
611.58
534.86
497.47
457.32
415.33

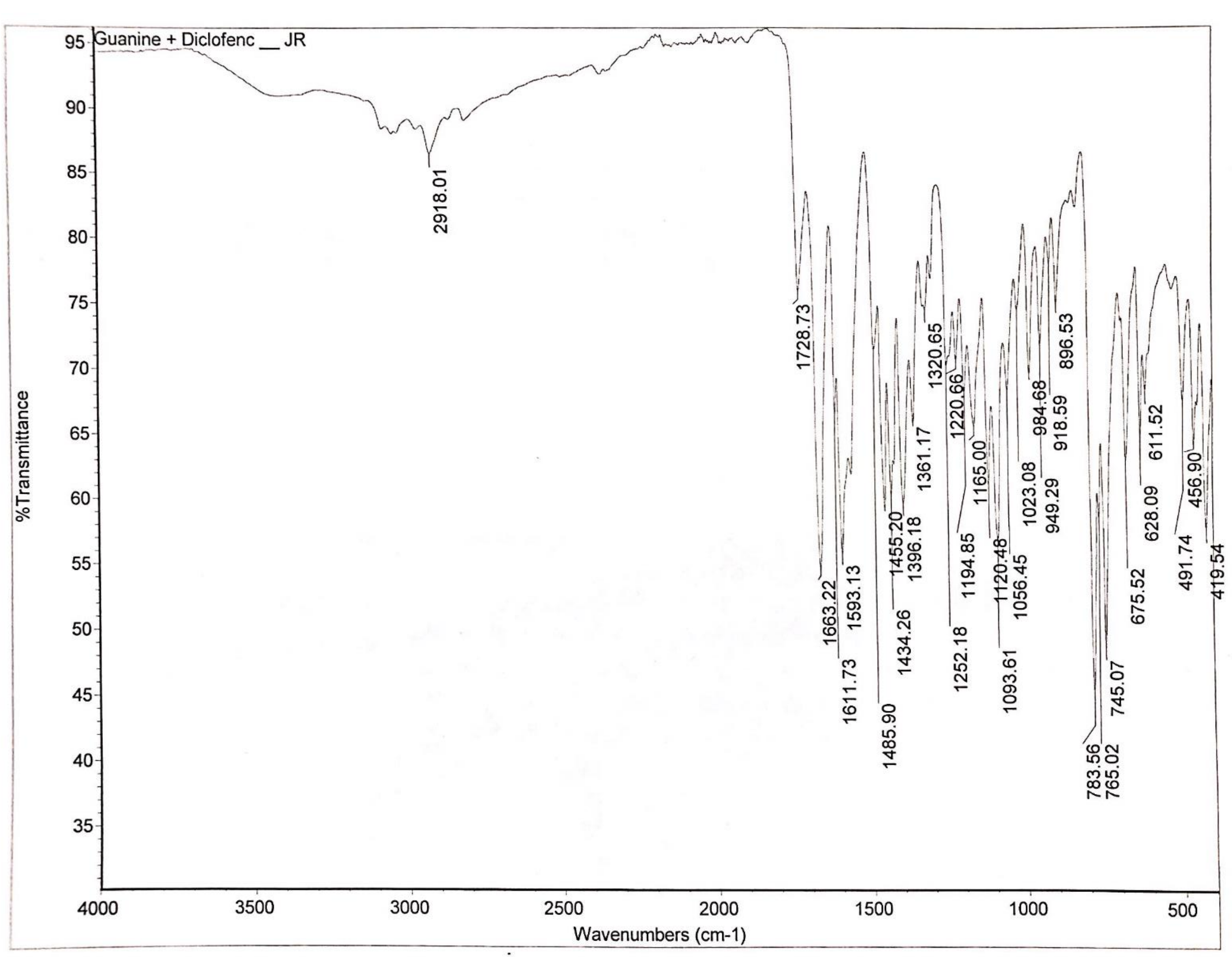
P-anisidine __JR

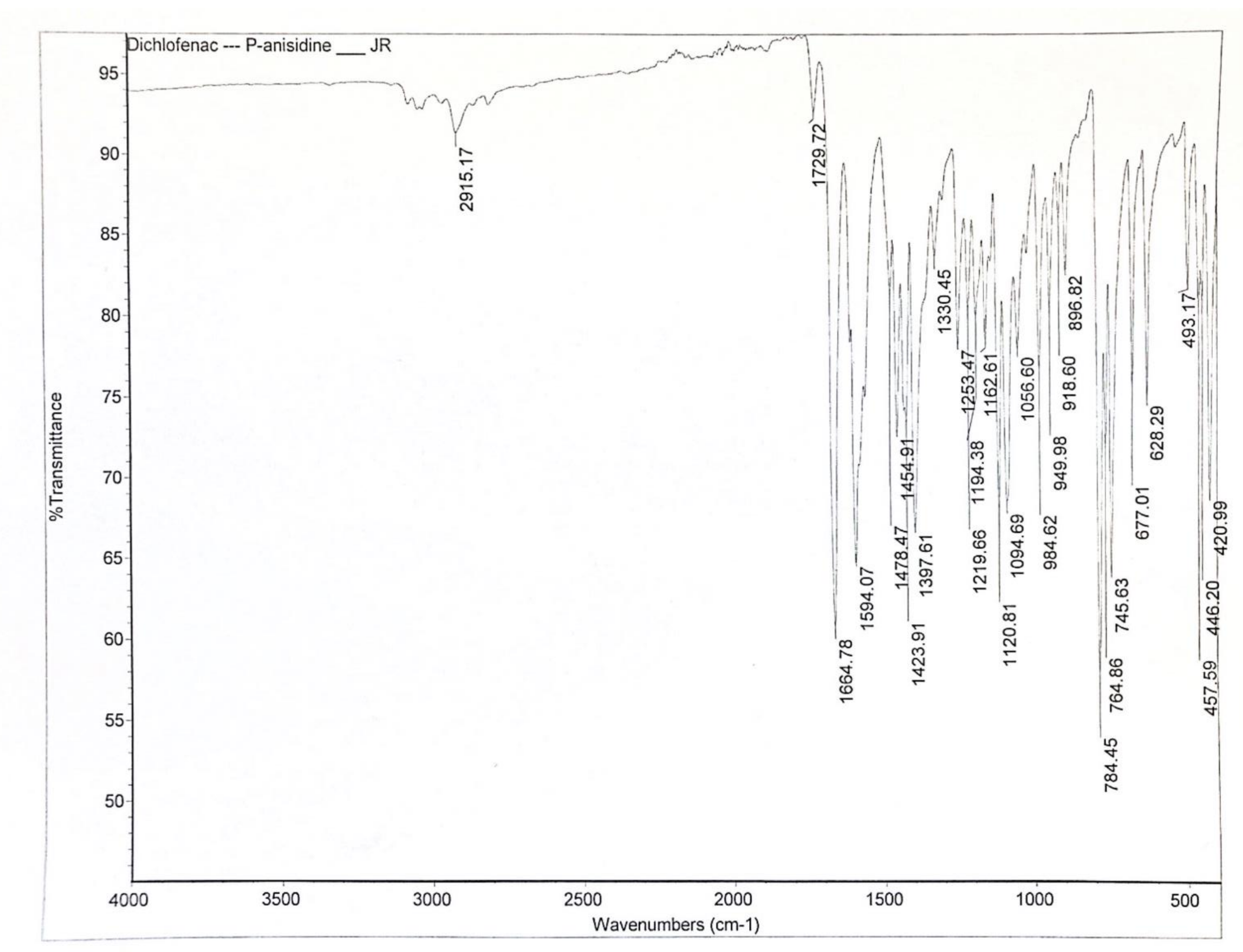
%Transmittance

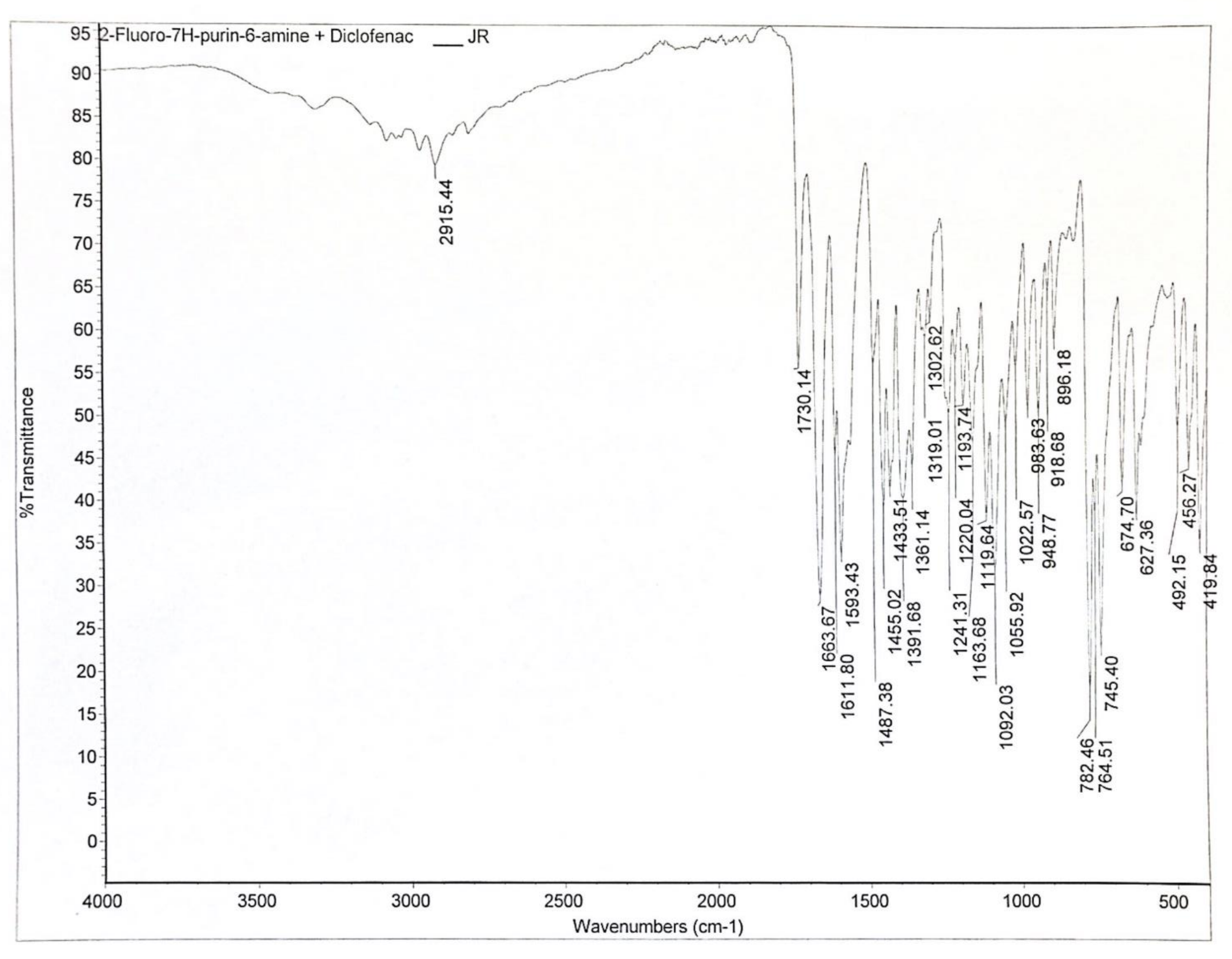
Wavenumbers (cm-1)

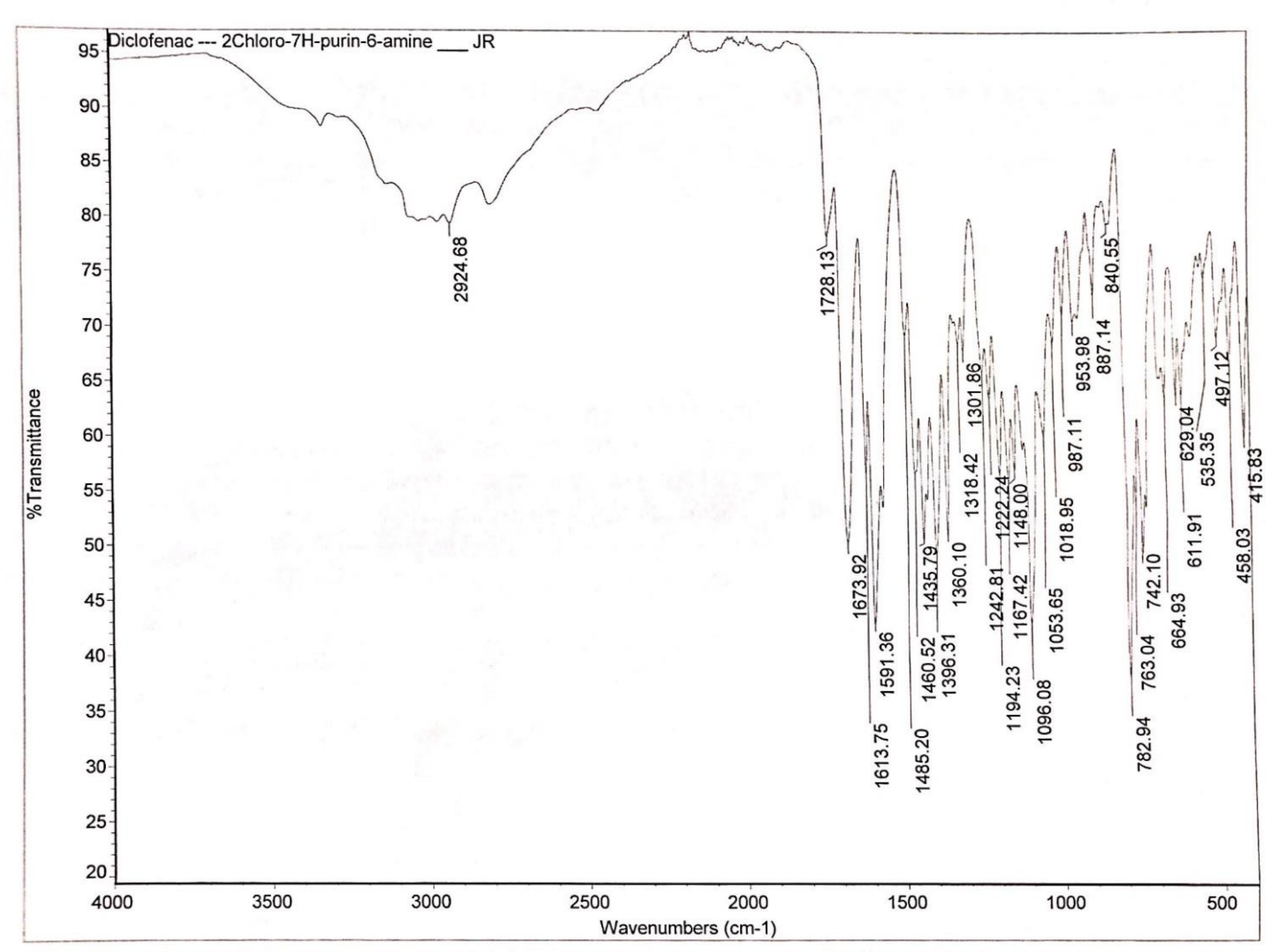
Peak labels: 3421.10, 3344.84, 3219.98, 3006.71, 2964.21, 2838.73, 1859.71, 1629.43, 1502.36, 1463.28, 1439.61, 1333.61, 1297.35, 1273.31, 1229.90, 1176.65, 1128.82, 1029.69, 918.45, 824.01, 728.36, 708.78, 590.94, 514.58, 413.88

CH_3O–C6H4–NH_2

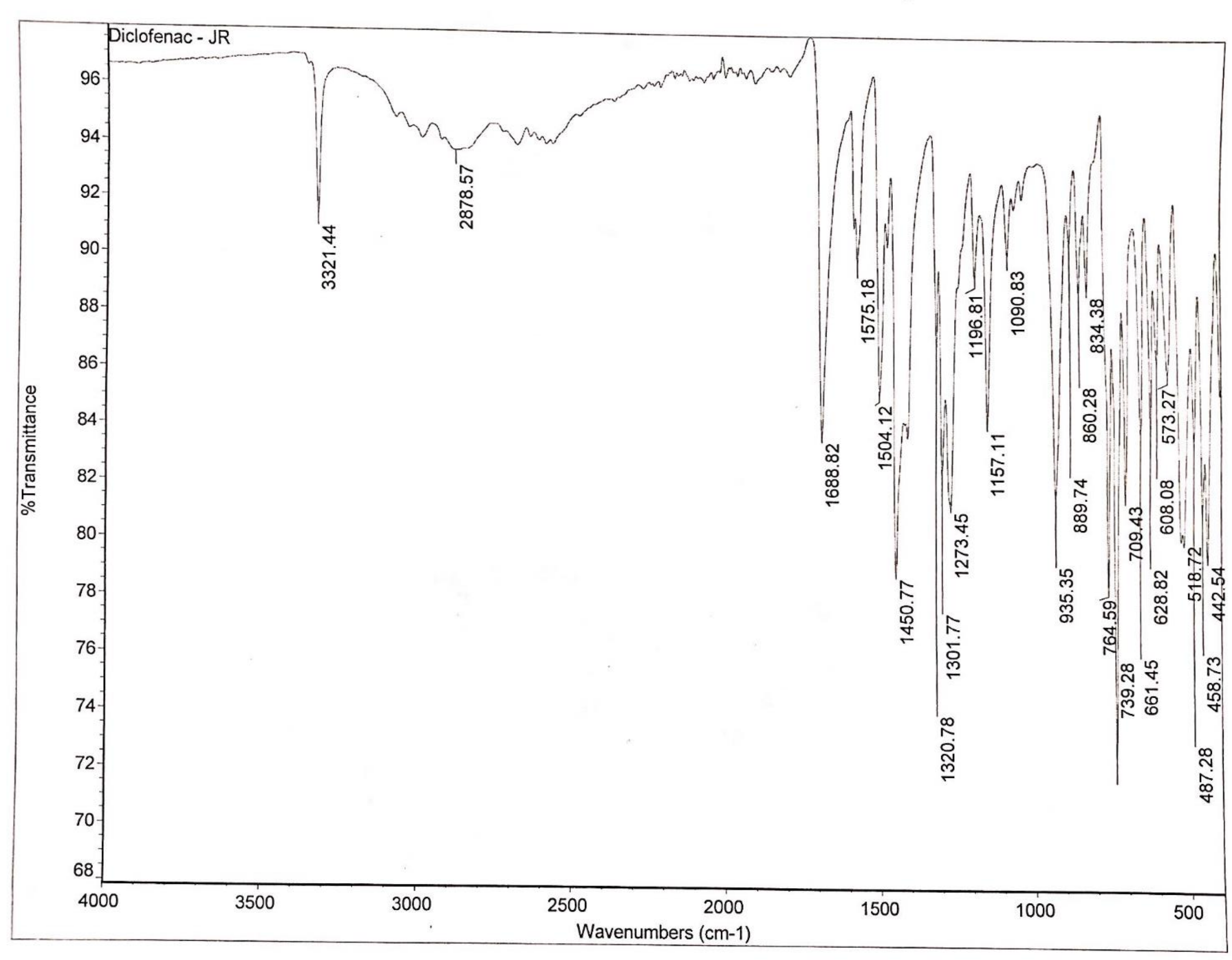

Guanine + Diclofenc __ JR
%Transmittance
Wavenumbers (cm-1)
95
90
85
80
75
70
65
60
55
50
45
40
35
4000
3500
3000
2500
2000
1500
1000
500
2918.01
1728.73
1663.22
1611.73
1593.13
1485.90
1455.20
1434.26
1396.18
1361.17
1320.65
1252.18
1220.66
1194.85
1165.00
1120.48
1093.61
1056.45
1023.08
984.68
949.29
918.59
896.53
783.56
765.02
745.07
675.52
628.09
611.52
491.74
456.90
419.54


Dichlofenac --- P-anisidine ___ JR
%Transmittance
Wavenumbers (cm-1)
2915.17
1729.72
1664.78
1594.07
1478.47
1454.91
1423.91
1397.61
1330.45
1253.47
1219.66
1194.38
1162.61
1120.81
1094.69
1056.60
984.62
949.98
918.60
896.82
784.45
764.86
745.63
677.01
628.29
493.17
457.59
446.20
420.99

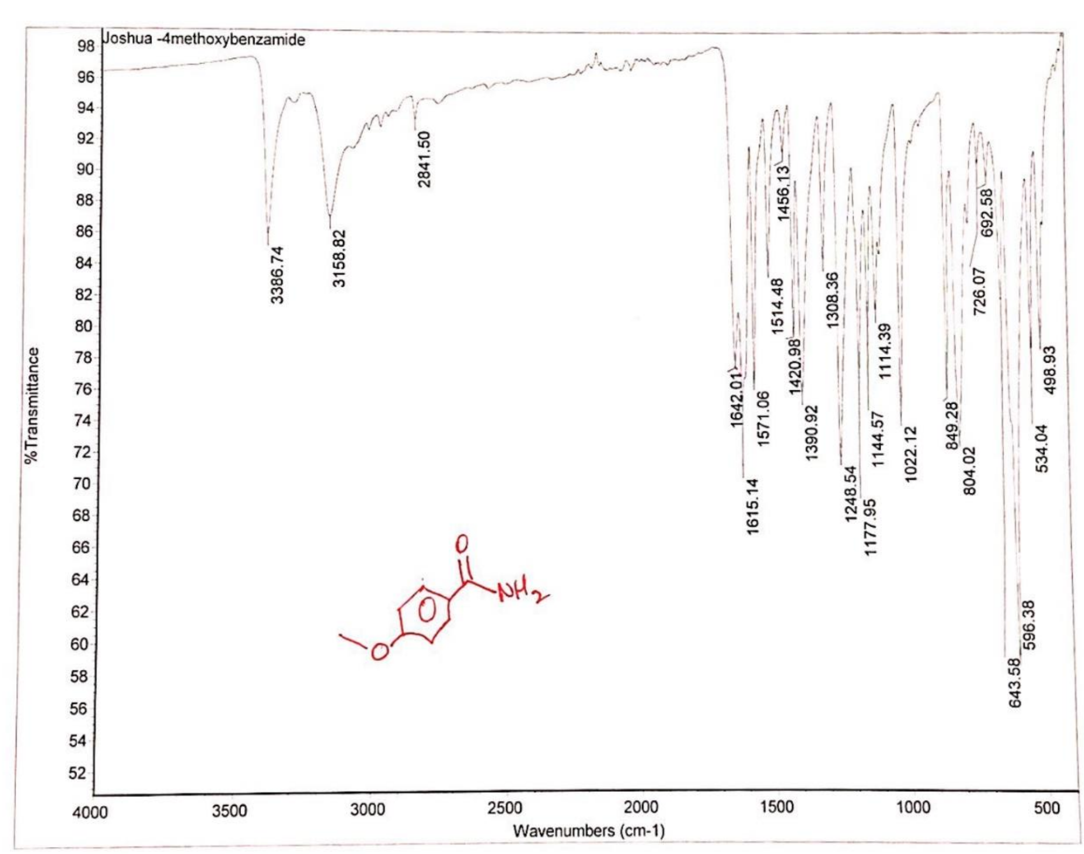

2-Fluoro-7H-purin-6-amine + Diclofenac ___ JR
%Transmittance
Wavenumbers (cm-1)
2915.44
1730.14
1663.67
1611.80
1593.43
1487.38
1455.02
1433.51
1391.68
1361.14
1319.01
1302.62
1241.31
1220.04
1193.74
1163.68
1119.64
1092.03
1055.92
1022.57
983.63
948.77
918.68
896.18
782.46
764.51
745.40
674.70
627.36
492.15
456.27
419.84

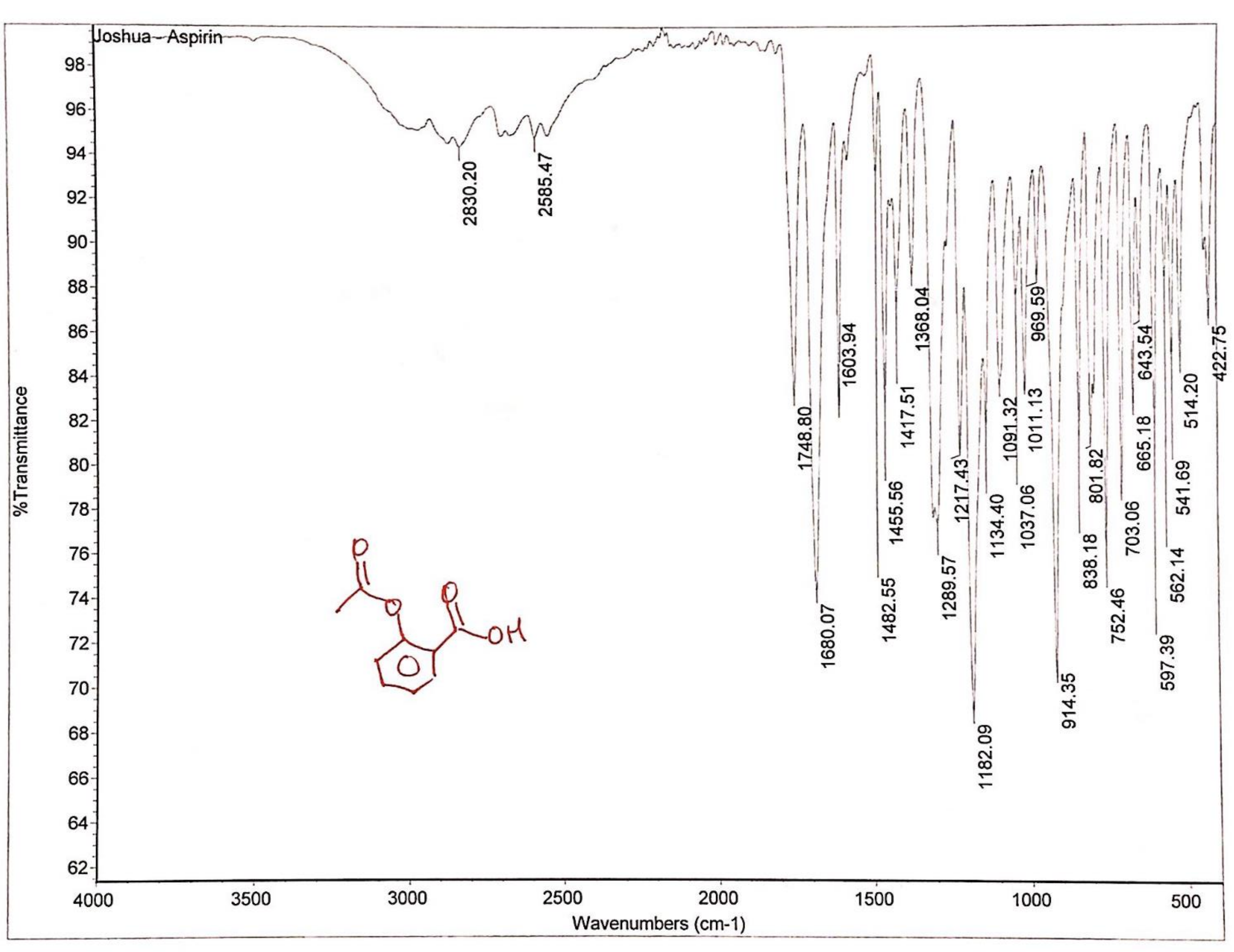


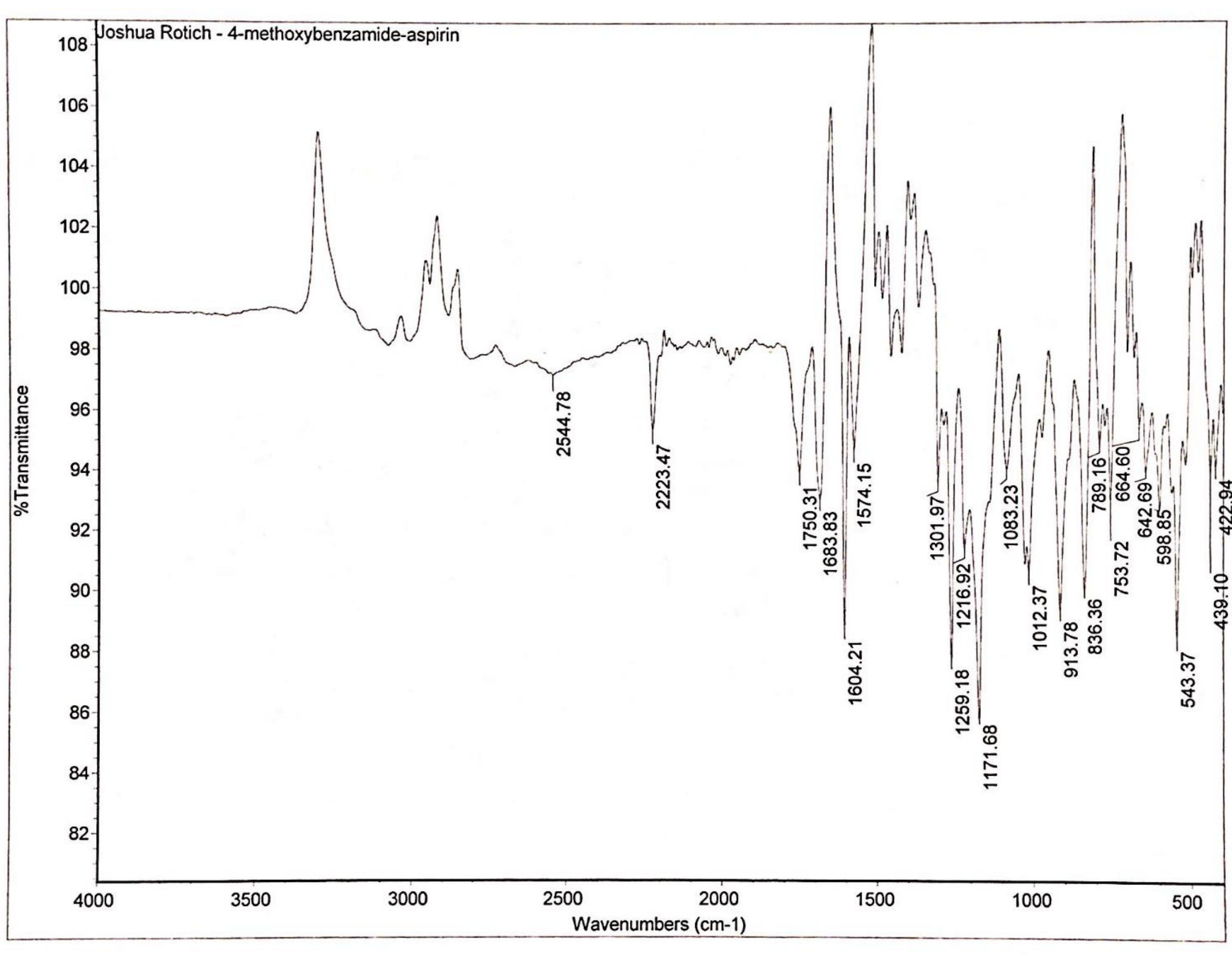
Diclofenac --- 2Chloro-7H-purin-6-amine ___ JR
%Transmittance
Wavenumbers (cm-1)
2924.68
1728.13
1673.92
1613.75
1591.36
1485.20
1460.52
1435.79
1396.31
1360.10
1318.42
1301.86
1242.81
1222.24
1194.23
1167.42
1148.00
1096.08
1053.65
1018.95
987.11
953.98
887.14
840.55
782.94
763.04
742.10
664.93
629.04
611.91
535.35
497.12
458.03
415.83

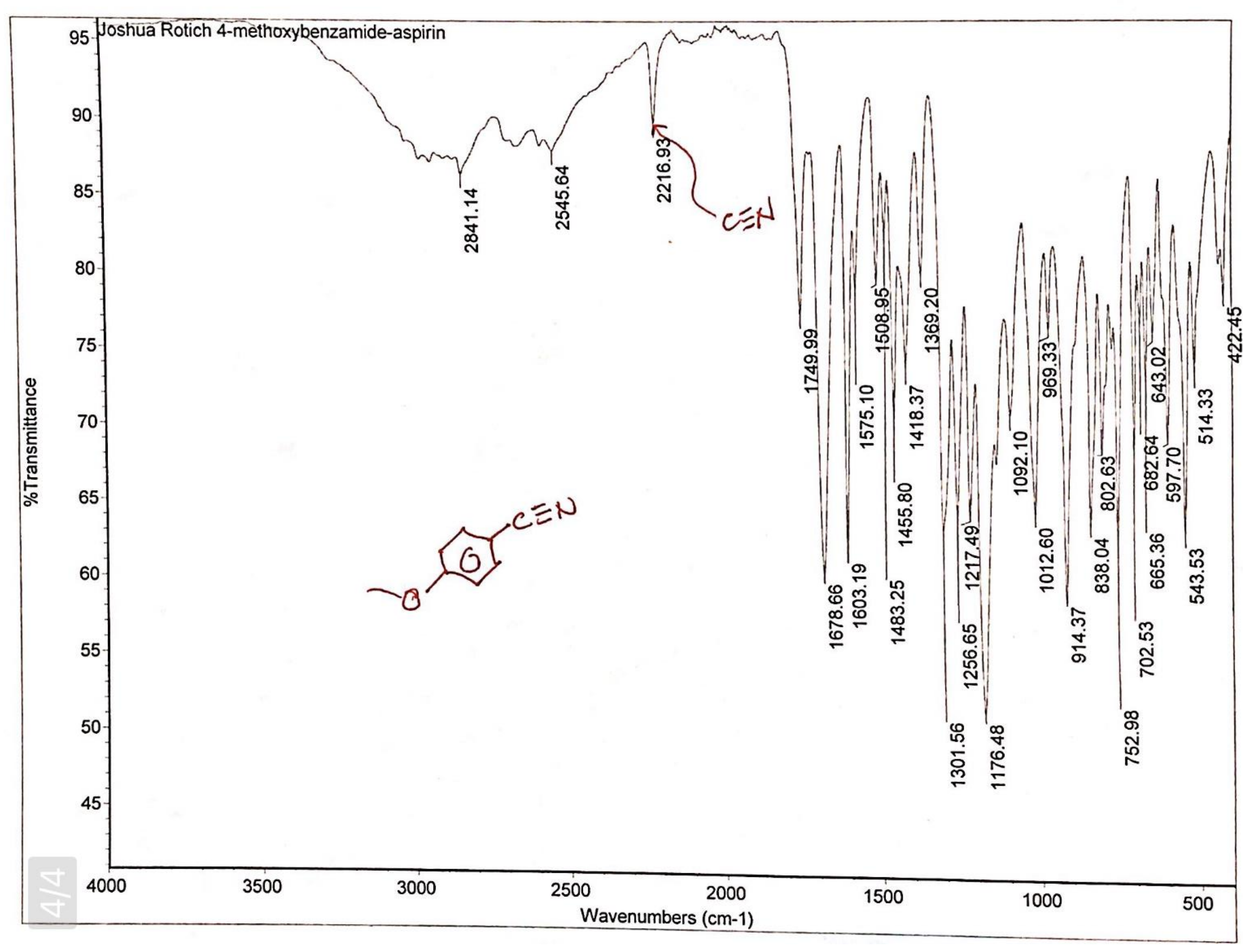


Diclofenac - JR
%Transmittance
Wavenumbers (cm-1)
3321.44
2878.57
1688.82
1575.18
1504.12
1450.77
1320.78
1301.77
1273.45
1196.81
1157.11
1090.83
935.35
889.74
860.28
834.38
764.59
739.28
709.43
661.45
628.82
608.08
573.27
518.72
487.28
458.73
442.54


Joshua -4methoxybenzamide
%Transmittance
Wavenumbers (cm-1)
3386.74
3158.82
2841.50
1642.01
1615.14
1571.06
1514.48
1456.13
1420.98
1390.92
1308.36
1248.54
1177.95
1144.57
1114.39
1022.12
849.28
804.02
726.07
692.58
643.58
596.38
534.04
498.93
NH2


Joshua - Aspirin
%Transmittance
Wavenumbers (cm-1)
2830.20
2585.47
1748.80
1680.07
1603.94
1482.55
1455.56
1417.51
1368.04
1289.57
1217.43
1182.09
1134.40
1091.32
1037.06
1011.13
969.59
914.35
838.18
801.82
752.46
703.06
665.18
643.54
597.39
562.14
541.69
514.20
422.75
OH


Joshua Rotich - 4-methoxybenzamide-aspirin
%Transmittance
Wavenumbers (cm-1)
108
106
104
102
100
98
96
94
92
90
88
86
84
82
4000
3500
3000
2500
2000
1500
1000
500
2544.78
2223.47
1750.31
1683.83
1604.21
1574.15
1301.97
1259.18
1216.92
1171.68
1083.23
1012.37
913.78
836.36
789.16
753.72
664.60
642.69
598.85
543.37
439.10
422.94


Joshua Rotich 4-methoxybenzamide-aspirin
%Transmittance
Wavenumbers (cm-1)
2841.14
2545.64
2216.93
C≡N
1749.99
1678.66
1603.19
1575.10
1508.95
1483.25
1455.80
1418.37
1369.20
1301.56
1256.65
1217.49
1176.48
1092.10
1012.60
969.33
914.37
838.04
802.63
752.98
702.53
682.64
665.36
643.02
597.70
543.53
514.33
422.45
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