IMPROVING DELIVERY OF ELACRIDAR TO ENHANCE Efficacy of MolEcularLy-targeted aGEnts in treatment of glioblastoma

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Dedication

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Abstract

Treatment of glioblastoma with new molecularly-targeted agents has been largely ineffective in clinical trials. Many of these molecularly-targeted agents are substrates for the efflux transporters P-gp and BCRP, and therefore, one of the reasons for the lack of efficacy could be the limitations to drug delivery to the target site. P-gp and BCRP are efflux transporters that are expressed at the blood-brain barrier, which acts as a protective mechanism and prevents chemotherapeutics from reaching the brain parenchyma. P-gp and BCRP are also expressed at the tumor cell surface. These two sequential barriers could restrict the access of chemotherapeutics to the target site and therefore could reduce their efficacy. The main objective of this work was to overcome these sequential barriers by use of a pharmacological inhibitor of P-gp and BCRP, elacridar. The ultimate aim was to develop a chronic dosage regimen of a molecularly-targeted agent with elacridar as an adjuvant to enhance drug delivery to the brain in preclinical models of glioma.

We demonstrated that the bioavailability of elacridar is limited due to its poor physicochemical properties. We also showed that the distribution of elacridar into the brain is limited by the presence of P-gp and BCRP and is governed by a saturable efflux process that can be overcome by increasing the dose of elacridar. We developed a microemulsion formulation of elacridar that improved its bioavailability several-fold and allowed us to decrease the dose of elacridar required to show an inhibitory effect. We examined the effect of elacridar as an adjuvant to erlotinib in treatment of tumor-bearing mice. It was found that while the co-administration of elacridar definitely improved the
brain distribution of erlotinib, it did not offer any advantage in improving overall survival of the tumor bearing animals.

These observations show that improving the distribution of a single molecularly targeted agent may not be sufficient in order to effectively target a heterogenous tumor such as glioma. To effectively treat an aggressive disease such as glioblastoma, a combination of drugs that target a number of growth pathways; combined with a pharmacological inhibitor of transporters could help formulate an effective strategy to target tumor cells.
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CHAPTER I:

ENHANCING DRUG DISTRIBUTION TO THE BRAIN TO IMPROVE EFFICACY OF MOLECULARLY-TARGETED AGENTS IN TREATMENT OF GLIOBLASTOMA
Glioblastoma (GBM) is one of the most aggressive cancers with poor prognosis, and poor survival rates. Survival rates remain poor in GBM despite advances in radiotherapy, chemotherapy and surgical techniques. One of the reasons for poor response to chemotherapy in glioma is the barriers to drug delivery posed by the blood-brain barrier and the brain-tumor barrier. An additional problem in the effective treatment of glioma is the recurrence of the disease despite aggressive treatment. In this chapter, we examine the role played by the efflux transporters P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) in limiting the efficacy of chemotherapy, and the difficulties in treating GBM as a whole-brain disease. The difficulties in treatment of glioma arise from the poor distribution of molecularly targeted agents to the invasive tumor cells that are protected by an intact BBB. The limitations to drug delivery by P-gp and BCRP can be overcome by use of pharmacological inhibitors. Elacridar is a dual inhibitor of P-gp and BCRP that has been shown to be effective in enhancing delivery of molecularly-targeted agents. The difficulties in developing a chronic dosage formulation of elacridar can be overcome by developing a microemulsion formulation of elacridar.
1.1 Introduction:

An estimated 24,620 new cases of primary malignant brain and CNS system tumors are expected to be diagnosed in the United States in 2013 (Dolecek, 2012). Glioblastoma (GBM) is a Grade IV astrocytoma as classified by the World Health Organization (WHO) (Louis et al., 2007). The WHO grade IV is assigned to lesions that are malignant, necrotic, rapidly proliferating tumors that result in death. The treatment for glioblastoma is aggressive comprises of surgical resection followed by radiation therapy and chemotherapy, and while the improvement in overall survival have been significant, the expected survival of a patient detected with GBM is only about 12-15 months post diagnosis (Stupp et al., 2005). Therefore there remains scope for improvement in the the treatment paradigm for GBM. Several molecularly targeted therapies along with anti-angiogenic therapies have been examined for use in clinical trials of GBM (Rich et al., 2004; Razis et al., 2009; Thiessen et al., 2010; Reardon et al., 2011; Uhm et al., 2011; De Bonis et al., 2012; Kesavabhotla et al., 2012; Kreisl et al., 2012). However, none of the treatment regimens have offered a significant improvement in overall survival.

A major issue in treatment of glioblastoma and other brain metastases to the brain is the barrier to delivery of targeted agents. The blood-brain barrier (BBB) is a physiological barrier that prevents the distribution of chemotherapeutics to the central nervous system. Transport proteins at the BBB actively efflux drugs out of the brain, and thus limit the exposure of tumor cells to drugs. In addition to this, the tumor cells themselves have been known to express transport proteins. Thus, the drug needs to overcome sequential barriers in order to reach the target site. The efficacy of many chemotherapeutics is therefore
hindered by their limited ability to reach the site of action. This chapter looks at the possible mechanisms for limited efficacy of chemotherapeutics in treating GBM and brain metastases, including the barriers that limit drug distribution, and resistance to drug therapy. We also examine possible mechanisms of recurrence of GBM and brain metastases, and a possible role of pharmacological inhibitors in improving drug delivery across the blood-brain barrier and at the tumor cells.

1.2. Brain tumors:

1.2.1 Glioblastoma

Brain tumors can be classified into primary and secondary brain tumors based on their origin. Primary brain tumors start “de novo”, without any evidence of a precursor tumor and can be either benign (non-malignant) or malignant tumors. Benign tumors have cells that look relatively normal and grow slowly and do not metastasize (Ohgaki and Kleihues, 2007). More than 50% of all primary brain tumors are non-malignant (Dolecek, 2012). Despite their name, non-malignant tumors are not entirely harmless and can cause seizures, hemorrhage and increased pressure in the brain due to mass of tumor (Piepmeier and Baehring, 2004).

Primary malignant brain tumors are tumors that originate in the brain itself, however, they have the capacity to metastasize to the rest of the brain tissue and grow rapidly (Ohgaki et al., 2004). Gliomas account for about 80% of all malignant primary tumors. Gliomas are tumors that arise from glial cells, and include astrocytoma, glioblastoma, oligodendrogloma, ependymoma, mixed glioma, malignant glioma NOS, and a few more rare histologies (Dolecek, 2012). Gliomas have been classified by the World Health
Organization (WHO) into 4 grades based on their malignancy and prognosis. Grade I and II tumors are slow growing, least malignant type of tumors, while grade III and IV tumors are highly malignant and associated with a poor prognosis (Louis et al., 2007). Glioblastoma Multiforme is a grade IV astrocytoma that is characterized by malignant, necrotic, infiltrative, rapidly proliferating tumors that result in death. The median survival time of patients with glioblastoma multiforme is about 14 months despite aggressive treatment including surgical resection, radiation therapy and chemotherapy (Stupp et al., 2005). The difficulty in treating glioblastomas arises due to the presence of the blood brain barrier, heterogeneity of the tumors and invasiveness of tumors (Van Meir et al., 2010) and recurrence even after completion of treatment. Glioblastoma cells are highly invasive and have the capacity to move away from tumor site and then give rise to secondary tumors (Molina et al., 2010); therefore surgical debulking is only partially effective in slowing down the progression of the disease. The treatment of glioma is made challenging due to these properties of tumor cells.

1.2.2 Metastases

Metastatic brain tumors are secondary tumors that originate elsewhere in the body or in the brain. Secondary glioblastomas may originate from tumors elsewhere in the body like lungs, breast or skin, or a progression from a low grade astrocytoma (Ohgaki and Kleihues, 2007). Only about 10% of all glioblastomas are secondary tumors (Ohgaki and Kleihues, 2007). The most common primary tumors that result in brain metastasis are lung, breast, kidney and melanomas of the skin and colon.
Metastases from the lung as the primary site are the most common. Approximately 10% to 18% of patients present with brain metastases at the time of initial diagnosis, and an about 40% to 50% will develop metastases during the course of their disease (Yamanaka, 2009; Reveiz et al., 2012). Brain metastasis occurs in about 5 to 15% of breast cancer patients (Freedman and Anders, 2012). These rates continue to increase due to increase in survival rates resulting from better treatment and imaging techniques. In breast cancer, there are several risk factors for development of CNS metastasis including expression of HER2 (Human epidermal growth factor receptor) positive breast tumors, patient age (less than 50 years), tumors that are hormone receptor negative and higher disease burden (Evans et al., 2004; Lin et al., 2004). Brain metastasis occurs in about 10-40% of melanoma patients. While metastases from the lung are seen more frequently; melanomas are more likely to give rise to metastases and are possibly more resistant to chemotherapy (Fisher and Larkin, 2012). In several cases of melanoma, brain metastases are likely to be discovered only at time of autopsy (Fonkem et al., 2012).

The treatment paradigm for metastases is the same as that for glioblastoma; surgical resection followed by radiation therapy and chemotherapy (Kristensen et al., 1992; Yamanaka, 2009; Fisher and Larkin, 2012; Fonkem et al., 2012; Reveiz et al., 2012). As in glioblastoma, the delivery of chemotherapeutics to the tumor site is a limiting factor in efficacy of the drugs.

**1.3 Treatment:**

The treatment of glioblastoma and brain metastases follows an aggressive multi-step approach. Patients first undergo surgical resection of the tumor section with the help of
intraoperative-MRI or fluorescene-guided imaging (Carapella et al., 2011). The tumor de-bulking is carried out only on the visualized tumor core, and therefore, invasive tumor cells that give rise to recurrence cannot be excised completely as they might be present in the surrounding functional tissue (Carapella et al., 2011). This limits the possibility of removal of the entire tumor mass. There are inherent difficulties in complete excision of the tumor due to its diffuse nature or its location (Carapella et al., 2011). The tumor cells that are left behind post-surgery are then treated by a combination of radiotherapy and chemotherapy (Johnson and Chang, 2012). Stupp et.al. performed a clinical trial where they observed the effect of addition of temozolomide to the treatment regimen post radiotherapy and surgery. In this influential paper, they found that addition of chemotherapy to radiotherapy and surgery improved the overall survival rate significantly (Stupp et al., 2005). However, the actual increase in the overall survival was only about 2 months. Despite this modest increase, temozolomide is now an accepted standard of care of GBM (Johnson and Chang, 2012) and has been used in treating other brain metastases (Fonkem et al., 2012). Another approved FDA approved therapy is Gliadel ®, a BCNU loaded wafer that is implanted at the site of the tumor, however this has been shown to result in toxicity without any benefit in terms of the overall survival (De Bonis et al., 2012). Avastin ® (bevacizumab) is a monoclonal antibody that has been approved by for treatment of GBM despite only modest improvement in overall survival in high grade glioma (Verhoeff et al., 2010; Narayana et al., 2012). While these therapies have received FDA approval, their efficacy in treating GBM is limited. This underscores the urgent
need for developing treatment strategies that are effective in treating glioblastoma and brain metastases.

Several molecularly targeted agents including erlotinib, imatinib, gefitinib, sorafenib, lapatinib and more recently vandetanib have been examined clinically for use in glioblastoma. However, none of them have shown to be effective in improving the overall survival rate (Rich et al., 2004; Razis et al., 2009; Thiessen et al., 2010; Reardon et al., 2011; Uhm et al., 2011; De Bonis et al., 2012; Kesavabhotla et al., 2012; Kreisl et al., 2012). Despite aggressive treatment of glioma and brain metastases, patients have a poor prognosis and survival, mainly due to resistance to chemotherapy as well as recurrence of the disease post treatment.

1.4. Recurrence and Resistance:

The development of multidrug resistance (MDR), is defined as simultaneous resistance of cancer cells towards a broad spectrum of structurally unrelated cytotoxic drugs that have different modes of action, and it is a major obstacle to effective treatment of primary and metastatic brain tumors is (Bredel, 2001; Dean et al., 2005). Clinically, MDR may be either intrinsic resistance (at the beginning of chemotherapy) or acquired resistance (acquired over the period of treatment), and the latter is attributed to genetic mutations and the selection of the therapy (Kroll, 1995). The possible mechanisms that lead to both types of chemoresistance in treating gliomas are complex and multifactorial.

One of the greatest hurdles in effectively treating glioma is the recurrence of the disease. This recurrence post-treatment is seen in primary brain tumors as well as brain metastases (Wen and Brandes, 2009) and nearly all patients of glioma show a recurrence of the
There could be several possible reasons for this recurrence of the tumor. Firstly, the resection of the tumor is carried out only at the site of the broken down BBB. One of the defining characteristics of glioma cells is that they are invasive and move away from site of occurrence into healthy tissue, sometimes even as far away as the contralateral hemisphere (Berens and Giese, 1999). These invasive cells escape resection since the tumor that is behind an intact BBB is not resected and cannot be detected by contrast enhanced MRI. The invasive cells could result in a relapse of the disease.

1.4.1. Cancer stem-like cells hypothesis:

The ‘cancer stem-like cell’ hypothesis is one of the hypotheses put forward to explain tumorigenesis. Cancer stem-like cells are described as a small population of cells within the tumor that have the ability to drive tumor growth through limitless self-renewal (Dean et al., 2005). Ignatova et al, were the first to isolate clonogenic, neurosphere-forming stemlike cells from human glioblastoma multiforme (GBM) (Ignatova et al., 2002). These stem-like cells have the ability to were shown to differentiate into multi-lineage progeny cells and had the ability to form tumors in immunocompromised mice (Bleau et al., 2009a; Bleau et al., 2009b). The discovery of these stem-like cells has an enormous implication in the treatment of glioblastomas.

Cancer stem-like cells in the brain are referred to as brain tumor stem-like cells since they share several similarities with neural stem cells (Singh et al., 2004). The identification of cancer stem-like cells could be done by identifying their genotype or phenotype. Brain tumor stem-like cells are known to express a variety of proteins including Nestin (a cytoplasmic marker for neural stem cells) (Dell'Albani, 2008) and CD
133 (a cell surface marker) (Singh et al., 2003). The cancer-stem cells have been known to over-express ABCG2, which contributes to their chemoresistance, and also is a trait that can be used to isolate these cells (Bleau et al., 2009a; Bleau et al., 2009b). In an experimental setting, these stem-like cells have been sorted from the tumor by flow cytometry, using a dye, Hoechst 33342. The stem-like cells are known to express BCRP on their cell surface, and that on treatment with the dye, these cells will efflux the dye out and remain unstained, while the bulk of the tumor cells is stained (Bleau et al., 2009a). Generally, the stem-like cell population is a very small fraction of the entire tumor bulk. However, capacity of endless self-renewal possessed by these cells, is a major driving force for the growth and recurrence of tumors (Dean et al., 2005).

The importance of cancer stem-like cells in the recurrence of brain tumors is underscored by the discovery that these cells are more resistant to radiotherapy and chemotherapy. Bao et.al, showed that ionizing radiation was ineffective in killing cancer stem-like cells. Ionizing radiation causes apoptosis by damaging DNA of tumor cells. However, cancer stem-like cells have a finely developed DNA damage check-point response that allows them to repair DNA and prevent apoptosis (Bao et al., 2006). The silver-lining to this result could be that the DNA check-point mechanism could be targeted to overcome resistance in radiation resistant tumors.

Therapy with temozolomide is the standard of care in glioma therapy. Temozolomide is a DNA alkylating agent that causes apoptosis by alkylating DNA and causing double strand breaks. DNA repair protein O\(^6\)-methylguanine-DNA methyl transferase (MGMT) can reverse these strand breaks and prevent apoptosis. GBM stem-like cells have been
known to have an increased expression of MGMT (Liu et al., 2006; Chua et al., 2008). Pretreatment of neurospheres derived from GBM stem cells with temozolomide prior to their implantation in mice, enhanced their ability to give rise to tumors. Therefore, treatment with temozolomide would be less effective in targeting these tumor-initiating cells. Therefore, other targets and chemotherapeutic agents must be explored in order to effectively eliminate these cells.

Tumor-initiating cells are also known to express enzymes like matrix metalloproteinases that digest extracellular matrix and allow cells to move away from the tumor site and resulting in invasion into healthy tissue (Deryugina and Quigley, 2006; Inoue et al., 2010), which would allow them to escape into brain tissue that is protected by an intact BBB. This would prevent their removal into surgery and also protect them from chemotherapy.

Cancer stem-like cells are also known to express P-gp and BCRP on their cell surface. In fact the expression of these transporters is one of the defining characteristics of stem-like cells (Bleau et al., 2009a; Nakai et al., 2009). These transporters can efflux a variety of drugs and thus act as a protective barrier. Several anti-cancer agents and molecularly targeted agents are substrates for P-gp and BCRP. Therefore, the cancer stem-like cells are effectively resistant to the chemotoxic and chemostatic effect of these drugs. This problem is compounded by the fact that these cancer stem-like cells could be protected by an intact BBB as well. One way of targeting these cells is the use of pharmacological inhibitors of P-gp and BCRP. These inhibitors are discussed in detail later on in the chapter.
According to the cancer stem-cell theory, therapy that targets all proliferating cells spares the resistant CSCs, results in tumor recurrence, even after tumor response to treatment (Jones et al., 2004). This is referred to as the ‘dandelion hypothesis’, similar to weeds, where the bulk of the tumor (leaves) are treated but the tumor initiating cells (roots) cause recurrence (Rahman et al., 2011).

The recurrence of tumor despite aggressive treatment is a major obstacle in curing GBM and other brain metastases. To overcome this limitation we must radically change the way that GBM and brain metastases are treated. GBM should be considered as a whole brain disease (Agarwal et al., 2011a), and that therapy should be targeted not only to the bulk of the tumor, but also the invasive tumor cells and tumor initiating cells that are responsible for recurrence of the disease. The role of the invasive tumor cells in recurrence of the disease has been well explained by Berens and Giese in their 1999 review (Berens and Giese, 1999). The targeting of the invasive and tumor initiating cells is difficult due to the presence of the BBB in addition to the expression of transporters on the tumor cells (see Figure 1.1) To effectively overcome these two barriers we must first understand their nature and structure.

1.5. Barriers to drug delivery:

1.5.1. Blood Brain Barrier:

The blood-brain barrier is comprised of neurovascular units that are made up of a number of heterogeneous cells including endothelial cells. The endothelial cells surrounding the capillary lumen are joined together by tight junctions, which prevent passive diffusion of drug into the brain parenchyma (Loscher and Potschka, 2005a). The endothelial cells
express a large variety of transporters that express transport proteins on their apical and basolateral surface that can either influx or efflux substrates into or from the brain parenchyma (Loscher and Potschka, 2005a). These transporters are ATP-dependent, that is they actively transport substrates from the brain. The BBB, is thus a physiological barrier that protects the brain parenchyma from xenobiotics. Some drugs that have low molecular weight (<500 daltons), high lipophilicity, polar surface area between 60-70 Å, positive charge with a pH of 7-8; have better permeability into the brain (Pajouhesh and Lenz, 2005; Broccatelli et al., 2012) despite the presence of tight junctions. However, a large number of drugs do not possess these characteristics and show lower permeability into the brain. In addition to this, several drugs with a CNS target are substrates for the ATP-dependent transporters, which actively efflux drugs from the brain back into the capillary lumen.

ABC-cassette transporters are widely expressed at the BBB and function as a protective barrier against xenobiotics. However, this protective function also extends to drugs that are intended for a CNS delivery, thus limiting their distribution and efficacy (Loscher and Potschka, 2005b). Of the transporters in the ABC superfamily of transporters, P-glycoprotein (P-gp) and Breast cancer resistance protein (BCRP), are two of the most widely expressed at the human BBB. These transporters have also received a lot of attention for their role in limiting the distribution of a number of drugs to the CNS. P-glycoprotein (P-gp, ABCB1, MDR1) is one of the most widely expressed transport proteins at the BBB. It is known to be expressed on the luminal side of endothelial cells, thus acting as an efflux transporter (Loscher and Potschka, 2005a). P-gp is widely studied
for its role in development of multidrug resistance in brain tumor cells (Abe et al., 1998; Spiegl-Kreinecker et al., 2002; Walsh et al., 2010) It has also been implicated in the limited brain distribution of many anti-cancer drugs that are substrates for P-gp in several preclinical studies (de Vries et al., 2007; Agarwal et al., 2010; Elmeliegy et al., 2011). P-gp, thus plays an important role in limiting the efficacy of drugs that are targeted to the CNS, in a wide variety of CNS disorders such as epilepsy, HIV, and also glioma (Edwards et al., 2002; Shaik et al., 2007; Agarwal et al., 2011a; Zhang et al., 2012).

Breast cancer resistance protein (BCRP, ABCG2) is another transport protein that is expressed widely on the human BBB (Nicolazzo and Katneni, 2009). Preclinical studies show that several anti-cancer drugs, including sorafenib, imatinib are substrates for BCRP (Breedveld et al., 2005; Lagas et al., 2010; Agarwal et al., 2011b). Expression of BCRP on the BBB is responsible for limiting their brain distribution. Several anti-cancer drugs are dual substrates for P-gp and BCRP. More importantly, several reports suggest that P-gp and BCRP act in tandem to limit the distribution of anti-cancer drugs. This ‘synergistic’ effect has been observed in case of several dual substrates of P-gp and BCRP including lapatinib, erlotinib and sorafenib (Polli et al., 2009; Kodaira et al., 2010; Agarwal and Elmquist, 2012). Therefore, we can conclude that simultaneous pharmacological inhibition of both the transporters would be more effective than selective inhibition of either one of these transporters.

Several reports argue that the BBB at the tumor site shows greater permeability than in the healthy brain, and therefore transporters may not hamper the delivery of drugs to the tumor (Stewart, 1994). Several preclinical studies also show that the tumor
concentrations of drugs such as erlotinib, gefitinib are higher in the tumor core as compared to the surrounding brain. Despite this evidence of lack of transporter effect, we must remember that the tumor core, where drug delivery is not an issue, is generally excised from the brain. The tumor cells which are the targets for chemotherapy reside several centimeters away from the tumor core (Piepmeier and Baehring, 2004; Sanai and Berger, 2012). Blakely et al., in their microdialysis study with methotrexate showed that, in patients with solid tumors, the drug concentrations in the tumor are several orders of magnitude higher than in the healthy tissue (Blakeley et al., 2009). This underscores the fact that drug delivery to tumor cells behind an intact BBB is still limited and strategies that could overcome this need to be explored in greater detail. Therefore, glioma should be treated like a ‘whole-brain disease’, rather than restrict therapy strictly only to the site of first occurrence (Agarwal et al., 2011a).

1.5.2. Brain Tumor Barrier:

One of the reasons for developing multi-drug resistance is the expression of ATP-dependent efflux proteins on the tumor cell surface. The expression of mdr1 is higher in high grade gliomas as compared to low grade tumors (Spiegl-Kreinecker et al., 2002). Overexpressed P-gp was detected in 2% of 67 human primary and metastatic brain tumours including 31 gliomas (Mousseau et al., 1993). It was also demonstrated that even though there were only 4–23% P-gp positive cells in human gliomas before chemotherapy, P-gp expression was shown to be increased in patients examined after treatment, implying that P-gp may be responsible for both intrinsic and acquired MDR in gliomas (Abe et al., 1998). The overexpression of P-gp was correlated with a reduced
overall survival rate for patients with lowgrade glioma (Andersson et al., 2004).

Melanoma metastases to the brain have also been shown to express P-glycoprotein on their cell surface (Walsh et al., 2010). P-gp is also found to be overexpressed on the glioma tumor-initiating or cancer stem-like cells (Nakai et al., 2009). This property of cancer stem-like cells was useful in isolating them from other cancer cells. P-gp expressed on the tumor-initiating cells also helps to protect them from anti-cancer drugs by effluxing drug out of the tumor cells, rendering them resistant to chemotherapy (Lu and Shervington, 2008).

Tumor-initiating cells have been shown to overexpress BCRP on their cell surface (Bleau et al., 2009b). It is one of the identifying characteristics of tumor-initiating cells. Bleau and coworkers have described the isolation of the side population from a PDGFR driven tumor, where the side population was separated by flow cytometry using a dye that is a substrate for BCRP (Bleau et al., 2009a; Bleau et al., 2009b). ABCG2 expression in human brain vessels extracted from glioblastoma vessels and parenchymal tissue displayed a higher level than normal brain tissue (Zhang et al., 2003). While enhanced ABCG2 expression was detected in human glioma tissues, low levels were found in normal brain tissue. Moreover, high-grade gliomas tended to have a higher expression of ABCG2 compared for low-grade gliomas (Jin et al., 2009).

P-gp and BCRP expression on tumor cells and tumor-initiating cells is one of the mechanisms of development of chemoresistance. Several pharmacological inhibitors of these transporters are available and have been used extensively in vitro. Elacridar (GF120918), a dual inhibitor of P-gp and BCRP, and its possible applications as an
adjuvant in the treatment of glioblastoma and brain metastases has been discussed in
detail later.

1.6. Clinical significance:

Temozolomide is currently the standard of care in treatment of glioblastoma, despite the
fact that the addition of temozolomide increases the overall survival only by about 2
months. Several new molecularly targeted drugs are being examined for use in treatment
of glioblastoma. Clinical trials that have examined the effect of molecularly targeted
agents including erlotinib, imatinib, lapatinib, sorafenib, gefitinib and vandetanib either
alone or in combination with other agents have found that these drugs have little to no
effect on the overall survival of glioma patients (Rich et al., 2004; Razis et al., 2009;
Thiessen et al., 2010; Reardon et al., 2011; Uhm et al., 2011; De Bonis et al., 2012;
Kesavabhotla et al., 2012; Kreisl et al., 2012).

One of the possible reasons for the failure of these potent drugs could be the barrier to
drug delivery that is presented by the BBB and the Brain-tumor barrier. This lack of
response in glioma patients is especially significant since gefitinib, imatinib, lapatinib,
erlotinib, sorafenib and vandetanib are substrates for P-gp and Bcrp and their brain
distribution has been shown to be limited in several preclinical studies due to the
presence of these transporters (Bihorel et al., 2007; Marchetti et al., 2008; Polli et al.,
2009; Agarwal et al., 2010; Lagas et al., 2010; Agarwal et al., 2011b; Elmeliegy et al.,
2011; Minocha et al., 2012). This phenomenon was discussed in detail in an extensive
review by Agarwal et.al. (Agarwal et al., 2011a). It is of utmost importance that we
understand the full effect of active transporters at both the BBB as well as on the tumor
cells, especially since several promising drugs that are being considered for use in GBM and treatment of brain metastases have been found to be substrates for both P-gp and BCRP (see Table 1.1).

1.7 Overcoming barriers to drug delivery:

The discovery of the BBB has spurred on researchers to improve the delivery of drugs into the brain. Several different strategies have been implemented so far with varying degrees of success. It is possible to bypass the BBB altogether by implanting drug loaded wafers at the site of tumor sites. Gliadel ® wafers are loaded with BCNU and release the drug over an extended period of time (Buonerba et al., 2011). Intra-arterial administration of hyperosmolar mannitol has been used to disrupt the BBB to improve the brain distribution of chemotherapeutics (Neuwelt and Rapoport, 1984) such as methotrexate (Gumerlock et al., 1992) and bevacizumab (Boockvar et al., 2011), and have resulted in slowing down of the tumor progression (Burkhardt et al., 2012). The drawback of this technique is that it opens up the BBB and allows all xenobiotics to pass through. A more sophisticated technique for bypassing the BBB would be the administration of pharmacological inhibitors of P-gp and BCRP. Administration of these agents would selectively inhibit the transporters and allow drugs that are substrates to pass through.

1.7.1. Pharmacological inhibitors of transporters:

Most inhibitors of P-gp and BCRP were initially developed a means to overcome multi-drug resistance. One of the first clinical applications of P-gp inhibitors was in a study with cyclosporine in addition to chemotherapy in the treatment of retinoblastoma by
Chan et al. where they saw an cure rate of 91%, when cyclosporine was administered as an adjuvant to treatment (Chan et al., 1996). However, the use of cyclosporine has been limited due to its unfavorable drug-drug interactions and pharmacokinetics. 1st generation inhibitors of P-gp are defined as drugs already in clinical use or compounds under investigation for other therapeutic indications with an inhibitory activity on P-gp and BCRP as an important side-effect (Palmeira et al., 2012). Some examples of this class of inhibitors are cyclosporine, verapamil and ketoconazole (Siegsmund et al., 1994; Perrotton et al., 2007; de Souza et al., 2012). These compounds have been shown to have effectively reversed multi-drug resistance in drug resistant cell lines and have shown promise in potentiating effects of anti-cancer agents in pilot studies (Stewart et al., 1997). However, these compounds already are pharmacologically active, clinical applications of these compounds as inhibitors could possibly have undesired pharmacological effects.

Second generation P-gp inhibitors, are mostly analogues of first generation that do not possess the pharmacological activity of the original molecule but could inhibit P-gp with greater potency and lower toxicity (Palmeira et al., 2012). Valspodar (PS-833) is an example of second generation P-gp inhibitors. Valspodar has been shown to improve accumulation of the P-gp substrate mitoxantrone by inhibition of P-gp in MDA-MB-435 cancer cell lines (Decleves et al., 2008). Valspodar was also effective in inhibiting P-gp at the BBB and improving the brain distribution of paclitaxel, docetaxel and imatinib in preclinical studies (Tan et al., 2000; Kemper et al., 2003; Kemper et al., 2004). However, despite this, the clinical applications of valspodar as an adjuvant to reverse multidrug resistance were unsuccessful (Friedenberg et al., 2006; Pein et al., 2007; Lhomme et al.,
Phase III studies in patients with ovarian cancer treated with a combination of paclitaxel and carboplatin and valsapar as an adjuvant, showed a lack of effect of valsapar on the overall survival (Friedenberg et al., 2006). A huge disadvantage of the second generation of inhibitors is that they interact with CYP3A4 to contribute to unpredictable pharmacokinetics profiles of anti-cancer drugs (Fischer et al., 1998). Co-administration of valsapar with paclitaxel has been known to result in toxicity due to drug-drug interactions, requiring reduction in dose (Chico et al., 2001; O’Brien et al., 2010).

3rd generation inhibitors have been developed using quantitative structure activity relationship (QSAR). The most widely studied 3rd generation inhibitors are elacridar, zosquidar, tariquidar and laniquidar. The inhibitory potency and the duration of action of these inhibitors exceed those of first and second generation inhibitors (Palmeira et al., 2012). These inhibitors have been used as a tool to examine the accumulation of substrate molecules in P-gp and Bcrp overexpressing cell lines. Another use of these compounds is the visualization of P-gp and Bcrp expression in preclinical models using a combination of Positron emission tomography (PET) and radiolabeled inhibitors (Dorner et al., 2009; Kawamura et al., 2011a; Kawamura et al., 2011b). A list of commonly used pharmacological inhibitors is shown in Table 1.2.

1.7.2 A case for elacridar adjuvant therapy:

Elacridar (GF120918, Molecular Formula: C_{34}H_{33}N_{3}O_{5}, Molecular Weight: 563.64) is a dual inhibitor of P-gp and BCRP. It was initially developed as a reversal agent for multidrug resistance (Hyafil et al., 1993). Hyafil et.al. showed that treatment with elacridar re-
sensitized doxorubicin-resistant cell-line to doxorubicin, and enhanced uptake of daunorubicin in MDR cells (Hyafil et al., 1993). Elacridar has been studied in several clinical trials as a P-gp inhibitor to improve the oral absorption of drugs that are substrates for P-gp. Here, elacridar inhibits the P-gp present in the gut lumen, limiting the efflux of these substrates and thus improving their oral bioavailability. Improved bioavailability of toptecan and paclitaxel in humans was achieved through this strategy (Malingre et al., 2001; Kruijtzer et al., 2002; Kuppens et al., 2007).

Elacridar has also been examined for use in reversing drug resistance in clinical studies. A Phase II study with doxorubicin and elacridar as an adjuvant for treatment of drug resistant solid tumors did not show any positive results, however, they did observe that the plasma concentrations of elacridar were sufficient to effectively inhibit P-gp (Planting et al., 2005).

Elacridar has since then been used as an inhibitor of P-gp and BCRP at the BBB mostly to demonstrate the effect of these transporters on the brain penetration of various drugs in preclinical studies. Several anti-cancer drugs such as vinblastine, paclitaxel, doxorubicin, (Hughes et al., 1998; van der Sandt et al., 2001; Kemper et al., 2003) as well as the newer molecularly targeted agents such as gefitinib, sorafenib, imatinib, sunitinib (Chen et al., 2009; Agarwal et al., 2010; Lagas et al., 2010; Agarwal et al., 2011b; Tang et al., 2012a) are substrates for either, P-gp, BCRP or both; resulting in limited brain penetration. Several preclinical studies have shown that co-administration of elacridar with the substrate drugs was able to inhibit transporters at the BBB and improve their distribution into the brain. Elacridar being a dual inhibitor of P-gp and BCRP, can inhibit both
transporters at the same time, giving rise to greater than additive effect in the overall brain partitioning of the drug, which is seen in many substrates (Polli et al., 2009; Agarwal and Elmquist, 2012). This improved brain distribution of molecular targeted agents with co-administration of elacridar shows potential in improving their brain distribution, particularly in light of the several failed clinical trials for their use in glioma. Breedveld et.al. in their paper suggested the possibility of utilizing elacridar and other modulators of transporter function to improve oral bioavailability and drug delivery to the brain (Breedveld et al., 2006). Utilizing this concept, Hubensack and coworkers used elacridar as an adjuvant to improve brain distribution of paclitaxel in a mouse xenograft model of glioma (Hubensack et al., 2008). The brain distribution of paclitaxel was significantly improved and resulted in a 90% reduction in the tumor burden in glioma. However, there was unexpected toxicity due to modulation of peripheral P-gp by elacridar.

Use of elacridar can help overcome limited drug distribution due to the BBB and possibly enhance the efficacy of these drugs in treatment of brain tumors. Application of elacridar as an adjuvant can also help us target tumor cells and tumor initiating cells that are resistant to chemotherapy due to expression of P-gp and BCRP at their cell surface.

1.7.3 Limitations to application of elacridar as an adjuvant:

Elacridar is an extremely lipophilic molecule (logP : 5.6) with extremely poor solubility in most aqueous and organic solvents. Therefore, it is not surprising that the formulation of an injectable dosing solution of elacridar is extremely difficult. Ward et.al., studied the pharmacokinetics of elacridar in mice and rats post an oral dose, and found that the
bioavailability of elacridar is dissolution-rate limited (Ward and Azzarano, 2004). This reflects in the plasma exposure of elacridar in mice after an oral dose, as increase in the dose did not result in a corresponding increase in the plasma exposure (Ward and Azzarano, 2004; Planting et al., 2005). This phenomenon was observed in humans as well (Planting et al., 2005). In addition to unpredictability of the plasma exposure of elacridar, it has limited bioavailability. Our published study indicates that the absolute bioavailability of elacridar after oral and intraperitoneal dosing in mice is only about 22% and 1% respectively (Sane et al., 2012). Therefore, the plasma exposure of elacridar following an oral dose cannot be easily predicted, and different studies have used varying doses to achieve inhibition of the transporters at the BBB. Tang et.al., administered a very large oral dose of 100 mg/kg in a successful attempt to improve brain distribution of sunitinib and its active metabolite (Tang et al., 2012a; Tang et al., 2012b). Edwards and coworkers used an oral dose of 250 mg/kg elacridar with a three day pretreatment with elacridar to enhance the brain distribution of amprenavir in rats (Edwards et al., 2002). In a study that examined brain distribution of morphine, Letrent et.al., administered a 500 mg/kg oral dose of elacridar (Letrent et al., 1998). Other studies have utilized a variety of doses ranging from 25 mg/kg oral gavage to a 10 mg/kg intravenous bolus (Kemper et al., 2004; Chen et al., 2009). From a brief review of the preclinical applications of elacridar, we can see that the dosage regimen was set up to show a positive response and has not been optimized. We propose to utilize elacridar as an adjuvant to molecularly targeted agents, which are chemostatic by nature and therefore are to be administered daily. For purposes of development of a chronic dosage regimen, we need to optimize the
dose of elacridar. Administration of a very large dose can give us effective inhibition of the transporters at the BBB, however, it is also likely its chronic administration would result in adverse drug reactions. In order to do this, we have determined the bioavailability of elacridar post oral and intraperitoneal dose (Chapter II).

Since we propose to use elacridar in preclinical models of brain tumors to enhance delivery of drugs across both the BBB and BTB, we need to have an in depth understanding of the distribution of elacridar into the mouse brain. The brain distribution of elacridar in mice has not been studied in detail. In addition to this, the interaction of elacridar with P-gp and BCRP at the BBB, has also not been examined in detail. There have been some studies using PET and radiolabeled elacridar that indicate that elacridar could be a possible substrate for P-gp and BCRP (Dorner et al., 2009; Kawamura et al., 2011a; Kawamura et al., 2011b). In order to understand the interaction of elacridar at the BBB, we have studied the brain distribution of elacridar in wild-type, P-gp knockout, Bcrp knockout and triple knockout mice (Chapter III).

We know from our previously published study that the bioavailability of elacridar is limited by its poor solubility (Sane et al., 2012). To improve the bioavailability of elacridar, we have developed a novel microemulsion formulation of elacridar (Chapter IV), and examined its effect as an adjuvant in tumor bearing mice (Chapter V).

1.8. Application of microemulsion to improve drug delivery:

A successful example of improved oral bioavailability using a Cyclosporin A is a potent immunosuppressant that is used in organ transplant, and is a classic example for improvement of solubility and bioavailability of highly lipophilic drugs. The oral
bioavailability of cyclosporine was very poor due to its poor solubility and high lipophilicity, and there was considerable inter-patient variability. Formulation of cyclosporine as a microemulsion was found to improve its bioavailability (Hirunpanich and Sato, 2009) and further improvement in its formulation as a microemulsion concentrate even reduced inter-subject variability (Vonderscher and Meinzer, 1994). It is available as the marketed preparation Sandimmune and Sandimmune Neoral ®. Elacridar too, has poor bioavailability when it is administered as a suspension either orally or intraperitoneally (Sane et al., 2012). The dissolution-rate limited absorption is a limitation to oral absorption, resulting in poor systemic absorption (Ward and Azzarano, 2004). Elacridar is also highly lipophilic. All these characteristics make elacridar an ideal candidate for formulation as a microemulsion.

1.8.1. Definition and characteristics of microemulsions

Microemulsion is defined as a system of water, oil and amphiphile which is optically isotropic and thermodynamically stable liquid dispersion (Danielsson and Lindman, 1981). A microemulsion can be one of the three types depending on the relative ratios of its components: (a) oil-in-water (o/w) system, which consists of oil droplets dispersed in an aqueous continuous phase; (b) water-in-oil (w/o) system where water is the dispersed phase and oil is the continuous phase; and (c) a water and oil bicontinuous microemulsion where almost equal amounts of oil and water are present (Attwood, 1994). Microemulsions differ from ordinary emulsions in that, their droplet size is less than 100 microns, are transparent to translucent, and can be formed spontaneously, while ordinary emulsions have large droplet size (>200 microns), are milky or opaque and require
energy input like homogenization (Raid et al., 2008). The major advantage of preparing microemulsions over regular emulsions is that, (a) applied external force is not required; formation of microemulsion is a function of its composition (b) Microemulsions are thermodynamically stable as opposed to regular emulsions.

A simplified thermodynamic model has been proposed to explain the spontaneous formation of a microemulsion system (Raid et al., 2008),

\[ \Delta G_f = \gamma \Delta A - T \Delta S \]  

(Equation 1)

Where,

\[ \Delta G_f = \text{free energy of microemulsion formation} \]
\[ \gamma = \text{interfacial tension at the oil-water interface} \]
\[ \Delta A = \text{change in the interfacial area and is associated with decreasing droplet size} \]
\[ T = \text{Absolute temperature} \]
\[ \Delta S = \text{system entropy} \]

The formation of a microemulsion is driven by the entropy term \( \Delta S \), as randomness associated with the dispersion of two immiscible phases. This leads to higher entropy and free energy that is necessary to form a stable microemulsion. Interfacial tension is lowered by migration of surfactant molecules to the interface of the two immiscible phases. At the critical micelle concentration (CMC), there is no further decrease in the interfacial tension, however addition of a second surfactant (co-surfactant), decreases the interfacial tension further, resulting in a thermodynamically stable microemulsion.

Formation of a microemulsion is also associated with a reduction in droplet size. There is an increase in \( \Delta A \), due to reduction in droplet size; however this is offset by a decrease
in $\gamma$, due to use of a high concentration of amphiphiles. This results in a negative $\Delta G_f$, leading to a spontaneous formation of the microemulsion (Lawrence and Rees, 2000).

1.8.2. Formulation of microemulsion system:

1.8.2.1 Selection of components:

Pharmaceutically acceptable microemulsion systems need to be composed using components that are generally regarded as safe (GRAS). Microemulsions are generally composed of surfactants, co-surfactants or co-solvents and oil phase. Most widely used pharmaceutically acceptable oily components are triglycerides (vegetable oils), mixture of mono and diglycerides as well as fatty esters such as isopropyl myristate, isopropyl palmitate and ethyl oleate (Tenjarla, 1999). Zwitterionic and nonionic surfactants are commonly used. Nonionic surfactants include sucrose esters (Thevenin et al., 1996), polyglycerol fatty acid esters (Ho et al., 1996), polyoxyethylene hydrogenated castor oil (Araya et al., 2005). Lecithin and other zwitterionic phospholipids are also used; their biocompatibility is an added advantage (Cilek et al., 2005). Co-surfactants are weak surfactants that are combined with surfactants to enhance their ability to decrease the surface tension (Tenjarla, 1999). Co-solvents are weak amphiphiles that promote microemulsion formation by making the interfacial film more flexible and decreasing the lipophilicity of the oil phase (Aboofazeli et al., 1994; Kahlweit et al., 1995). Commonly used co-surfactants are ethanol (Hirunpanich and Sato, 2009), medium chain mono and di-glycerides and 1,2 alkanediols (Aboofazeli et al., 1994).

1.8.2.2: Phase behavior studies:
The phase behavior of the selected components is studied by constructing a ternary phase diagram. This is important because mixing these components can result in a variety of systems including coarse emulsions, gels, vesicles or micellar systems (see Figure 1.2). A ternary phase diagram is prepared with a triangular diagram where each corner represents 100% of a component, and each side represents a binary system. Generally there are more than three components in each microemulsion system, therefore, we simplify the phase behavior studies by fixing the mass ratio of two components (eg: surfactant:co-surfactant or oil:co-surfactant) and creating a “pseudoternary” system. A ternary phase diagram can be constructed by either (a) preparing and large number of samples with different composition or (b) titrating a binary or pseudobinary mixture with the third component (Raid et al., 2008).

Microemulsion can be characterized by visual inspection. The individual systems are formulated in transparent glass vials and inspected for clarity and phase separation. Microemulsions are visually clear due to their small droplet size. Polarizing light microscopy could be used to determine if the system is isotropic (i.e. has only one refractive index).

1.8.3. Improved bioavailability of drugs in microemulsion formulation

The proposed mechanisms for improved bioavailability of drugs in a microemulsion system include:

1. Protection of protein and peptide drugs from metabolizing enzyme (Cilek et al., 2005)
2. Improved permeability across the gastrointestinal membranes due to presence of surfactant molecules that disrupt the lipid bilayer of endothelial cells (Scott Swenson and Curatolo, 1992)

3. Enhanced solubility of drugs (Araya et al., 2005)

4. Inhibition of P-gp transport in the gut lumen by surfactants such as Cremophor EL, Tween 80, and PEG (Hugger et al., 2002).

There are several commercially available microemulsions including cyclosporine (Neoral®), ritonavir (Norvir®) and saquinavir (Fortovase®) (Raid et al., 2008).

However, wide scale commercialization of this technique have not materialized due to limitations in use of co-surfactants, unpredictable improvement in oral bioavailability and possible precipitation of drug on dilution with aqueous solvent.
1.9 Statement of the problem:

The treatment of glioblastoma and other metastatic brain tumors with chemotherapy is hampered by limited distribution of the drug to the site of action in the tumors as well as to the invasive tumor cells present behind an intact BBB. New molecularly targeted drugs are being explored for use in glioma and brain metastases. In preclinical studies, many of these newer molecules have been discovered to be substrates for P-gp and BCRP. From previous experience, it is known that substrates of P-gp and BCRP are not effective in crossing the BBB and that the molecules that show these properties have not been very effective in clinical trials for the treatment of glioma. In addition to this, the transporters expressed at the tumor cell surface also efflux these drugs from the tumor cells (Brain-tumor barrier or BTB). It is very important to consider these sequential barriers that the drug faces in order to reach the site of action in order to design a successful treatment regimen. We know that the BBB as well as the expression of P-gp and BCRP on tumor cells limit the drug from reaching the target site. In order to overcome these barriers, a pharmacological inhibitor of these transport proteins could be utilized.

Elacridar has been used in preclinical models to improve drug distribution to the brain by inhibiting P-gp and BCRP. Therefore, we must engineer a treatment regimen using elacridar such that its use as an adjuvant can help overcome the barriers due to transport proteins. Unfortunately, there is limited data available on the pharmacokinetics of elacridar especially its behavior at the BBB. In order to inhibit P-gp and BCRP at the tumor cell surface, elacridar itself must first cross the BBB, and reach the site of the tumor, or invasive tumor cells at a concentration that is sufficient to result in inhibition of
the transporters on the tumor cells. To compound this problem, elacridar itself is very lipophilic and is very poorly soluble. This makes it a difficult molecule to formulate as a dosage form that can be administered on a chronic basis.

The problems in devising a treatment regimen with elacridar as an adjuvant are two-fold:

1) Pharmacokinetic parameters of elacridar, its oral bioavailability and brain distribution in the mouse model have not been studied in detail. Without prior knowledge of all these variables, it would be impossible to design an intelligent dosing regimen that would effectively enhance drug delivery across the BBB as well as the BTB.

2) The poor physicochemical properties of elacridar including poor solubility and high lipophilicity prevent preparation of a simple solution or a readily soluble suspension that can be administered on a chronic basis. Improving the solubility of elacridar in a new formulation can help improve the pharmaceutical elegance of its dosage form, and provide a convenient formulation for chronic dosage.

**1.10 Research Objective:**

The objectives of this research project were:

1. To determine the bioavailability of elacridar following administration by oral gavage and intraperitoneal injection
2. To investigate the effect of expression of P-gp and BCRP at the BBB on the brain distribution of elacridar
3. To develop a strategy to improve the bioavailability of elacridar following oral or intraperitoneal administration
4. To examine the effect of elacridar as an adjuvant for enhancing drug delivery to brain tumors in xenograft model in mice

1.1. Research Plan:

2. We first determined the bioavailability of elacridar following administration by oral gavage and intraperitoneal administration and examined its brain distribution. (Chapter II)

3. We examined the effect of P-gp and BCRP expression at the BBB on the brain distribution of elacridar, as well as the effect of dose on the saturation of the transporters at the BBB (Chapter III)

4. To improve the poor bioavailability of elacridar we observed in Chapter I, we developed a novel microemulsion formulation of elacridar to improve the bioavailability of elacridar and characterized it in vitro and in vivo (Chapter IV)

5. To conclude the study, we administered elacridar microemulsion as an adjuvant to erlotinib treatment and examined its effect on distribution and efficacy of erlotinib in treatment of glioblastoma in a xenograft model
Table 1.1: List of molecularly targeted agents that have been examined for use in treatment of glioma or brain metastases and are substrates for P-gp and BCRP

<table>
<thead>
<tr>
<th>Molecularly targeted agent</th>
<th>Substrate Status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-gp</td>
<td>BCRP</td>
</tr>
<tr>
<td>Imatinib</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Tandutinib</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Cediranib</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Vemurafenib</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Vandetanib</td>
<td>-</td>
<td>✓</td>
</tr>
<tr>
<td>Pazopanib</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Compound</td>
<td>Pgp</td>
<td>BCRP</td>
</tr>
<tr>
<td>----------------</td>
<td>-----</td>
<td>------</td>
</tr>
<tr>
<td><strong>First generation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verapamil</td>
<td>✓</td>
<td>-</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Quinidine</td>
<td>✓</td>
<td>-</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>✓</td>
<td>-</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td><strong>Second generation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexverapamil</td>
<td>✓</td>
<td>-</td>
</tr>
<tr>
<td>Cinchonine</td>
<td>✓</td>
<td>-</td>
</tr>
<tr>
<td>Valspodar</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td><strong>Third generation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zosuquidar</td>
<td>✓</td>
<td>-</td>
</tr>
<tr>
<td>Elacridar</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Tariquidar</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Laniquidar</td>
<td>✓</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 1.1. Pictorial representation of the multiple barriers to drug delivery, where BBB is the blood-brain barrier, and BTB is the brain-tumor barrier. The expression of transporters such as P-gp and BCRP on both these barriers, limit the distribution of chemotherapeutic agents to the target site.
Figure 1.2: A hypothetical pseudo-ternary phase diagram of an oil-in-water system representing the various systems that can be observed when oil, surfactant mixture and water are blended in various proportions (Constantinides, 1995)
Figure 1.3: Chemical structures of third generation inhibitors: tariquidar (A), laniquidar (B) and elacridar (C)
CHAPTER II:

BRAIN DISTRIBUTION AND BIOAVAILABILITY OF ELACRIDAR AFTER DIFFERENT ROUTES OF ADMINISTRATION IN THE MOUSE

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The objective of this study was to characterize the bioavailability and disposition of elacridar (GF120918) in mouse plasma and brain after various routes of administration. Elacridar is a potent inhibitor of P-gp and BCRP and has been widely used to examine the influence of these efflux transporters on drug distribution to the brain. FVB mice were administered 100 mg/kg elacridar either orally or intraperitoneally. The absolute bioavailability of elacridar after the oral or intraperitoneal dose was determined with respect to an intravenous dose of 2.5 mg/kg. The absolute bioavailability was 0.22 for oral administration and 0.01 for intraperitoneal administration. The half-life of elacridar was approximately 4 hours after intraperitoneal and intravenous administration, while it was nearly 20 hours after oral dosing. The brain-to-plasma partition coefficient (Kp,brain) of elacridar increased as plasma exposure increased, suggesting saturation of the efflux transporters at the blood-brain barrier. The Kp,brain after intravenous, intraperitoneal, and oral dosing was 0.82, 0.43 and 4.31, respectively. The low aqueous solubility and high lipophilicity of elacridar result in poor oral absorption, which most likely is dissolution-rate limited. The results from this study illustrate the importance of the route of administration and dose in achieving effective plasma and brain concentrations of elacridar, and can be used as a guide for future studies involving elacridar administration, and in developing formulation strategies to overcome the poor absorption.
2.1 Introduction:

Elacridar (GF 120918) is a potent inhibitor of P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) (Witherspoon et al., 1996; Allen et al., 1999). It is a third generation inhibitor (Tan et al., 2000) and was initially described as a multi-drug resistance reversal agent (Hyafil et al., 1993), where it restored the sensitivity of multidrug resistant tumors to doxorubicin.

Elacridar has been used extensively *in vitro* and *in vivo* as a P-gp and BCRP inhibitor. When co-administered with P-gp substrates such as topotecan and paclitaxel, elacridar improved their oral absorption by inhibiting intestinal P-gp thereby preventing efflux of substrate drugs into the intestinal lumen (Kruijtzer et al., 2002; Bardelmeijer et al., 2004). The role of P-gp and BCRP in limiting the distribution of substrates drugs for the efflux proteins across the blood-brain barrier has been examined using elacridar as a dual inhibitor of P-gp and BCRP. Co-administration of elacridar improved the brain penetration of several substrate molecules such as morphine and amprenavir (Letrent et al., 1998; Edwards et al., 2002). Elacridar also significantly increased brain distribution of several tyrosine kinase inhibitors (TKIs) including imatinib, dasatinib, gefitinib, sorafenib (Bihorel et al., 2007; Chen et al., 2009; Lagas et al., 2009; Agarwal et al., 2010; Lagas et al., 2010; Agarwal et al., 2011; Tang et al., 2012a). More recently, it was shown that brain penetration of the tyrosine kinase inhibitor sunitinib and its active metabolite is limited by the P-gp and BCRP at the blood-brain barrier. The oral administration of elacridar improved the brain penetration of sunitinib in wild-type mice by 12- fold such that it was equal to that seen in *Mdr1a/b(-/-)Bcrp1(-/-)* mice (Tang et al., 2012a; Tang et
Elacridar has also been administered to glioma xenograft bearing mice to enhance the brain penetration of paclitaxel (Hubensack et al., 2008).

The common objective of many of the above studies was to use P-gp and Bcrp inhibition by elacridar as a strategy to enhance distribution of substrate drugs to the brain. One such therapeutic area where this could be particularly useful is in the treatment of devastating brain tumors such as glioma. Many molecularly targeted tyrosine kinase inhibitors that are currently being evaluated in glioma do not effectively cross an intact BBB due to P-gp- and Bcrp-mediated efflux (Breedveld et al., 2005; Bihorel et al., 2007; Chen et al., 2009; Lagas et al., 2009). Co-administration of elacridar with TKIs that do not effectively cross the BBB due to P-gp and Bcrp mediated efflux could lead to improved efficacy of these drugs in glioma as a result of their enhanced delivery across the blood-brain barrier. Since most of these TKIs are intended for chronic administration, for elacridar to effectively improve delivery, it must also be administered chronically. To accomplish this effectively, we must have a better understanding of the factors that could affect its systemic bioavailability and brain distribution.

Chronic administration of elacridar has several difficulties, mainly arising due to its unfavorable physicochemical properties. Elacridar is practically insoluble in water, poorly soluble in most other aqueous solvents and it is extremely lipophilic (log P = 5.67) (Padowski and Pollack, 2010). This makes it difficult to formulate elacridar as an injectable. These poor physico-chemical properties also mean that its oral absorption will most likely be dissolution-rate limited (Ward and Azzarano, 2004) that can contribute to observed variability in variability in plasma and tissue concentrations in preclinical
studies. Intersubject variability in exposure following oral dosing has been observed in clinical trials (Planting et al., 2005). Brain penetration of elacridar in mice has been shown to be dose dependent and influenced by the presence of P-gp and BCRP at the BBB. This was elegantly demonstrated using radiolabelled elacridar PET imaging (Kawamura et al., 2011a; Kawamura et al., 2011b). All of these factors contribute to a very variable adsorption and disposition of elacridar.

While elacridar has been used to alter the brain penetration of a wide variety of drugs, the factors influencing the brain distribution of elacridar itself have not been carefully elucidated. The objective of this study was to describe the pharmacokinetics of elacridar in plasma and brain after different routes of administration and to estimate the systemic bioavailability of elacridar.

The results from this study can be helpful in determining the dose and route of administration of elacridar in future studies that may involve chronic administration, particularly in preclinical studies using the mouse model.
2.2 Materials and Methods

2.2.1 Chemicals:

Elacridar (GF 120918) [N-(4-(2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinoloinyl)ethyl)phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide] of molecular weight 563.64 was purchased from Toronto Research Chemicals (Ontario, Canada). Hydroxypropylmethylcellulose (Methocel E5LV) was obtained from Dow Chemical Company (IL). All other chemicals used were reagent grade or HPLC grade from Sigma-Aldrich (St. Louis, MO).

2.2.2 Animals:

In vivo studies were conducted in FVB wild-type mice (Taconic Farms, Germantown, NY). All animals were 8-10 weeks old at the time of the experiment. All mice were maintained under a 12 hour light/dark cycle and had unlimited access to food and water and were maintained under a temperature controlled environment. All studies were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

2.2.3 Intravenous administration of elacridar:

Elacridar intravenous dosing solution was prepared on the day of the experiment by dissolving elacridar in a vehicle containing DMSO, propylene glycol and saline (2:2:1 v/v/v) at a concentration of 1.25 mg/ml. FVB wild-type mice were injected with an intravenous dose of 2.5 mg/kg (2ul volume/g of bodyweight) into the tail vein. Blood and brain were collected at 0.5, 1, 2, 4 and 8 hours, post dose (n = 4 at each time point). Animals were euthanized by use of a carbon dioxide chamber. Blood was collected by
cardiac puncture and plasma was obtained by centrifugation at 7500 rpm for 10 min at 4 °C. The whole brain was quickly removed from skull and rinsed with ice-cold saline. Brains were immediately flash frozen with liquid nitrogen. The specimens were stored at -80°C until analysis by LCMS-MS.

2.2.4 Intraperitoneal and oral administration of elacridar:

Elacridar for intraperitoneal and oral dosing was prepared on the day of the experiment by preparing a stable suspension of elacridar, using 0.5% HPMC and 1% Tween 80 to obtain a 10 mg/ml formulation. Mice received an intraperitoneal dose of 100 mg/kg by injection into the peritoneal cavity. For oral administration, mice received a dose of 100 mg/kg by oral gavage. Blood and brain were sampled at 15 min, 0.5, 1, 2, 4, 8 hours after intraperitoneal dosing and at 0.5, 1, 2, 4, 8, 17 and 24 hours after oral dosing. Plasma and brain samples were collected and processed in the manner described above.

2.2.5 Analysis of elacridar by LCMS-MS:

The concentration of elacridar in mouse plasma and brain was determined by HPLC coupled with mass spectrometry. Frozen brain samples were thawed and homogenized with three volumes of 5% bovine serum albumin using a tissue homogenizer (Thermo Fisher Scientific, Waltham MA). 50 µl of plasma and 100 µl of brain homogenate were spiked with 20 ng of internal standard, tryphostin (AG 1478) and 100 µl of a pH 11 buffer (0.1 M sodium hydroxide and 0.04 M sodium bicarbonate). Samples were extracted by vigorously vortexing with 1 ml of ethyl acetate for 5 min and then centrifugation at 7500 rpm for 15 min at 4°C. 600 µl of organic layer was transferred to microcentrifuge tubes and dried a gentle stream of nitrogen. Samples were reconstituted
in 100 µl of mobile phase and transferred to autosampler vials. A 5 µl volume was injected using a temperature controlled autosampler maintained at 10°C.

Chromatographic analysis was performed using an Agilent Technologies (Santa Clara, CA) Eclipse XDB-C18 RRHT threaded column (4.6 mm id X 12.5 mm, 5 µ). The mobile phase was composed of acetonitrile: 20mM ammonium formate (with 0.1 % formic acid) (42:58 v/v) with a flow rate of 0.25 ml/min. The eluent was monitored using a Thermo Finnigan TSQ Quantum 1.5 detector (Thermo Fisher Scientific, Waltham, MA). The instrument was equipped with an electrospray interface. The samples were ionized by the electrospray probe and analyzed in the positive ionization mode operating at a spray voltage of 4500 V for both elacridar and the internal standard. The spectrometer was programmed to allow the [MH]+ ion of elacridar at m/z 564.6 and that of internal standard at 316.67 to pass through the first quadrupole (Q1) and into the collision cells (Q2). The collision energy was set at 39 V for elacridar and 9V for tryphostin. The product ions for elacridar (m/z 252.9) and internal standard (m/z 300.9) were monitored through quadrupole 3(Q3). The scan width and scan time for monitoring the two product ions were 1.5 m/z and 0.5 s resp. The assay was precise and linear over a range of 2.5 ng/ml to 1500 ng/ml (% CV less than 10).

2.2.6 Pharmacokinetic calculations:

Pharmacokinetic parameters from the concentration time profile in plasma and brain were calculated by non-compartmental analysis using Phoenix WinNonlin 6.1 (Mountain View, CA). The terminal rate constants were determined using the last three data points for plasma and brain. The areas under concentration-time curve for plasma (AUC_{plasma})
and brain (AUC\textsubscript{brain}) from time 0 to infinity were calculated using the linear trapezoidal method. The AUC\textsubscript{extrapolated} (AUC from last measured time point to infinity) was estimated by dividing the last measured concentration by the elimination rate constant. After intravenous dosing, the concentration at time zero (C\textsubscript{0}) was back-extrapolated by log-linear regression of first two data points. The AUC\textsubscript{plasma} from time zero to infinity after intravenous injection was then determined as above. The absolute bioavailability after oral and intraperitoneal administration was calculated as

\[ F = \frac{\text{AUC}_{\text{plasma}} \text{ (IP or PO)}}{\text{AUC}_{\text{plasma, intravenous}}} \times \frac{\text{Dose}_{\text{intravenous}}}{\text{Dose (IP or PO)}} \]

The partition coefficient of elacridar into the brain (Kp, brain) after different routes of administration were calculated as a ratio of AUCs (AUC\textsubscript{brain} / AUC\textsubscript{plasma}). The brain to plasma ratio at each time point was calculated as a ratio of brain concentration to plasma concentration (C\textsubscript{brain} / C\textsubscript{plasma}).

### 2.2.7 Statistical Analysis:

SigmaPlot for Windows version 11.0 (Systat Software, Inc., San Jose, CA) was used to determine if a difference between two groups was statistically significant. One-way analysis of variance with the Holm-Sidak post hoc test was used for multiple comparisons at a significance level of 0.05.
2.3 Results:

2.3.1 Intravenous administration of elacridar:

The disposition of elacridar in plasma and brain was studied in FVB wild type mice following an intravenous injection. The plasma concentrations showed a bi-exponential decline indicating distinct distribution and elimination phases (Figure 2.1.a). Concentrations rapidly reached peak levels in brain within 0.5 hours after IV dosing with the maximum concentration (Cmax) observed at 0.5 hours, the first measured time point. The brain-to-plasma concentration ratio was high at the initial time points (up to 2 hours) and decreased thereafter as concentrations in brain declined more rapidly compared to those in plasma (Figure 2.1.b). This is consistent with the observed terminal half-life of 4.4 hours in plasma and 1.5 hours in brain (Table 2.1). The total plasma clearance was estimated to be 0.46 ml/min (The blood flow to the liver for a 20g mouse is 1.8 ml/min (Davies and Morris, 1993)). The AUC₀–∞ was 161 ug*min/ml in plasma and 131 ug*min/ml in brain. The resulting Kp ratio was 0.82 indicating that after intravenous dosing there is approximately equal partitioning of elacridar into the brain as plasma.

2.3.2 Intraperitoneal administration of elacridar:

The plasma and brain concentrations after an intraperitoneal dose of 100 mg/kg were measured in FVB wild type mice. After intraperitoneal dosing, brain concentrations were significantly lower than plasma concentrations at all measured time-points, except at 4 hours post dose (Figure 2.2.a). The corresponding brain-to-plasma concentration ratios remained less than one at all measured time points (Figure 2.2.b). It is interesting to note that, the plasma concentrations after intravenous administration at which we observe a
greater than one brain-to-plasma concentration ratio are higher than the maximum plasma concentrations seen in intraperitoneal dosing. The brain distribution of elacridar could be dependent on its plasma levels. The observed $C_{\text{max}}$ in plasma after IP dose was $0.29 \pm 0.06 \, \mu g/ml$ and that in brain was $0.06 \pm 0.02 \, \mu g/ml$. The apparent plasma clearance (Cl/F) was estimated to be $33 \, \text{ml/min}$ by non-compartmental analysis. The $AUC_{0-\text{inf}}$ in plasma was $90.3 \, \mu g\cdot\text{min/ml}$ and $43.5 \, \mu g\cdot\text{min/ml}$ in the brain. The elimination phase half-life of elacridar after non compartmental analysis was estimated to be $4.3 \, \text{hours}$ in plasma and $9.2 \, \text{hours}$ in brain. The $K_p$ ratio was $0.48$, indicating that the partitioning into the brain was lower after intraperitoneal administration as compared to intravenous administration.

2.3.3 Oral administration of elacridar:

The brain and plasma pharmacokinetics of elacridar were studied in FVB wild type mice after a $100 \, \text{mg/kg}$ dose administered orally. The brain concentrations were lower than plasma concentrations until 1 hour post dose, after which the brain concentrations were several fold higher than plasma concentrations (Figure 2.3.a). The brain to plasma ratio after oral administration was less than one for initial time points, and then increased reaching a maximum of ~ 6 at 4 hours post-dose, before showing a slow decline (Figure 2.3.b). Non-compartmental analysis of plasma and brain concentration-time data showed that the plasma $AUC_{0-\text{inf}}$ was $1460 \, \mu g\cdot\text{min/ml}$ and brain $AUC_{0-\text{inf}}$ was $6296 \, \mu g\cdot\text{min/ml}$ (Table 2.1). The $C_{\text{max}}$ in brain after oral dose was $3877\pm410 \, \text{ng/ml}$, significantly higher than that seen after intraperitoneal dosing. The time to reach $C_{\text{max}}$ in plasma was 4 hours, indicating a slow dissolution and absorption from the gut. Post oral dosing, the $K_p$ was found to be $4.31$, suggesting that a high dose of elacridar
administered orally gives high distribution in the brain. The half-life of the drug in the brain after the oral dose mirrored its plasma half-life, values being 19.8 and 15.6 hours, respectively.

2.3.4 Determination of bioavailability after intraperitoneal and oral administration:
Absolute bioavailability was determined as a ratio of dose normalized AUC after intraperitoneal or oral administration to dose normalized AUC after IV administration (Table 2.2). The bioavailability after intraperitoneal administration was 1.3% and that after oral administration was 22% with the suspension. The bioavailability after intraperitoneal was very poor, indicating that intraperitoneal may not a favorable route of administration to get reproducible plasma and brain exposure of elacridar. The bioavailability of elacridar after oral administration was higher than after an equivalent dose administered intraperitoneally, this could be due to enhanced dissolution in the gut due to presence of bile salts. The half-life of elacridar after oral administration was ~ 5 times longer than after IV or IP administration. There seems to be non-linearity in absorption, distribution and possibly elimination of elacridar. The bioavailability has been calculated with the assumption that there is no change in the clearance of the drug with increase in plasma exposure of the drug.
2.4 Discussion

Elacridar is a third generation inhibitor of the transporters P-gp and BCRP (Hyafil et al., 1993; Witherspoon et al., 1996). It has been used to determine the influence of P-gp and BCRP on the brain distribution of drugs that are substrates for P-gp and BCRP (Breedveld et al., 2005; Bihorel et al., 2007; Chen et al., 2009; Agarwal et al., 2010; Agarwal et al., 2011). It has also been suggested as a tool to improve the efficacy of drugs such as sunitinib in the treatment of glioma (Tang et al., 2012a). The objective of this study was to investigate the brain and plasma pharmacokinetics of elacridar in the mouse, and determine its bioavailability after oral and intraperitoneal administration.

The physicochemical properties of elacridar such as poor solubility and high lipophilicity could be responsible for the variability that is observed in plasma concentrations in this study. This inter-individual variability has been previously observed before in humans upon oral administration of elacridar (Kuppens et al., 2007) and was attributed to variability in dissolution of elacridar. In a study done in mice, the rate of dissolution of elacridar was also found to be a factor in limiting the plasma exposure of orally administered elacridar, despite increasing its dose (Ward and Azzarano, 2004). Oral dosing is a convenient route for drug administration in both rodents and humans, especially for chronic administration. Recently, it was shown that brain penetration of the tyrosine kinase inhibitor sunitinib, and its active metabolite is limited by P-gp and BCRP at the blood brain barrier. The oral administration of elacridar at a 100 mg/kg dose improved the brain penetration of sunitinib in wild-type mice by nearly 12-fold (Tang et al., 2012a; Tang et al., 2012b). Both plasma and brain exposure of elacridar after a 100
mg/kg oral dose were significantly higher than after intraperitoneal administration. The partition coefficient of elacridar in the brain after oral administration was 4.31, indicating good distribution of elacridar from systemic circulation into the brain. Interestingly, the elimination half-life after an oral dose of 100 mg/kg was ~ 20 hours, approximately 5-fold greater than the half-lives after intraperitoneal or intravenous dosing. There could be two possible reasons for this phenomenon. The increased oral half-life could be due to a slow dissolution rate (given the poor aqueous solubility) which limits the rate of absorption and thus leads to an extended release period. So the observed half-life might be a measure of a possible long absorption half-life (flip-flop kinetics). The finding that the observed Tmax in plasma was 8 hours post dose supports this hypothesis. Another reason for the extended half-life could be a change in hepatic or renal clearance of the drug due to the higher plasma exposures of elacridar. However, elacridar is not a potent inhibitor of any P450 enzymes in vitro (Ward and Azzarano, 2004), with the IC$_{50}$s in micromolar range. Irrespective of this finding, the results show that high dose administration of elacridar by the oral route can yield high exposures in plasma and brain. Tang et al., suggested that a 100 mg/kg dose could represent an effective dose in mice and indicated that there is no significant toxicity associated with administration of this dose (Tang et al., 2012a). Although toxicity was not evaluated in the current study, there was no visible elacridar related adverse findings after a single dose in mice. However, if a chronic dosing regimen is to be considered, the concerns for toxicity cannot be dismissed, especially due to the high plasma concentrations and the extended half-life of elacridar that are seen with this dose.
Intraperitoneal dosing is also an attractive option for chronic dosing, especially in preclinical species. The mice that received elacridar intraperitoneally, showed a much lower plasma exposure as compared to oral administration after an equivalent dose. This may most likely be due to poor dissolution and/or absorption of the drug from the peritoneal cavity. The plasma and brain concentrations also showed inter-animal variability. The intraperitoneal route of administration, while convenient to dose chronically in mice, resulted in a reduced plasma exposure of elacridar, therefore, the applications of an intraperitoneal dosing regimen with the current suspension formulation are limited. The Kp (AUC\text{brain}/AUC\text{plasma}) ratio after an intraperitoneal dose of 100 mg/kg was 0.48. In a similar study by Padowski and Pollack (Padowski and Pollack, 2010), an intraperitoneal dose of 10 mg/kg yielded a Kp of 0.0784. Together, these findings indicate the presence of non-linearity in plasma and brain exposures with increasing dose, especially after IP dosing.

Intravenous dose was used as the reference for calculating the bioavailability after the other two routes of administration. After an intravenous dose of 2.5 mg/kg, the AUC in plasma and brain of elacridar were approximately equal yielding a Kp ratio of 0.82 (Table 2.1). Despite observing good brain distribution of elacridar after intravenous administration it is not a viable option for chronic dosing in mice due to difficulties in performing repeated tail vein injections. Moreover, the dosing solution prepared for IV administration is unstable and prone to precipitation of the drug.

The bioavailability of elacridar appears to be limited by its poor physicochemical properties. The bioavailability of elacridar after oral administration was about 22%, while
that after intraperitoneal administration was only about 1%. The higher AUC_{plasma} after oral administration could be due to the solubilizing effect of bile salts in the gut. Oral administration of elacridar appears to be the most effective way to achieve plasma exposures necessary to effectively inhibit P-gp and BCRP at the BBB.

There were a couple of significant findings in this study, related to the brain penetration of elacridar after the different routes of administration. First, K_p ratio for elacridar (a measure of its brain distribution) was found to be dependant on its plasma exposure (Figure 2.4). When the plasma exposure was high, as seen after oral and IV administration, the K_p ratio was greater than one, with the highest K_p ratio of ~ 5 seen after oral dosing which yielded the highest plasma exposure. The K_p ratio after the intraperitoneal route of administration, that resulted in a low plasma exposure, was less than one. Second, the brain-to-plasma concentration ratios plotted as a function of time for all routes of administration showed an increase to a maximum value followed by a decrease (Figures 2.1.b., 2.2.b., 2.3.b.). This was unexpected because after reaching a steady-state in the tissue of distribution (pseudo distributional equilibrium), the brain to plasma concentration ratio should remain constant. One explanation for both these findings can be the active efflux of elacridar from the brain by P-gp and BCRP. Elacridar inhibits both these transporters at the BBB and it is possible that the mechanism behind the inhibitory action might be competitive (due to it being a substrate for the two transporters). This has been shown to be true in a study that used PET imaging and transporter knockout mice to study the influence of P-gp and BCRP on elacridar distribution at the BBB (Kawamura et al., 2011a; Kawamura et al., 2011b). The above
finding is currently being investigated in another study. However it does explain why brain exposures are dependent on plasma concentrations. As the plasma concentrations drop below levels that are required to saturate efflux at the BBB, the net efflux from the brain could become larger than the passive diffusion into the brain. This would result in the brain concentrations decreasing more rapidly than the corresponding plasma concentration, ultimately resulting in a decreasing trend in brain to plasma ratios with respect to time.

Concurrent administration of elacridar with drugs that are substrates for P-gp and BCRP improves their distribution across the BBB, and could lead to improved efficacy. The study of elacridar pharmacokinetics in the brain is important if we consider the issue of target cells that are present behind an intact BBB in the invasive rim of a brain tumor or the normal brain. Distribution of elacridar in the brain tissue could possibly address the issue of target cells that express BCRP and P-gp and are therefore resistant to chemotherapy (Lu and Shervington, 2008). Elacridar in the brain could be effective in inhibiting P-gp and BCRP present on these cells, allowing the chemotherapeutic agents to act on them, possibly preventing recurrence of the tumor. A targeted approach for CNS delivery that employs elacridar, could also reduce the dose required to achieve effective brain concentrations thus reducing systemic toxicity.

The use of elacridar has been limited in preclinical and clinical situations partly due its poor solubility and poor bioavailability. It is an unmet need to improve the bioavailability and solubility of elacridar. There are several possible methods that can be explored,
including synthesis of water soluble prodrugs, preparation of solid dispersions, use of surfactants, cyclodextrins, and permeation enhancers.

In summary, this study has examined the pharmacokinetics of elacridar in plasma and brain in mice and determined its bioavailability after different routes of administration. The results from these experiments can be used to determine doses and routes of administration of elacridar for future studies.
2.5 Footnotes

This work was supported by National Institutes of Health - National Cancer Institute [CA138437] (W.F.E.) and a Faculty Development grant at the University of Minnesota (W.F.E.). Financial support for Sagar Agarwal was provided by the Doctoral Dissertation Fellowship from the University of Minnesota. Financial support for Ramola Sane was provided by the Ronald J. Sawchuk Fellowship and Rowell Fellowship.
Figure 2.1.a.: Elacridar concentrations in plasma and brain after a single intravenous dose of 2.5 mg/kg in FVB wild type mice.

Brain concentrations are higher at corresponding higher plasma concentration and decrease as plasma concentrations decrease. *, p<0.05. Mean ± S.D. (n = 4 at each time point)
Figure 2.1.b.: Brain to plasma concentration ratio for elacridar after a single intravenous dose of 2.5 mg/kg in FVB wild type mice.

The brain-to-plasma concentration ratio is greater than one at initial time points and then drops below unity as plasma concentration decreases.
Figure 2.2.a.: Elacridar concentrations in plasma and brain after a single intraperitoneal dose of 100 mg/kg in FVB wild type mice.

Brain concentrations are significantly lower than plasma concentrations indicating that brain delivery of elacridar after intraperitoneal administration is limited. * p<0.05. Mean ± SD (n = 4 at each time point)
Figure 2.2.b.: Brain to plasma concentration ratio for elacridar after a single intraperitoneal dose of 2.5 mg/kg in FVB wild type mice.

The brain-to-plasma concentration ratio is less than unity at all time-points indicating poor brain distribution. Data presented as mean ± S.D. (n = 4 at each time point)
Figure 2.3.a: Elacridar concentrations in plasma and brain after a single oral dose of 100 mg/kg in FVB wild type mice.

Brain concentrations are significantly lower than plasma concentrations at early time points and higher than plasma at later time points. *, p<0.05. Mean ± SD (n = 4 at each time point)
Figure 2.3.b.: Brain to plasma concentration ratios for elacridar after a single oral dose of 100 mg/kg in FVB wild type mice.

The brain-to-plasma concentration ratio is less than unity at initial times, but increase with time. Data presented as mean ± S.D. (n = 4 at each time point).
Figure 2.4: Kp ratio (AUCbrain/AUCplasma) of elacridar after different routes of administration as a function of the AUCplasma

The brain penetration of elacridar in mice is a function of the plasma AUC; as the plasma AUC increases, the brain partitioning ratio of elacridar increases to greater than 1.
Table 2.1: Plasma and brain pharmacokinetic parameters calculated by noncompartmental analysis after administration of a single dose of elacridar in FVB wild type mice

<table>
<thead>
<tr>
<th>Method</th>
<th>Plasma</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>Half life (hr)</td>
<td>Cmax (ug/ml)</td>
</tr>
<tr>
<td>Plasma</td>
<td>4.4</td>
<td>0.5 ± 0.14</td>
</tr>
<tr>
<td>Brain</td>
<td>1.5</td>
<td>1.1 ± 0.38</td>
</tr>
<tr>
<td>IP</td>
<td>Half life (hr)</td>
<td>Cmax (ug/ml)</td>
</tr>
<tr>
<td>Plasma</td>
<td>4.3</td>
<td>0.29 ± 0.1</td>
</tr>
<tr>
<td>Brain</td>
<td>9.2</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>PO</td>
<td>Half life (hr)</td>
<td>Cmax (ug/ml)</td>
</tr>
<tr>
<td>Plasma</td>
<td>20</td>
<td>0.78 ± 0.1</td>
</tr>
<tr>
<td>Brain</td>
<td>16</td>
<td>4.3 ± 0.79</td>
</tr>
</tbody>
</table>
Table 2.2: Plasma pharmacokinetics and bioavailability calculated by noncompartmental analysis of data from IV, IP and PO administration

<table>
<thead>
<tr>
<th>Route</th>
<th>Dose (mg/kg)</th>
<th>CL/F (ml/min)</th>
<th>Vd/F (L)</th>
<th>T_{1/2} (hr)</th>
<th>AUC_{(0-inf)} (ug*min/ml)</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
<td>100</td>
<td>2.05</td>
<td>3.5</td>
<td>20</td>
<td>1460</td>
<td>0.22</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>100</td>
<td>33.2</td>
<td>12.3</td>
<td>4.3</td>
<td>90.3</td>
<td>0.013</td>
</tr>
<tr>
<td>Intravenous</td>
<td>2.5</td>
<td>0.46</td>
<td>0.17</td>
<td>4.4</td>
<td>161.4</td>
<td>1</td>
</tr>
</tbody>
</table>
CHAPTER III:
SATURABLE ACTIVE EFFLUX BY P-GP AND BCRP AT THE BLOOD-BRAIN BARRIER LEADS TO NON-LINEAR DISTRIBUTION OF ELACRIDAR TO THE CENTRAL NERVOUS SYSTEM

This manuscript is currently under review in the Journal of Pharmacology and Experimental Therapeutics.
The objective of this study was to investigate the factors that affect CNS distribution of elacridar. Elacridar is a P-gp and BCRP inhibitor that has been used to study the influence of P-gp and BCRP on brain distribution of chemotherapeutics. Adequate distribution of elacridar across the BBB and into the brain parenchyma is necessary to target tumor cells that may over-express these transporters and reside behind an intact BBB. We examined the effect of P-gp and BCRP on brain penetration of elacridar using FVB wild-type, Mdr1a/b(-/-), Bcrp1(-/-), and Mdr1a/b(-/-)Bcrp1(-/-) mice. Initially, mice were administered 2.5 mg/kg elacridar intravenously, and plasma and brain concentrations were determined. The brain-to-plasma partition coefficient of elacridar in wild-type mice was 0.82, as compared to 2.1 in Mdr1a/b(-/-) mice, 6.6 in Bcrp1(-/-) mice and 15 in Mdr1a/b(-/-)Bcrp1(-/-) mice, indicating that both P-gp and BCRP limit the brain distribution of elacridar. The four mouse genotypes were then administered increasing doses of elacridar and the observed brain partitioning of elacridar was modeled as a function of dose. The observed and model predicted maximum brain-to-plasma ratio (E_{max}) was the same across the four genotypes. However, the ED_{50} was lower for Mdr1a/b(-/-) mice compared to Bcrp1(-/-) mice. This correlates to the relative expression of P-gp and Bcrp at the BBB in these mice. The results from this study demonstrates the quantitative enhancement in elacridar CNS distribution as a function of its dose and provides useful information for its rational use as adjuvant therapy to improve targeting of chemotherapeutic agents to tumor cells in the brain parenchyma.
3.1 Introduction:

The blood-brain barrier (BBB), also known as the neurovascular unit, is a complex anatomical and biochemical system of capillary endothelial cells, pericytes, astrocytes and neurons that together perform a barrier function to protect the CNS (Hartz and Bauer, 2010). P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) are two efflux transporters present in the BBB endothelial cells that have been implicated in limiting the brain distribution of several drugs. Consequently, inhibition of these transporters may improve the efficacy of substrate drugs that are intended for a CNS target. Many molecularly-targeted anti-tumor agents, including several tyrosine kinase inhibitors such as sorafenib, gefitinib, imatinib, dasatinib, cediranib, sunitinib and vemurafenib have limited brain distribution due to efflux by P-gp and BCRP (Breedveld et al., 2005; Bihorel et al., 2007; Chen et al., 2009; Lagas et al., 2009; Agarwal et al., 2010; Lagas et al., 2010; Agarwal et al., 2011b; Mittapalli et al., 2012; Tang et al., 2012; Wang et al., 2012). Elacridar is a potent inhibitor of P-gp and BCRP (Hyafil et al., 1993; Allen et al., 1999) and its co-administration with many of these drugs, and other P-gp/BCRP substrates, has been shown to increase their brain distribution by several fold in mice (Bihorel et al., 2007; Chen et al., 2009; Lagas et al., 2009; Agarwal et al., 2010; Lagas et al., 2010; Agarwal et al., 2011b; Tang et al., 2012). Lack of adequate drug distribution to brain tumors has been thought to hamper effective treatment of lethal malignancies like gliomas (Agarwal et al., 2011a). Effective chemotherapeutic treatment of tumors in the brain requires that the drug traverse an intact BBB and achieve sufficient concentration in the cell of interest in the brain. However, as
mentioned above, several anti-cancer agents used for treatment of brain tumors fail to effectively enter the brain, primarily due to P-gp- and BCRP-mediated active efflux at the BBB.

Elacridar has been used to improve distribution of paclitaxel in brain tumor models (Hubensack et al., 2008). It could potentially be used along with other therapeutic molecules to enhance their CNS distribution and thus improve efficacy, especially in tumor cells that reside behind an intact BBB (Agarwal et al., 2011a). Another important aspect of treatment of brain tumors is the development of resistance to drugs. Expression of P-gp and BCRP in the tumor cells and tumor initiating cells forms a second barrier to drug delivery. This causes efflux of drugs from the tumor cells, further lowering their efficacy (Lu and Shervington, 2008). Adequate distribution of elacridar itself into the brain parenchyma would be necessary to inhibit efflux of drugs from such tumor cells and thus overcome this second barrier. This could lead to improved efficacy and possibly reduce recurrence of the tumor. Given that elacridar must effectively inhibit the transporters at both the BBB and the invasive tumor cells that reside behind an intact BBB, the primary objective of this paper is to examine the dose-dependent factors that influence the distribution of elacridar across the BBB into the brain.

Elacridar efflux by P-gp and BCRP at the BBB is a saturable process and therefore its brain distribution would be predicted to be concentration-dependent. This was suggested by experiments using radiolabeled elacridar and PET imaging in mice with genetic deletions of P-gp or BCRP or both (Dorner et al., 2009; Kawamura et al., 2011a; Kawamura et al., 2011b). Moreover, elacridar distribution into the brain has been
suggested to be influenced by the administered dose (Kawamura et al., 2011b). In a previous study from our group, (Sane et al., 2012) we showed that the brain partitioning of elacridar in wild-type mice was a function of its plasma exposure. We hypothesized that P-gp and BCRP at the BBB could be instrumental in affecting the brain distribution of elacridar. In the current study, we examine this phenomenon by using the FVB mouse model with genetic deletions of P-gp (Mdr1a/b(-/-)) or BCRP (Bcrp1(-/-)) or both (Mdr1a/b(-/-)Bcrp1(-/-)).

The brain distribution of elacridar has not been carefully characterized despite its widespread use in preclinical studies examining its effects on transport of substrate drugs across the BBB. The aim of this study was to examine in detail the factors, including transporters at the BBB and administered dose, which would work in concert to influence the distribution of elacridar across the BBB into the brain. The results from this study will be useful to formulate an elacridar dosing strategy that could be used to successfully target drug molecules that are substrates for efflux proteins, to invasive tumor cells that reside behind an intact BBB.
3.2 Materials and Methods:

Elacridar [GF120918; N-[4-[(6,7-dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)ethyl]-5-methoxy-9-oxo-10H-acridine-4-carboxamide]] was purchased from Toronto Research Chemicals, Inc. (Ontario, Canada). Radiolabeled ^14^C dasatinib was obtained from Bristol Myers-Squibb (New Jersey). All other chemicals used were HPLC or reagent grade and were obtained from Sigma-Aldrich (St. Louis, MO).

3.2.1 Cell culture studies:

Intracellular accumulation of elacridar was studied in epithelial Madin-Darby canine kidney II (MDCKII) cells expressing either human P-gp (MDCKII-MDR1 cell line) or murine Bcrp (MDCKII Bcrp1 cell line) that were kindly provided by Dr. Piet Borst and Dr. Alfred H. Schinkel (The Netherlands Cancer Institute). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 ug/ml), and amphotericin B (250 ng/ml) and maintained at 37°C with 5% CO2 under humidifying conditions. Confluent monolayers of cells were obtained by seeding at a cell density of 10^5^ cells/well and incubation for 3 days. On the day of the experiment, the cells were washed twice with pre-warmed cell assay buffer at 37°C.

3.2.2 Accumulation of elacridar in MDCKII cells:

The accumulation of elacridar was examined in MDCKII wild-type, MDR1 transfected and Bcrp1 transfected cells. The experiment was initiated after a 30 minute pre-incubation during which the cells were equilibrated with 1 ml of cell assay buffer. The cells were then incubated with buffer containing increasing concentrations of elacridar,
ranging from 50 nM to 10 uM, for 1 hour in an orbital shaker at 37°C. The experiment was terminated by aspirating the drug solution and washing with ice-cold phosphate-buffered saline. A 1% Triton X-100 solution (0.5 ml) was added to each well to solubilize the cells, and the protein concentration in the solubilized cell fractions was determined by the BCA protein assay (Thermo Fisher Scientific). Concentrations of elacridar in 200 µl aliquot of cell lysate were determined by LC-MS/MS. The amounts of elacridar in the cell lysates were normalized by the protein concentration in the respective wells and expressed as ng/microgram of protein. The amount accumulated in the cells was described as function of the concentration of elacridar in the incubating medium. The accumulation of elacridar in the wild-type cells was described by a linear model. The intracellular accumulation of elacridar in MDRI and Bcrp1 transfected cells was described by an $E_{\text{max}}$ model. Phoenix WinNonlin 6.2 (Pharsight, Mountain View, CA) was used to fit a simple $E_{\text{max}}$ model to the data using the following equation;

$$E = \frac{E_{\text{max}} \times C}{EC_{50} + C},$$  \hspace{1cm} \text{Equation (1)}$$

where, $E$ is the cellular accumulation of elacridar (ng of elacridar/µg of protein), $E_{\text{max}}$ is the maximum accumulation predicted at concentration of elacridar that saturate the efflux process. $EC_{50}$ is the concentration of elacridar at which half maximal accumulation is seen and $C$ is the concentration of elacridar in µM in the incubating medium.

3.2.3 Accumulation of $^{14}$C dasatinib with increasing concentrations of elacridar:

The efficacy of elacridar in inhibiting the efflux of a prototypical dual substrate, radiolabeled dasatinib, by P-gp and BCRP was determined by measuring the accumulation of dasatinib in MDCKII-MDRI cells and MDCKII-Bcrp1 cells.
respectively. The experiment was initiated by pre-incubating the cells for 30 min with increasing concentrations of elacridar from 50 nM to 20 µM. The cells were then incubated with trace amounts of $[^{14}\text{C}]$ dasatinib in the presence of varying concentrations of elacridar for 1 hour. The experiment was terminated as described in the previous section. The radioactivity (disintegrations per minute, dpm) associated with 100 µl of cell lysate was determined by liquid scintillation counting (LS-6500; Beckman Coulter, Fullerton, CA). The observed radioactivity was normalized by protein concentration in each well and dasatinib accumulation was expressed as dpm/µg of protein. Dasatinib accumulation in absence of elacridar was considered as control, and the accumulation in presence of elacridar was expressed as percent of control.

The nonlinear relationship of dasatinib accumulation in the transfected cells as a function of elacridar concentration was quantitated. Phoenix WinNonlin 6.2 (Pharsight, Mountain View, CA) was used to fit a simple $E_{\text{max}}$ model to the data, using equation (1); where, $E$ is the accumulation of dasatinib (ng of dasatinib/µg of protein), $E_{\text{max}}$ (ng/µg) is the maximum accumulation at concentrations of elacridar that saturate the efflux process, $EC_{50}$ (µM) is the concentration of elacridar at which half maximal inhibition is seen, and $C$ is the concentration of elacridar in the incubating medium (µM).

3.2.4 In vivo studies:

In vivo studies were carried out in FVB (wild-type) mice, $Mdr1a/b(-/-)$ (P-gp knockout), $Bcrp1(-/-)$ (Bcrp knockout) and $Mdr1a/b(-/-)Bcrp1(-/-)$ (triple knockout) that were 8-10 weeks old (Taconic Labs, Hudson, NY). Animals were maintained under a 12-hour light/dark cycle, temperature-controlled environment and free access to food and water at
all times. All studies were carried out in accordance with guidelines set by Principles of Laboratory Animal Care (National Institutes of Health, Bethesda, MD) and approved by the Institutional Animal Care and Use Committee of University of Minnesota.

3.2.5 Plasma and brain pharmacokinetics of elacridar in FVB mice:
An elacridar dosing solution for intravenous administration was prepared in DMSO, propylene glycol, and water (4:4:2 v/v/v) at a concentration of 1.25 mg/ml. FVB mice (P-gp knockout, Bcrp knockout, triple knockout) received a dose of 2.5 mg/kg elacridar intravenously via tail vein injection. Animals were sacrificed at 0.5, 1, 2, 4, and 8 hours post dose (n=4 at each time point) by using a carbon dioxide chamber, and blood and brain were rapidly collected. Blood was collected by cardiac puncture and transferred to heparinized tubes. Brain was removed from the skull, washed with ice-cold saline and flash-frozen with liquid nitrogen. Plasma was separated by centrifugation at 7500 rpm for 10 minutes at 4°C. Both plasma and brain specimens were stored at -80 °C until analysis by LC-MS/MS. Plasma and brain concentration-time profiles from wild-type animals for comparison were obtained from our previously published study.

The pharmacokinetic parameters for elacridar in plasma and brain for all mouse genotypes were determined by non-compartmental analysis using Phoenix WinNonlin 6.2. The tissue partition coefficient (Kp) for the brain was calculated as the ratio of the area under the concentration-time curve for brain (AUC_{brain}) to the area under the concentration-time curve for plasma (AUC_{plasma}). Comparisons between the AUCs in plasma and brain were carried out using the method described by Nedelman et al. for AUCs measured after destructive sampling (Bailer, 1988; Nedelman et al., 1995).
3.2.6 Dose dependent distribution of elacridar in FVB mice:
Dosing solutions for intravenous administration of elacridar were prepared in DMSO, propylene glycol, and water (4:4:2 v/v/v) at elacridar concentrations of 0.25 mg/ml, 0.5 mg/ml, 1.25 mg/ml, 2.5 mg/ml and 5 mg/ml. All four mouse genotypes (wild-type, P-gp knockout, Bcrp knockout, triple knockout) received intravenous doses of 0.5 mg/kg, 1 mg/kg, 2.5 mg/kg, 5 mg/kg and 10 mg/kg (n= 4 for each dose and for each genotype) by injection into the tail vein. Animals were sacrificed at 1 hour post dose by a carbon dioxide chamber. Blood and brain were collected as described in the previous section.

3.2.7 Calculation of ED$_{50}$:
The effective dose for half-maximal brain penetration of elacridar was estimated by measuring brain and plasma concentrations of elacridar at various doses and calculating a mean brain-to-plasma ratio at each dose in all mouse genotypes. A simple E$_{max}$ model was fit to the mean brain-to-plasma ratio data as a function of elacridar dose (mg/kg) using Phoenix WinNonlin 6.2,

$$E = \frac{E_{max} \times D}{ED_{50} + D}, \quad \text{Equation (2)}$$

where, E is the brain-to-plasma ratio, $E_{max}$ is the maximum brain-to-plasma ratio, D is the administered elacridar dose in mg/kg, and ED$_{50}$ is the dose required to produce a half-maximal brain-to-plasma ratio.

3.2.8 Analysis of elacridar by liquid chromatography / mass spectrometry
The concentration of elacridar in plasma and brain homogenate specimens was determined by high-pressure liquid chromatography with tandem mass spectrometric detection as described in Chapter II. Frozen samples stored at -80°C were thawed at room
temperature. Brain samples were homogenized with a tissue homogenizer (Thermo
Fisher Scientific) along with addition of three volumes of ice-cold 5 % bovine serum
albumin solution in distilled water. A 50 µl aliquot of plasma and 100 µl aliquot of brain
homogenate were spiked with 50 ng of the internal standard, tyrphostin (AG1478). The
samples were extracted by addition of 100 µl of a pH 11 buffer (0.1 M sodium hydroxide
and 0.04 M sodium bicarbonate) and 1 ml of ice-cold ethyl acetate. Samples were
centrifuged for 15 min at 7500 rpm at 4 ºC and 700 µl of the organic supernatant layer
was transferred to fresh microcentrifuge tubes and dried under a gentle stream of
nitrogen. The dried extracts were reconstituted in mobile phase (20 mM Ammonium
formate and 1% formic acid: acetonitrile in a ratio of 58:42) and transferred to glass
autosampler vials. 5 µl volumes of reconstituted extract were injected by an autosampler
system that was maintained at 10 ºC into the HPLC system. Analytes were separated
using an Agilent Technologies Eclipse XBD-C18. The LCMS/MS analysis of samples for
elacridar concentration were carried out as described earlier in Chapter II.

3.2.9 Modeling and Simulation:

A two-compartment pharmacokinetic model with “plasma” (central compartment) and
“brain” (peripheral compartment) compartments was created using STELLA (iSEE
sytems, Lebanon, NH, USA). The simulations were performed to examine the effect of
simultaneous passive diffusion and active efflux processes from the brain on the
distribution of a dual substrate, such as elacridar, to the brain. The effect of active efflux
processes from brain into the plasma was examined. Plasma and brain compartments
were linked by distributional flows which represent the rates of drug transfer between compartments.

The two compartments were connected by a bidirectional passive clearance (CL\textsubscript{pass}) and two active efflux processes (denoted as A and B) that could represent clearance due to two efflux transporters such as P-gp and BCRP. The “plasma” compartment has an additional clearance due to systemic elimination (CL\textsubscript{elim}).

The dose was administered as an intravenous bolus into the central compartment at time zero. The rate of elimination from the central compartment is expressed as,

\[
\text{Rate}_{\text{elimination}} = \text{CL}_{\text{elim}} \times C_{\text{plasma}}. \quad \text{Equation (3)}
\]

The passive diffusion into the brain compartment is described by,

\[
\text{Rate}_{\text{passive diffusion}} = \text{CL}_{\text{pass}} \times (C_{\text{plasma}} - C_{\text{brain}}). \quad \text{Equation (4)}
\]

The CL\textsubscript{pass} was assumed to be a constant in the models with two active efflux clearances from the brain as well as models that described either one active efflux clearance or no active efflux clearance.

The active efflux from the brain compartment was modeled using the Michaelis-Menten equation,

\[
\text{CL}_{\text{active}} = \frac{V_{\text{max}} \times C_{\text{brain}}}{K_m + C_{\text{brain}}}, \quad \text{Equation (5)}
\]

Where, CL\textsubscript{active}, is a non-linear, saturable efflux clearance, \( V_{\text{max}} \) reflects the transporter capacity or expression of the transporter, \( K_m \) is the affinity of the transporter for the compound. The two processes A and B were assigned a unique \( V_{\text{max}} \) and \( K_m \) for the two transporters, P-gp and Bcrp; based on their relative expression and relative affinity from in vivo results.
Four models were defined and simulations performed:

Model 1: Passive diffusion and two distinct active efflux processes (A and B) from the brain

Model 2: Passive diffusion and one active efflux process (A) from the brain

Model 3: Passive diffusion and one active efflux process (B) from the brain

Model 4: Only passive diffusion to and from the brain.

These models assume that total concentrations in the plasma and brain are available for transport. Moreover, it is further assumed that inhibition or genetic deletion of the transport systems do not influence the systemic elimination (CL_{elim}) of the substrate. This is often the case with substrates that are primarily metabolized, as in the case of the tyrosine kinase inhibitors.

For model 1, the rate of change of amount in the plasma can be represented as:

\[
V_p \times \frac{dC_p}{dt} = C_b \times \left[ CL_{pass} + \left( \frac{V_{max_A}}{Km_A + C_b} \right) + \left( \frac{V_{max_B}}{Km_B + C_b} \right) \right] - C_p \times \left[ CL_{pass} - CL_{elim} \right] \quad \text{Equation (6)}
\]

And the rate of change of amount in the brain can be described as,

\[
V_b \times \frac{dC_b}{dt} = (CL_{pass} \times C_p) - \left[ CL_{pass} + \left( \frac{V_{max_A}}{Km_A + C_b} \right) + \left( \frac{V_{max_B}}{Km_B + C_b} \right) \right] \times C_b \quad \text{Equation (7)}
\]

The brain distribution of a prototypical compound (such as elacridar) that is a substrate for two transporters (such as P-gp and BCRP) was simulated using this model. The values for \( V_{max} \) (\( V_{max} \) A = 50, \( V_{max} \) B = 150) were chosen to represent differences in the relative magnitude of the capacity of transporters such as P-gp and BCRP. \( K_m \) values for A and B were chosen to reflect possible differences in affinity for the two transporters A and B (\( K_m \) A = 0.1, \( K_m \) B = 10), based on results from in vivo studies. Simulations were
run to reflect the changes in the active efflux from the brain with change in dose administered, and how that would result in dose-dependent changes in brain distribution. In essence, the effect of dose escalation, and the subsequent increase in plasma concentrations on the brain-to-plasma ratios and the active clearance from the brain in all four models was examined.

3.2.10 Statistical Analysis
Comparison between groups were made using SigmaStat, version 3.1 (Systat Software, Inc., San Jose, CA). Statistical difference between two groups was tested by using the two-sample t-test, and significance was declared at $p < 0.05$. Multiple groups were compared by one-way analysis of variance with the Holm-Sidak post hoc test for multiple comparisons at a significance level of $p < 0.05$. 
3.3 Results:

3.3.1 Intracellular accumulation of elacridar in MDCKII cells

Intracellular accumulation of elacridar was examined in MDCKII wild-type cells as well as polarized epithelial MDCKII cells that overexpressed the transporter proteins, P-gp or BCRP. In wild-type cells, the accumulation of elacridar increased linearly with the concentration of elacridar in the incubating medium (Figure 3.1). In MDCKII MDRI and Bcrp1 transfected cells, the cellular accumulation showed a non-linear increase to a maximum value with increase in concentration in incubating medium (Figure 3.1). The accumulation of elacridar in MDRI and Bcrp1 transfected cells was plotted as a function of elacridar concentrations in the incubating medium, and was described by a simple E\text{max} model. At 50 nM, the accumulation of elacridar in the three cell lines was not significantly different from each other, however, at higher concentrations, the elacridar accumulation was significantly lower in the cell lines overexpressing P-gp and BCRP as compared to the wild-type cells (p < 0.05). This indicates that P-gp and BCRP both limit the intracellular accumulation of elacridar. The maximum accumulation of elacridar (E\text{max}) in the P-gp and BCRP over-expressing cell lines was calculated to be 26.82 ± 3.07 ng/µg of protein and 30.7 ± 7.3 ng/µg of protein, respectively. The EC\text{50} values were 10.65 ± 2.03 µM and 17.4 ± 5.9 µM for P-gp overexpressing and BCRP overexpressing cell lines, respectively.

3.3.2 Intracellular accumulation of dasatinib in presence of elacridar

Dasatinib is a tyrosine kinase inhibitor that is a substrate for both P-gp and BCRP (Chen et al., 2009; Lagas et al., 2009). We used radiolabeled dasatinib as a probe substrate to
observe the change in accumulation of dasatinib with increasing concentrations of elacridar. The accumulation of $[^{14}C]$ dasatinib increased non-linearly with increases in the concentration of elacridar (Figure 3.2). The maximal dasatinib accumulation ($E_{\text{max}}$) in $MDR1$ transfected cells was $1323 \pm 126 \%$ as compared to $1083 \pm 154 \%$ for $Bcrp1$ cells. The EC$_{50}$ for both the cell lines were equivalent, $1.01 \pm 0.37 \mu$M for $MDR1$ transfected cells and $1.01 \pm 0.58 \mu$M for $Bcrp1$ transfected cells. These results indicate the non-linearity in the inhibitory effect of elacridar on dasatinib accumulation with increasing elacridar concentration, most likely due to saturation of the efflux inhibitory process.

### 3.3.3 Plasma and brain disposition of elacridar in FVB mice

The plasma disposition and the brain distribution of elacridar were studied in FVB mice to understand the role of P-gp and BCRP on the distribution of elacridar to the brain. Mouse models used were FVB $Mdr1a/b$ (-/-), $Bcrp1$ (-/-) and $Mdr1a/b$(-/-)$Bcrp1$(-/-) and results from our previously published study using wild-type mice (Sane et al., 2012) were used for comparison.

Elacridar plasma concentrations after a 2.5 mg/kg intravenous dose were different between the four genotypes (Figure 3.3). This difference in plasma concentrations is reflected in the area under the plasma concentration-time curve (AUC$_{\text{plasma}}$). The AUC$_{\text{plasma}}$ in triple knockout mice was significantly (p<0.001) lower than the area under the curve for the other mouse genotypes (Table 3.1). In wild-type mice, the AUC$_{\text{plasma}}$ was significantly higher than both the single knockout mice [$Mdr1a/b$ (-/-) and $Bcrp1$ (-/-)]. Absence of P-gp and BCRP may allow elacridar to reach sites of metabolism in tissues
that are otherwise limited by the presence of efflux proteins. This could be one of the possibilities why the knockout mice have lower plasma exposure than wild-type mice. As expected for a P-gp and BCRP substrate, the brain concentration-time profiles differed markedly between the mouse genotypes (Figure 3.4). The maximum concentration observed (C$_{\text{max brain}}$) was different for various genotypes despite administration of the same dose. The C$_{\text{max}}$ for $Mdr1a/b(-/-)$ (1.6 ± 0.2 µg/g) was higher than that in wild-type mice (1 ± 0.3 µg/g) but lower than $Bcrp1(-/-)$ mice (2.9 ± 0.2 µg/g) and $Mdr1a/b(-/-)Bcrp1(-/-)$ mice (3.2 ± 0.3 µg/g) (Table 3.2). This difference in peak brain concentrations was also reflected in the area under the brain concentration-time profiles (AUC$_{\text{brain}}$). The absence of transporters at the BBB influenced the area under the curve; the AUC$_{\text{brain}}$ in $Mdr1a/b(-/-)Bcrp1(-/-)$ mice was nearly 10-fold higher than wild-type mice, AUC$_{\text{brain}}$ in $Bcrp1(-/-)$ mice was approximately 8-fold higher than wild-type and AUC$_{\text{brain}}$ in $Mdr1a/b(-/-)$ mice was about 3-fold higher than wild-type mice. The tissue-to-plasma partition coefficient for brain (Kp) reflects this, the values being 0.82 for wild-type, 3.5 for $Mdr1a/b (-/-)$ mice, 6.6 for $Bcrp1 (-/-)$ mice and 15 for $Mdr1a/b(-/-)Bcrp1(-/-)$ mice. These results are similar to the ‘greater than additive’ efflux effect seen for several drugs that are substrates for both P-gp and BCRP (Polli et al., 2009; Agarwal and Elmquist, 2012) and confirm that the two transporters, P-gp and BCRP, work together to keep elacridar out of the brain.

The half-life of elimination from the brain was found to be longer in $Mdr1a/b(-/-)Bcrp1(-/-)$ mice (5.4 hr) compared to the $Mdr1a/b(-/-)$ (3.2 hr), $Bcrp1(-/-)$ (2.9 hr) and wild-type mice (1.5 hr). The half-life in the brain corresponds to the brain C$_{\text{max}}$ in the
different genotypes. A longer half-life generally corresponds to a higher $C_{\text{max}}$ in the brain (see Table 3.2). The presence of P-gp and BCRP at the BBB decreases the half-life of elacridar in the brain. Figure 3.5 shows the brain and plasma concentration time profiles in the four different genotypes, plotted on different graphs with the same scale for visual comparison. The brain concentrations in the $Mdr1a/b(-/-)Bcrp1(-/-)$ mice were higher than the wild-type or mice with either $Mdr1a/b(-/-)$ or $Bcrp1(-/-)$ mice.

The brain-to-plasma concentration ratios with respect to time for the four genotypes are shown in Figure 3.6. These brain-to-plasma concentration ratios, when plotted with respect to time, showed an increase and then plateau to a distributional steady state in $Mdr1a/b(-/-)Bcrp1(-/-)$ mice. However, in wild-type, $Mdr1a/b(-/-)$ and $Bcrp1(-/-)$ animals, the brain-to-plasma ratios increased to a maximum value, and then decreased with time. Although, this may be counter-intuitive for a peripheral (brain) compartment in a linear system, it can be explained by the involvement of non-linear active processes in the distribution of elacridar to the mouse brain. P-gp and BCRP at the mouse BBB limit the distribution of elacridar into the mouse brain. The peculiar brain-to-plasma ratio versus time profiles can be explained by the active efflux of elacridar out of the brain. The concentrations in the brain decrease more rapidly compared to the plasma concentrations due to the active efflux of elacridar at the BBB, thus resulting in decreasing brain-to-plasma concentration ratios with time. This decrease in brain concentration is also enhanced at later times when the efflux clearance is not saturated by high plasma concentrations, as outlined in the next section.

3.3.4 Dose dependent brain distribution of elacridar in FVB mice
These experiments were carried out to determine if the efflux of elacridar from the BBB leads to non-linear distribution with increasing elacridar concentrations. The brain penetration of elacridar in various mouse genotypes was studied at one hour post intravenous injection. Mice were administered doses ranging from 0.5 mg/kg to 10 mg/kg, brain and plasma concentrations were measured, and brain-to-plasma concentration ratios were determined (Table 3.3). Elacridar brain-to-plasma concentration ratios increased non-linearly with dose in all four mouse genotypes (Figure 3.7). In the wild-type mice, the mean brain-to-plasma concentration ratio was 0.4 ± 0.1 at the lowest dose of 0.5 mg/kg, and 12 ± 1.1 at the highest dose of 10 mg/kg (Figure 3.7A). This shows that at lower doses of elacridar the brain penetration in wild-type mice is restricted, but with increasing doses, plasma concentrations that are sufficient to saturate the efflux transporters at the BBB are achieved, thus enhancing its brain distribution. Similar profiles were observed in the Mdr1a/b(-/-) mice (Figure 3.7C) and Bcrp1(-/-) mice (Figure 3.7B). In Mdr1a/b(-/-)Bcrp1(-/-) mice, brain-to-plasma concentration ratios were higher than those observed in the other genotypes even at the lower doses (Figure 3.7D). The brain-to-plasma concentration ratios in all genotypes showed an increasing trend reaching a maximum value at higher doses, except for wild-type mice. In the wild-type mice, higher doses that may have achieved brain-to-plasma concentration ratios closer to the maximum value could not be tested due to solubility limitations of elacridar which prevented higher dosing using the same vehicle. At the lowest dose of 0.5 mg/kg, the brain-to-plasma ratio in Mdr1a/b(-/-)Bcrp1(-/-) mice was significantly higher than the other genotypes ($p<0.05$). However at 10 mg/kg, this
difference was abolished and the brain-to-plasma ratios were not significantly different from each other \((p<0.05)\), suggesting that at higher plasma concentrations of elacridar the distribution limiting effect of P-gp and BCRP is overcome (due to their saturation), and there are no significant differences in brain penetration between the different mouse genotypes. These results confirm our hypothesis that brain distribution of elacridar is limited by saturable active efflux mediated by P-gp and BCRP at the mouse BBB. The brain-to-plasma concentration ratios versus dose profiles in all genotypes were described by a simple \(E_{\text{max}}\) model (Figure 3.7, Table 3.4). The model predicted maximum brain-to-plasma ratio \((E_{\text{max}})\) was approximately 20, 10, 14 and 12 in the wild-type, \(Mdr1a/b(-/-)\), \(Bcrp1(-/-)\) and \(Mdr1a/b(-/-)Bcrp1(-/-)\) mice, respectively.

### 3.3.5 Simulation of Brain Distribution and Impact of Saturable Active Efflux Processes

Simulations were carried out to examine the factors that affect the brain distribution of a compound that is a substrate for two separate active processes (Figure 3.8). Model 1 (equivalent to wild-type mice) had two distinct active efflux processes in addition to a passive process between the central and the peripheral compartment. Models 2 and 3 (equivalent to \(Mdr1a/b(-/-)\) or \(Bcrp1(-/-)\) mice) had one active process and passive diffusion that linked the brain and plasma compartments. Model 4 (equivalent to \(Mdr1a/b(-/-)Bcrp1(-/-)\) mice) had only passive diffusion between the plasma and the brain compartment. The effect of increasing dose on the active clearance was examined in model 1 (Figure 3.9). The active clearance from the brain is dependent on the concentration of the compound in the brain, when the clearance is a saturable process of
the Michaelis-Menten type (see Equation 5). The increase in dose, and the consequent increase in the brain concentration, saturates the efflux from the brain compartment, leading to a lower CL active from the brain as illustrated in Figure 3.11.

The brain-to-plasma ratios were also simulated for the same dose across the four models (Figure 3.10). The brain-to-plasma concentration ratios in model 4 showed an increase to a distributional steady state level. This is consistent with observed data for the Mdr1a/b(-/-)Bcrp1(-/-) mice and is expected for distribution into a peripheral compartment with only passive diffusion. The brain-to-plasma ratios in models 1, 2 and 3, when plotted against time, show an increase to a maximum value, followed by a decrease to an eventual steady state distribution. This corresponds to the values obtained experimentally for wild-type Mdr1a/b(-/-) mice and Bcrp1(-/-) mice (Figure 3.6). The distinctive pattern of the observed brain-to-plasma ratios in these mice is therefore a function of the active efflux out of the brain.

The brain-to-plasma concentration ratios at peak brain concentrations for models 1, 2, 3 and 4, with an increasing dose, were modeled as a function of time (Figure 3.11). The brain-to-plasma concentration ratios for models 1, 2 and 3 could be described by a simple E max model. This correlates well to the data obtained experimentally for wild-type, Mdr1a/b(-/-) mice and Bcrp1(-/-) mice. This simple nonlinear distribution model is therefore successful in describing the distribution of a compound with multiple active effluxes and passive diffusion between two compartments, such as elacridar.
3.4 Discussion:

The objective of this study was to characterize the influence of P-gp and BCRP at the mouse BBB on the non-linear CNS distribution of elacridar. This compound has been widely used as a P-gp and BCRP inhibitor, both in vitro and in vivo. However, the brain distribution of elacridar has not been fully characterized. Earlier studies using radiolabeled elacridar and positron emission tomography were the first to qualitatively examine the CNS distribution of elacridar-associated radioactivity in wild-type, P-gp knockout, Bcrp knockout, and triple knockout mice (Dorner et al., 2009; Kawamura et al., 2011a; Kawamura et al., 2011b). The results from those studies indicated that P-gp and Bcrp could play a role in limiting the brain distribution of elacridar. Our previous study (Sane et al., 2012) was the first to quantitatively examine the brain distribution of elacridar in wild-type mice using a specific assay (LC-MS/MS). The current study examined various factors, including P-gp and Bcrp expression at the BBB and the dose of elacridar; each of which could influence the nonlinear brain distribution of elacridar. Results from this study will enable us to understand the mechanisms that influence the brain distribution of this important transport inhibitor.

Elacridar has been used to improve brain distribution of paclitaxel in mouse brain tumor models (Hubensack et al., 2008). Co-administration of elacridar with chemotherapeutic drugs such as topotecan and paclitaxel, has been found to improve the brain distribution of these drugs (Kemper et al., 2003; de Vries et al., 2007). As such, elacridar could be used as an adjuvant in glioma chemotherapy, and could be particularly useful in targeting invasive tumor cells that reside behind an intact BBB (Lu and Shervington, 2008;
Agarwal et al., 2011a). Some tumor cells also express P-gp and Bcrp in the cell plasma membrane which contributes to the development of resistance to chemotherapy by active efflux of drugs from the tumor cells (Lu and Shervington, 2008). Therefore, elacridar must inhibit the transporters at the BBB as well as at the tumor cells in the brain parenchyma to effectively improve drug delivery to brain tumors. In order to do this, the distribution of elacridar itself into the brain parenchyma is important.

In vitro studies conducted in MDCKII cells confirmed that elacridar is a substrate for P-gp and Bcrp. The active efflux of elacridar due to P-gp and Bcrp is saturable, and increase in elacridar concentration, causes a decrease in the efflux clearance (See Figure 1), leading to increase in cellular accumulation. The inhibitory effect of elacridar is also dependent on its concentration non-linearly. A maximal possible inhibition of the transporters can be achieved only with high concentrations of elacridar (see Figure 3.2).

Dasatinib was chosen as a probe substrate since it is a dual substrate for P-gp and Bcrp (Chen et al., 2009; Lagas et al., 2009), and is also being considered as a candidate tyrosine kinase inhibitor for the treatment of glioblastoma (Reardon et al., 2012).

Elacridar pharmacokinetic studies in mice indicate lower plasma exposure in the transporter deficient mice compared to wild-type. Plasma concentrations of elacridar after an intravenous dose of 2.5 mg/kg in the wild-type were significantly higher than the concentrations in other mouse genotypes (See figure 3.3). While this result is unexpected regarding effects on systemic clearance, it is possible that plasma concentrations are lower in the knockout models, because genetic deletion of the transporters could allow elacridar to reach sites of metabolism in tissues that are otherwise restricted. The specific
mechanisms behind this finding are beyond the scope of the current study, but may be of interest for future work.

Elacridar brain concentrations in the knockout mice were significantly higher than in the wild-type mice. The half-lives of elacridar in the brains of wild-type, P-gp knockout and Bcrp knockout mice were shorter than the half-life in plasma (Table 3.1). This can be attributed to the active efflux of elacridar from the brain into the plasma. In the absence of either, or both, of these transporters, the half-life in the brain was prolonged, as seen in the triple knockout mice. The ratio of \( \text{AUC}_{\text{brain}} \) to \( \text{AUC}_{\text{plasma}} \) (Kp, or tissue partition coefficient) in the wild-type mice was lower than in the knockout mice. The Kp for P-gp knockout mice was 3.5 as compared to 6.6 for Bcrp knockout mice and 15 for triple knockout mice. This greater than additive effect for brain distribution seen in mice with genetic deletion or pharmacological inhibition of both transporters has been observed before with several dual substrates of P-gp and Bcrp such as sorafenib, gefitinib, laptinib, dasatinib (Chen et al., 2009; Lagas et al., 2009; Polli et al., 2009; Agarwal et al., 2010; Lagas et al., 2010; Agarwal et al., 2011b) Together, these results confirm that P-gp and BCRP work in concert at the mouse BBB to restrict the brain distribution of elacridar.

In our previous study, we observed that the brain-to-plasma concentration ratio in wild-type mice reached a maximum and then decreased with time after different routes of administration (Sane et al., 2012). We hypothesized that the decrease in the brain-to-plasma concentration ratios occurred because the brain concentrations of elacridar decrease faster than the corresponding plasma concentrations, and that this decrease could be due to a concentration-dependent (and hence time dependent following a single
dose) change in the active efflux clearance of elacridar from the brain by P-gp and Bcrp at the BBB. To test this hypothesis, we examined the brain-to-plasma concentration ratios in the various mouse genotypes. The brain-to-plasma ratios in wild-type, P-gp knockout and Bcrp knockout mice showed an increase to a maximum value and then decreased with time (Figure 3.6). However, in the triple knockout mice, the brain-to-plasma ratio increased to a maximum and reached a plateau at the distributional steady state value. This confirms our hypothesis that the decrease in brain-to-plasma ratios is a function of the active efflux clearance, processes mediated by P-gp and BCRP, and that the distribution of elacridar into the brain could be a non-linear process, which is saturable by changes in concentration.

Saturable efflux of elacridar from brain was then evaluated by studying the dose-dependent brain distribution of elacridar after intravenous administration in the four mouse genotypes. The observed brain-to-plasma concentration ratio was modeled as a response to elacridar dose (Figure 3.7). An interesting result from the experiment was that the maximum brain distribution ($E_{\text{max}}$) seen in all mouse genotypes was similar (Table 3.3 and 3.4). This indicates that at higher plasma concentrations there is a saturation of the relevant efflux transporter(s) leading to an increased distribution of elacridar into the brain, i.e., the maximum brain distribution when efflux clearances are at their minimum, where brain distribution is determined by passive diffusion which is theoretically equivalent amongst the genotypes. The $ED_{50}$ for the different mouse genotypes was varied significantly. It was approximately 0.3 mg/kg for triple knockout mice, indicating that a very low dose is required to achieve the half-maximal effect (i.e.,
brain distribution coefficient of elacridar at one hour post dose). For P-gp knockout mice, the ED$_{50}$ was approximately 0.77 ± 0.2 mg/kg, while for Bcrp knockout mice it was greater (1.7 ± 0.72 mg/kg) (Table 3.4). This difference in the ED$_{50}$ values could be attributed to the relative expression of P-gp and Bcrp at the mouse BBB. In mouse brain microvessel endothelial cells isolated from FVBn mice, the expression of P-gp is greater (~ 5-fold) relative to BCRP, as determined by quantitative proteomics (Agarwal et al., 2012). As a result, a lower dose is required to achieve half maximal effect when only BCRP is present at the BBB (P-gp knockout mice) whereas a larger dose is required to overcome the P-gp efflux in the Bcrp knockout mice. In wild-type mice, the ED$_{50}$ is the greatest among all mouse genotypes. In the wild-type mice, both P-gp and Bcrp work in tandem to restrict elacridar distribution to the brain.

A compartmental model was created in STELLA® to explain the dose-dependent and saturable behavior of the transporters at the BBB. This simple non-linear model can be instructive because the low solubility of elacridar limits the number of doses and the amount of elacridar that can be administered to mice, and therefore a simulation will be helpful in exploring distribution behavior over a wide range of doses and concentrations. The brain distribution of a compound that is a dual substrate for two transporters was modeled as a function of dose and time. Under these conditions and assumptions, model simulations show that the active efflux from the brain is a function of the dose administered, and, that at higher doses; the active efflux can be saturated (resulting in behavior depicted in Figure 3.9). The brain-to-plasma concentration ratio profiles in the four genotypes that were determined experimentally, showed a distinctive pattern for
each genotype (Figure 3.6). We expected that these patterns would be a result of active transport of elacridar from the brain. The compartmental models helped explain the brain-to-plasma concentration profile. If one compares Figure 3.6 (experimentally derived brain-to-plasma ratios for the four genotypes) with Figure 3.10 (model predicted brain-to-plasma ratios), it can be readily seen that the simple dual transporter model can easily explain the changes in brain distribution of elacridar as the dose increases. From experimental data, brain-to-plasma coefficient ratio at maximum brain concentration with increasing dose could be described by the Emax equation in the various mouse genotypes (Figure 3.7). These simulations also correspond to data obtained that was described by an Emax model (compare figures 3.7 and 3.11), indicating that the equilibrium distribution coefficient (Kp) of a substrate, while limited by the presence of the efflux transporters, can be increased by increasing the dose. Our model was useful in understanding the various transport processes and the role played by the dose, and hence concentration, in influencing brain distribution.

In summary, this study shows that brain distribution of elacridar is influenced by P-gp and Bcrp mediated active efflux at the BBB. We show that there is a greater than additive increase in elacridar transport across the BBB when these two transporters are absent. Moreover, we show that efflux of elacridar out of brain is a saturable process such that maximal brain penetration and targeting are seen at higher doses, i.e., doses that yield systemic concentrations high enough to saturate P-gp and Bcrp at the BBB. Thus the results from this study delineate the various distributional clearance mechanisms that regulate the brain distribution of elacridar. The dose of administered elacridar and the
expression of the efflux pumps at the BBB are two important factors that influence the rate and extent of distribution of elacridar into the brain. The results from this study contribute to our understanding of how the distribution of elacridar at the BBB is affected by efflux transport processes, and will be useful in the formulation of elacridar dosing strategies that could be used to enhance the distribution and targeting of molecularly-targeted agents, that are substrates for both P-gp and Bcrp, to the sites of action inside the invasive tumor cells that reside behind an intact, functional BBB.
3.5 Footnotes

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Figure 3.1: Accumulation of elacridar in MDCKII wild-type cells, *MDR1* transfected and *Bcrp1* transfected cells lines with increasing concentrations of elacridar.

Accumulation of elacridar is expressed as ng of elacridar per µg of protein in cell lysate (n= 4 wells, Mean ± SD). The increase in accumulation of elacridar is linear with increase in elacridar concentration in the incubating media in wild-type cells. In the transfected cell lines, the accumulation of elacridar can be described by a simple $E_{max}$ model.
Figure 3.2: Percent accumulation of $^{14}$C dasatinib in MDCKII \textit{MDRI} overexpressing cells and \textit{Bcrp1} overexpressing cells with varying concentrations of elacridar.

The accumulation of radiolabeled dasatinib increases non-linearly with increase in concentration of elacridar in the incubating media and can be described by a simple Emax model (n= 4 wells) Mean ± S.D.
Figure 3.3: Elacridar concentrations in plasma after a single intravenous dose of 2.5 mg/kg in FVB Wild-type, Mdr1a/b (-/-) mice, Bcrp1 (-/-) mice and Mdr1a/b(-/-)Bcrp1(-/-) mice,

Mean ± S.D. (n = 4). Plasma concentrations in Mdr1a/b(-/-)Bcrp1(-/-) mice are lower than wild-type mice.
Figure 3.4: Elacridar concentrations in the brain after a single intravenous dose of 2.5 mg/kg in FVB Wild-type, Mdr1a/b (-/-) mice, Bcrp1 (-/-) mice and Mdr1a/b(-/-)Bcrp1(-/-) mice,

Mean ± S.D. (n = 4). Brain concentrations in wild type mice are much lower than Mdr1a/b(-/-)Bcrp1(-/-) mice.
Figure 3.5: Elacridar concentrations in the plasma and brain after a single intravenous dose of 2.5 mg/kg in FVB Wild-type (A), Bcrp1 (-/-) mice (B), Mdr1a/b (-/-) mice (C) and Mdr1a/b(-/-)Bcrp1(-/-) mice (D), Mean ± S.D. (n = 4).
Figure 3.6: Elacridar brain-to-plasma concentration ratios after a single intravenous dose of 2.5 mg/kg in FVB Mdr1a/b(-/-) mice, Bcrp1 (-/-) mice and Mdr1a/b(-/-)Bcrp1(-/-) mice.

Mean ± SD (n = 4). The brain-to-plasma ratio in wild-type, Mdr1a/b(-/-) mice and Bcrp1(-/-) mice show an increase followed by a decrease. In Mdr1a/b(-/-)Bcrp1(-/-) mice the brain-to-plasma ratio shows an increase to a steady state.
Figure 3.7: Brain-to-plasma concentration ratios in wild-type (A), Bcrp1 (-/-) mice (B), Mdr1a/b(-/-) mice (C), and Mdr1a/b(-/-)Bcrp1(-/-)(D) mice after intravenous dosing of increasing doses.

The brain-to-plasma concentration ratios are lower at low doses and increase with increase in dose in a non-linear fashion and can be described by a simple $E_{\text{max}}$ model. Mean ± SD at 1 hour post dose.
Figure 3.8: Schematic representation of transport processes between plasma (central compartment) and brain (peripheral compartment) for a compound that is a substrate for two different transporters.
Figure 3.9: Change in active clearance (CL_{active}) with respect to time, and with increase in dose for Model 1.

The different symbols indicate different doses as per the legend. Total clearance from the brain due to active transport, is a function of the dose administered. At higher doses, the transporters are saturated, and the CL_{active} from the brain is inhibited. At lower doses, with increase in time, the brain concentrations decrease and the CL_{active} increases.
Figure 3.10: Simulated Brain-to-plasma ratio with respect to time for a compound in Model 1, 2, 3 and 4 that is a substrate for two transporters at a dose of 1000 units. The brain-to-plasma ratio shows an increase followed by a decrease in models 1, 2 and 3 where there is one or more active efflux from the brain compartment. In model 4, the transport is due to only passive diffusion, the brain-to-plasma ratio shows an increase to a maximum plateau.
Figure 3.11: Simulated brain-to-plasma ratio with increase in unit dose at time 1 hour post dose for a compound that is a substrate for two transporters, in Model 1, 2, 3 and 4.

The brain-to-plasma ratios show a gradual increase to a maximum value for models 1, 2 and 3, and can be described by a simple $E_{\text{max}}$ model.
Table 3.1: Plasma pharmacokinetics determined by noncompartmental analysis after 2.5 mg/kg intravenous bolus dose of elacridar in wild-type, Mdr1a/b(-/-), Bcrp1(-/-) and Mdr1a/b(-/-) Bcrp1(-/-) mice. (†, Data for wild-type mice from previously published study)

<table>
<thead>
<tr>
<th></th>
<th>Elimination rate constant (hr⁻¹)</th>
<th>Half-life (hr)</th>
<th>Volume of distribution (ml)</th>
<th>Clearance (ml/min)</th>
<th>AUC₉₉ (ug*min/ml)</th>
<th>AUC₉₉ (ug*min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (†)</td>
<td>0.155</td>
<td>4.4</td>
<td>170</td>
<td>0.46</td>
<td>138 ± 6.5</td>
<td>161</td>
</tr>
<tr>
<td>Mdr1a/b(-/-)</td>
<td>0.17</td>
<td>4.1</td>
<td>306</td>
<td>0.86</td>
<td>64.3 ± 7.8</td>
<td>86.7</td>
</tr>
<tr>
<td>Bcrp1 (-/-)</td>
<td>0.139</td>
<td>4.9</td>
<td>248</td>
<td>0.57</td>
<td>87.4 ± 6</td>
<td>130.4</td>
</tr>
<tr>
<td>Mdr1a/b(-/-) Bcrp1(-/-)</td>
<td>0.173</td>
<td>3.9</td>
<td>374</td>
<td>1.08</td>
<td>51 ± 1.3</td>
<td>69.4</td>
</tr>
</tbody>
</table>
Table 3.2: Brain pharmacokinetics of elacridar as determined by noncompartmental analysis after 2.5 mg/kg intravenous bolus dose of elacridar in wild-Type, *Mdr1a/b(-/-), Bcrp1(-/-) and Mdr1a/b(-/-) Bcrp1(-/-) mice. (†, Data for wild-type mice from previously published study)

<table>
<thead>
<tr>
<th></th>
<th>Elimination rate constant (hr⁻¹)</th>
<th>Half-life (hr)</th>
<th>C_{max} (ug/g)</th>
<th>AUC_{tlast} (ug*min/ml)</th>
<th>AUC_{inf} (ug*min/ml)</th>
<th>Kp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type(†)</td>
<td>0.474</td>
<td>1.5</td>
<td>1 ± 0.4</td>
<td>128 ±19</td>
<td>131</td>
<td>0.82</td>
</tr>
<tr>
<td>*Mdr1a/b(-/-)</td>
<td>0.213</td>
<td>3.3</td>
<td>1.6 ± 0.2</td>
<td>253 ± 12</td>
<td>303</td>
<td>3.5</td>
</tr>
<tr>
<td>*Bcrp1(-/-)</td>
<td>0.237</td>
<td>2.9</td>
<td>2.9 ± 0.2</td>
<td>727 ± 24</td>
<td>863</td>
<td>6.6</td>
</tr>
<tr>
<td>*Mdr1a/b(-/-) *Bcrp1(-/-)</td>
<td>0.126</td>
<td>5.4</td>
<td>3.2 ± 0.2</td>
<td>735 ± 27</td>
<td>1042</td>
<td>15.03</td>
</tr>
</tbody>
</table>
Table 3.3: Brain-to-plasma concentration ratios for Wild type, *Mdr1* (-/-), *Bcrp1* (-/-) and *Mdr1* (-/-) *Bcrp1* (-/-) mice at different doses

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Wild-type</th>
<th><em>Mdr1a/b</em> (-/-)</th>
<th><em>Bcrp1</em> (-/-)</th>
<th><em>Mdr1a/b</em> (-/-)<em>Bcrp1</em> (-/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.4 ± 0.1</td>
<td>5.4 ± 0.5</td>
<td>3.3 ± 1</td>
<td>6.4 ± 3</td>
</tr>
<tr>
<td>1</td>
<td>2.2 ± 0.6</td>
<td>4.6 ± 0.8</td>
<td>5.7 ± 0.1</td>
<td>8.4 ± 1.2</td>
</tr>
<tr>
<td>2.5</td>
<td>5.2 ± 0.7</td>
<td>7.3 ± 1.0</td>
<td>8.9 ± 2.9</td>
<td>14.9 ± 1.6</td>
</tr>
<tr>
<td>5</td>
<td>9.9 ± 0.5</td>
<td>8.8 ± 1</td>
<td>11.6 ± 3</td>
<td>10.6 ± 1.3</td>
</tr>
<tr>
<td>10</td>
<td>12.7 ± 2.8</td>
<td>10.1 ± 2.3</td>
<td>10.3 ± 2.3</td>
<td>10 ± 1.6</td>
</tr>
</tbody>
</table>
Table 3.4: Parameters for dose dependent brain penetration of elacridar in wild type, *Mdr1a/b(-/-)*, *Bcrp (-/-)*, and *Mdr1a/b(-/-)Bcrp1(-/-)* mice after intravenous doses ranging from 0.5 mg/kg to 10 mg/kg

<table>
<thead>
<tr>
<th></th>
<th>E&lt;sub&gt;max&lt;/sub&gt; (brain to plasma ratio)</th>
<th>ED&lt;sub&gt;50&lt;/sub&gt; (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-Type</td>
<td>20.7 ± 3.8</td>
<td>8.3 ± 2.5</td>
</tr>
<tr>
<td><em>Mdr1a/b(-/-)</em></td>
<td>9.8 ± 0.8</td>
<td>0.77 ± 0.2</td>
</tr>
<tr>
<td><em>Bcrp1(-/-)</em></td>
<td>14.2 ± 2.2</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td><em>Mdr1a/b(-/-)Bcrp1(-/-)</em></td>
<td>11.6 ± 1.1</td>
<td>0.3 ± 0.18</td>
</tr>
</tbody>
</table>
CHAPTER IV:

DEVELOPMENT AND EVALUATION OF NOVEL MICROEMULSION FORMULATION OF ELACRIDAR TO IMPROVE BIOAVAILABILITY

This manuscript is currently under review in the Journal of Pharmaceutical Sciences
The objective of this study was to develop a formulation of elacridar to overcome its poor solubility and improve its bioavailability. Elacridar is a P-gp and BCRP inhibitor that has been examined for use in improving the brain distribution of several drugs that have their brain distribution limited by active efflux by P-gp and BCRP. However, the use of elacridar is limited due to poor solubility and the resulting poor oral bioavailability. A microemulsion formulation using Cremophor EL, Carbitol and Captex 355 in the ratio 6:3:1 was developed by preparing pseudoternary phase diagrams. The microemulsion was effective in the \textit{in vitro} inhibition of P-gp and Bcrp resulting in an increase in the cellular accumulation of radiolabeled dasatinib several fold in MDCKII \textit{MDR1} and \textit{Bcrp1} transfected cells. FVB wild-type mice were used to determine the bioavailability of the elacridar after a 10 mg/kg dose of elacridar intraperitoneally and orally in the microemulsion formulation. Brain and plasma concentrations of elacridar were determined by LCMS/MS. The absolute bioavailability of elacridar after oral and intraperitoneal administration of the microemulsion was determined to be 0.47 and 1.3, respectively. The results from this study show that a microemulsion formulation of elacridar is effective in improving the bioavailability of elacridar and is an effective inhibitor of P-gp and Bcrp \textit{in vitro}. The microemulsion formulation offers a more practical alternative to the suspension formulation and allows a decrease in the dose required to achieve a significant inhibitory effect on these efflux transporters.
4.1 Introduction:

One of the major hurdles in effectively treating disorders in the brain is the protective barrier of the blood-brain barrier (BBB). Efflux transporters such as P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), work at the level of the BBB to actively efflux drugs from the brain, thus limiting distribution to their target site. Pharmacological inhibition of these efflux transporters may overcome this limitation to brain delivery.

Elacridar (GF 120918) was initially developed as a multi-drug resistance reversal agent to restore sensitivity of tumor cells to chemotherapeutics such as doxorubicin (Hyafil et al., 1993). It is a potent inhibitor of P-gp and BCRP (Witherspoon et al., 1996; Allen et al., 1999). This inhibitory effect has been used to improve the oral bioavailability of drugs in humans that are substrates for P-gp and BCRP, such as topotecan and paclitaxel, by limiting the efflux of substrate drugs into the intestinal lumen (Kruijtzer et al., 2002; Bardelmeijer et al., 2004; Kuppens et al., 2007). Elacridar has also been used to study the influence of P-gp and Bcrp in preclinical models on the brain distribution of substrate drugs such as morphine, amprenavir and several tyrosine kinase inhibitors (Letrent et al., 1998; Edwards et al., 2002; Breedveld et al., 2005; Chen et al., 2009; Agarwal et al., 2010). These studies have shown that inhibition of the efflux proteins at the BBB using elacridar is an effective way to enhance the brain distribution of drugs that are substrates for P-gp and Bcrp. This strategy could result in improved efficacy of several drugs that have a site of action in the brain, but have distribution limited by the BBB. Therefore, the use of elacridar as a drug delivery adjuvant has the potential to enhance efficacy of these drugs by improving their distribution to target sites within the CNS.
Co-administration of elacridar as an adjuvant treatment presents several challenges, in both preclinical and clinical applications. The bioavailabilities of elacridar in the mouse after an oral or intraperitoneal dose of elacridar are only about 22% and 1%, respectively (Sane et al., 2012). Unfavorable physicochemical properties, such as poor solubility and high lipophilicity, result in dissolution-limited absorption from the gut lumen (Ward and Azzarano, 2004). Therefore, remarkably large doses of elacridar, ranging from 100 mg/kg to 500 mg/kg, have been administered orally in preclinical species to achieve plasma concentrations that can be effective for inhibition of P-gp and Bcrp (Edwards et al., 2002; Tang et al., 2012). Moreover, given the magnitude of these doses, chronic multi-dose regimens are problematic. The poor solubility of elacridar also limits its development as an injectable. These are major obstacles in the practical use of elacridar in both preclinical animal models and in clinical applications. Since the oral absorption of elacridar is dissolution-rate limited (Ward and Azzarano, 2004), improving the dissolution rate would be an ideal strategy to overcome this limitation. Several approaches could be used to improve the dissolution rate of elacridar, including: the preparation of a microemulsion, a solid dispersion, as well as the use of surfactants, cyclodextrins or lipids. Given the success of some microemulsions in increasing bioavailability, and reducing variability in absorption (Talegaonkar et al., 2008) we chose the microemulsion approach to improve the absorption of elacridar. Microemulsions are thermodynamically stable, transparent or translucent, optically isotropic colloidal dispersions, with low viscosity and fine droplet size (<100 nm). Microemulsions could be generated using self
microemulsifying drug delivery systems (SMEDDS), which are comprised of a mixture of surfactant, co-surfactant and lipid, that on agitation with water, form microemulsions (Narang et al., 2007). Microemulsions improve the absorption of drugs by improving dissolution (Lawrence and Rees, 2000; Bagwe et al., 2001). SMEDDS and microemulsions have been widely used to improve the bioavailability of poorly soluble drugs. Neoral (cyclosporine A), Fortovase (saquinavir), and Norvir (ritonavir) are examples of commercially available SMEDDS, which have been employed to improve bioavailability of these poorly soluble drugs.

Elacridar is a good candidate for a microemulsion formulation, given its high lipophilicity and poor bioavailability. The objective of this study was to develop and characterize a microemulsion formulation of elacridar, assess its systemic bioavailability, and determine its pharmacokinetics in plasma and brain. An improvement in the bioavailability of elacridar will result in a practical dose, one that will allow chronic inhibition of the transporters at the BBB.

4.2 Materials and Methods:

4.2.1 Chemicals and reagents

Elacridar (GF 120918) [N-(4-(2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl)phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide] of molecular weight 563.64 g/L was purchased from Toronto Research Chemicals (Ontario, Canada). Cremophor EL, Cremophor RH40, Solutol HS, Captex 355, Captex 300 were obtained from Abitech (Janesville, WI). Carbitol [2-(2-Ethoxyethoxy)ethanol] was
purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used were reagent grade or HPLC grade from Sigma-Aldrich (St. Louis, MO).

4.2.2 Solubility:
The solubility of elacridar in various components was determined as follows: a small quantity of elacridar (~ 1 mg) was added to a 100 ul of vehicle in a closed microcentrifuge tube. The tubes were shaken on an orbital shaker at 37⁰ C at 200 rpm for 24 hours to allow for equilibration. At the end of 24 hours, the tubes were removed from the shaker and centrifuged at 10,000 rpm for 5 min to separate the solubilized fraction from the un-dissolved material. The supernatant was pipetted, diluted and concentration of elacridar was determined by LCMS-MS.

4.2.3 Construction of Phase diagrams:
A series of mixtures was prepared with various ratios of Solutol HS and Cremophor EL. The phase behavior of the system was studied at surfactant to co-surfactant ratios of 8:1, 4:1 and 2:1. Aliquots of each surfactant: co-surfactant mixtures were mixed with oil and then purified water. Mixtures were mixed by vortexing at room temperature. The samples were assessed visually and determined as being coarse emulsions, gels or clear and transparent microemulsions. The microemulsion region was plotted on a ternary diagram. The microemulsion region represents clear and optically isotropic systems. The non-microemulsion region represents the dispersed and turbid systems. These were identified by visual inspection.

4.2.4 Emulsion droplet size analysis:
The droplet size of the microemulsion was measured by the dynamic light scattering technique using a Delsa™Nano C (Beckman Coulter, Inc, Chaska, MN). The microemulsion formulation was diluted 50-fold in purified water prior to droplet size measurements.

**4.2.5 In vitro studies**

In vitro studies were conducted in epithelial Madin-Darby canine kidney II (MDCKII) cells over expressing either murine BCRP (MDCKII-\textit{Bcrp1}) or human P-gp (MDCKII-\textit{MDR1}) and were obtained from Dr. Piet Borst and Dr. Alfred H. Schinkel (The Netherlands Cancer Institute). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml) (all Sigma-Aldrich) and maintained at 37°C with 5% CO₂ under humidifying conditions.

**4.2.6 In vitro efficacy of elacridar microemulsion**

Intracellular accumulation of radiolabelled dasatinib in presence of the optimized elacridar microemulsion as well as elacridar as a solution was examined in wild-type and MDCKII \textit{MDR1} and \textit{Bcrp1} transfected cells using 24-well polystyrene plates (Thermo Fisher Scientific, Waltham, MA). Cells were seeded at a density of 1× 10⁵ cells per well. Fresh media was supplied every two days to the cells until a confluent monolayer was obtained. Prior to starting the experiment, the nutrient media was aspirated and cells were washed with pre-warmed cell assay buffer.

To study the effect of elacridar microemulsion on radiolabelled dasatinib cellular accumulation, the cells were preincubated for 30 minutes with one milliliter of treatment
buffer including elacridar solution in dimethyl sulfoxide (5 uM), elacridar microemulsion (1 uM) as well as equal volume of blank microemulsion components. One milliliter of tracer solution of $^{14}$C dasatinib added to the elacridar solution was added to the cells and the plates were incubated for 1 hour in an orbital shaker at 37°C. At the end of the incubation period, the radiolabelled drug solution was aspirated from the wells and the cells were washed with ice-cold phosphate buffered saline. 500 µl of 1 % Triton X solution was added to the wells to solubilize the cells. BCA protein assay (Thermo Fisher Scientific, Rockford, IL) was used to determine the protein concentrations in the solubilized cell fractions. Radioactivity in each 100 µl sample was determined by liquid scintillation counting (LS-6500; Beckman Coulter, Fullerton, CA). The radioactivity in the cell fractions was normalized to the protein concentrations in each well. The drug accumulation in the cells was expressed as amount of radioactivity (dpm) per microgram of protein.

4.2.7 Effect of increasing concentration of elacridar microemulsion on cellular accumulation of radiolabeled dasatinib

The intracellular accumulation of radiolabelled dasatinib with increasing concentrations of elacridar as microemulsion formulation was examined in MDCKII wild-type, MDR1, and Bcrp1 cells. The cells were pre-incubated for 30 minutes with one milliliter of increasing concentrations of elacridar microemulsion. A control experiment was carried out using blank microemulsion components. A volume of mixture of microemulsion components without elacridar equivalent to the volume used in the treatment groups was used. One milliliter of tracer solution of $^{14}$C dasatinib added to the elacridar solution was
added to the cells and the plates were incubated for 1 hour in an orbital shaker at 37 °C. At the end of the incubation period, the radiolabelled drug solution was aspirated from the wells and the cells were washed with ice-cold phosphate buffered saline. The rest of the experiment was carried out as described before. The accumulation of radiolabeled dasatinib in the cells was expressed as a percent of accumulation when elacridar microemulsion is absent, where the dpm of radiolabeled dasatinib is normalized to cell protein.

4.2.8 Animals:
In vivo studies were conducted in FVB wild-type mice (Taconic Farms, Germantown, NY). All animals were 8-10 weeks old at the time of the experiment. All mice were maintained under a 12-hour light/dark cycle, had unlimited access to food and water, and were maintained in a temperature-controlled environment. All studies were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

4.2.9 Determination of bioavailability
The dosing solution was prepared by dissolving elacridar in a mixture of the surfactants and oil at a ratio that would allow a 3 mg/ml solution. This solution was then diluted with saline to obtain a 1 mg/ml microemulsion. Mice were given a 10 mg/kg dose either by oral gavage or by intraperitoneal injection. Mice were administered elacridar microemulsion intravenously at dose of 2 mg/kg dose by tail vein injection. Animals were sacrificed at predetermined time points (n=4 at each time point) by carbon dioxide euthanasia. Plasma and brain were collected at 30 min, 1, 2, 4, 8 and 12 hours post-dose. Blood was collected by cardiac puncture and transferred to heparinized tubes. Plasma
was obtained by centrifugation of blood at 7000 rpm for 10 min. Whole brain was isolated from skull and rinsed with ice-cold saline and then flash frozen with liquid nitrogen. Both plasma and brain were stored at -80 °C before analysis by LCMS-MS.

4.2.10 Analysis of elacridar by LCMS-MS:

The concentrations of elacridar in mouse plasma and brain were determined by HPLC coupled with mass spectrometry. Brain samples were thawed and homogenized with three volumes of 5% bovine serum albumin using a tissue homogenizer (Thermo Fisher Scientific, Rockford IL). Twenty ng of internal standard, tyrphostin (AG1478) was added to 50 ul of plasma and 100 ul of brain homogenate along with 100 ul of pH 11 buffer. Liquid-liquid extraction was carried out by adding 1ml of ethyl acetate and vortexing the mixture, followed by centrifugation at 7500 rpm for 15 min at 4 °C. 600 ul of the organic phase supernatant was dried under nitrogen, and reconstituted in 100 ul of mobile phase and transferred to autosampler vials. A 5 µl volume was injected into the HPLC using a temperature-controlled auto sampler maintained at 10°C. The column used for chromatographic separation, purchased from Agilent Technologies (Santa Clara, CA), was an Eclipse XDB-C18 RRHT threaded column (4.6 mm id X 12.5 mm, 5 u). The mobile phase was composed of acetonitrile: 20 mM ammonium formate (with 0.1 % formic acid) (42:58 v/v) with a flow rate of 0.25 ml/min. A Thermo Finnigan TSQ Quantum 1.5 detector (Thermo Fisher Scientific) was used to analyze the eluent. The instrument was equipped with an electrospray interface. The samples were ionized by the electrospray probe and analyzed in the positive ionization mode operating at a spray voltage of 4500 V for elacridar and the internal standard. The spectrometer was
programmed to allow the [MH]+ ion of elacridar at m/z 564.6, and 316.67 for the internal standard to pass through the first quadrupole (Q1) and into the collision cells (Q2). The collision energy was set at 39 V for elacridar and erlotinib, 9V for tryphostin. The product ions for elacridar (m/z 252.9) and internal standard (m/z 300.9) were monitored through quadrupole 3 (Q3). The scan width and scan time for monitoring the two product ions was 1.5 m/z and 0.5 s, respectively. The assay was sensitive over a range of 2.5 ng/ml to 1000 ng/ml.

4.2.11 Pharmacokinetic Analysis:

Pharmacokinetic parameters obtained from the concentration-time profiles in plasma and brain were calculated by non-compartmental analysis using Phoenix WinNonlin 6.1 (Mountain View, CA). The area under the concentration-time curve for plasma and brain were calculated using the trapezoidal method. The terminal half-life was determined by using the last three data points in both plasma and brain concentration-time profiles. The AUCextrapolated (AUC from last measured time point) was estimated by dividing the last measured concentration by the terminal rate constant. For calculating the AUCplasma after an intravenous dose, a concentration at time zero that was extrapolated from the first few data points was used for determining concentration at time zero. The absolute bioavailability (F) was calculated as a ratio of dose-normalized AUCplasma after oral or intraperitoneal microemulsion administration to dose-normalized AUCplasma after intravenous dosing of elacridar solution as calculated in a previously published study (Sane et al., 2012).

\[
F = \frac{AUC_{plasma (IP or PO, microemulsion)}}{AUC_{plasma (IV, solution)}} \times \frac{Dose(IV, solution)}{Dose (IP or PO, microemulsion)}
\]
The brain-to-plasma partition coefficient of elacridar (Kp,brain) was calculated as the ratio of AUCs (AUC_{brain}/AUC_{plasma})

4.2.12 Statistical Analysis:
Comparison between groups were made using SigmaStat, version 3.1 (Systat Software, Inc., San Jose, CA). Statistical difference between two groups was tested by using the two-sample t-test, and significance was declared at p < 0.05. Multiple groups were compared by one-way analysis of variance with the Holm-Sidak post hoc test for multiple comparisons at a significance level of p < 0.05.

4.3 Results:
4.3.1 Solubility studies:
The components examined for use in the preparation of the microemulsion were examined for capability to dissolve elacridar. The results are listed in Table 1. The components selected for further development of the formulation were chosen on the basis of solubility of elacridar in those components. Cremophor EL provided the best solubilization capacity and was therefore chosen to formulate the microemulsion.
Cremophor itself is an inhibitor of P-gp (Regev et al., 2007); therefore appropriate controls have been used in all experiments.

4.3.2 Pseudoternary phase diagrams and droplet size:
Pseudoternary phase diagrams of oil, surfactant, co-surfactant and water were developed by preparing a number of mixtures at room temperature. Phase behavior of the system was studied using several surfactant to co-surfactant ratios (S_{mix} = 2:1, 4:1, 8:1, where the S_{mix} denotes the mixture of surfactant to co-surfactant) (Figure 4.1).
The microemulsion region was found to be present at high surfactant concentration and low oil concentrations at all $S_{\text{mix}}$ ratios. The microemulsion region was larger at $S_{\text{mix}}$ ratio 8:1 as compared to 4:1 and 2:1. The surfactant: co-surfactant ratio of 2:1 was chosen since the viscosity of the microemulsion was optimal for injectable formulations. The final formulation chosen was Cremophor EL: Carbitol: Captex in 6:3:1 ratio. A 3 mg/ml solution of elacridar was prepared in the microemulsion components in the absence of water. For the in vitro and in vivo studies, the 3 mg/ml solution was diluted with purified water or saline. To examine the droplet size of the microemulsion, the 1 mg/ml elacridar microemulsion was diluted 20-fold. The droplet size for the microemulsion without the drug was found to be 16.8 ± 0.8 nm. When the drug was present in the microemulsion, the droplet size was slightly larger, 19.1 ± 0.3 nm. The increase in droplet size with addition of drug was observed in a previously published study with Cremophor EL and cyclosporine (Gao Zhong-Gao, 1998), however, further exploration of this phenomenon was not carried out in this paper.

4.3.3 In vitro efficacy of elacridar microemulsion

This study was carried out to understand if the microemulsion formulation of elacridar is as effective in inhibiting P-gp and Bcrp in an in vitro model as the conventional solution formulation. The efficacy of elacridar microemulsion in inhibiting P-gp and Bcrp was measured using MDCKII cells over-expressing P-gp and Bcrp and wild-type cells were used as a control. Radiolabeled dasatinib was used in tracer quantities as a model substrate for P-gp and BCRP (Chen et.al 2010). The accumulation of dasatinib in MDR1-overexpressing cells increased several fold on treatment with elacridar microemulsion
There is a similar increase accumulation of radiolabeled dasatinib in $Bcrp1$-transfected cells on treatment with elacridar microemulsion (Figure 4.2.b). A solution of elacridar in DMSO was used as a control. The increase in radiolabeled dasatinib accumulation shows that the microemulsion formulation of elacridar is effective in inhibiting P-gp and Bcrp in vitro at a concentration of 1 uM. Microemulsion components without any added elacridar were used a control group because Cremophor EL has been shown to be an inhibitor of P-gp (Kemper et al., 2003; Regev et al., 2007). However, the microemulsion components did not show any inhibitory effect on the P-gp and Bcrp over-expressing cell lines. This would indicate that the Cremophor EL is present at a concentration that is well below its IC$_{50}$ for inhibition of either transport system.

4.3.4 Effect of increasing concentrations of elacridar as a microemulsion on cellular accumulation of radiolabeled dasatinib

The percent accumulation of radiolabeled dasatinib in MDCKII $MDR1$ and $Bcrp1$ cells showed unexpected results when the concentration of elacridar microemulsion was increased. The percent accumulation of radiolabeled dasatinib first showed an increase followed by a decrease in both sets of transfected cells (Figure 4.3). This was unexpected since previous experiments indicated that increase in elacridar concentrations result in a non-linear increase in the cellular accumulation of radiolabeled dasatinib (Submitted JPET August 2012). The control groups also showed a decrease in the accumulation from 100% to about 16% in both MDCKII $MDR1$ and $Bcrp1$ transfected cells. This phenomenon suggests that the Cremophor EL contributed by the
microemulsion system is well below a concentration that can inhibit P-gp. We hypothesize that the decrease in the cellular accumulation occurs due to a micellar trapping effect. At higher concentration of the microemulsion components, the surfactant mixture can create micelles that can entrap the radiolabeled drug. This phenomenon has been previously observed with a number of different monomeric surfactants including Pluronic 85 (Shaik et al., 2009). This unexpected phenomenon raises the possibility that greater concentrations of the microemulsion and its components may adversely affect the cellular accumulation of drugs due to the micellar effect in vivo.

**4.3.5 Intraperitoneal and oral administration of elacridar microemulsion**

The pharmacokinetics of elacridar microemulsion in plasma and brain were studied when the microemulsion was administered intraperitoneally and orally. The bioavailability of elacridar was calculated using equation 1, the area under the concentration time curve for plasma (AUC\textsubscript{plasma}) calculated after an intravenous dose of elacridar solution at 2.5 mg/kg, from a previously published study was used (Sane et al., 2012).

A 10 mg/kg dose of elacridar microemulsion was administered intraperitoneally to FVB mice. The plasma concentrations of elacridar showed a maximal concentration of 6.8 ± 0.7 ug/ml at 1 hour post dose (Figure 4.4.a). The brain concentrations showed slower rate of increase, with very low concentrations at the initial times, and the peak concentration of elacridar was reached at about 4 hours post dose. The brain-to-plasma concentration ratios of elacridar are very low at initial time points and then show an increase to 2.3 ± 0.6 at 4 hours post dose. The concentrations of elacridar achieved in the plasma and the brain are much higher than those seen after a 100 mg/kg dose.
administered as a suspension (Sane et al., 2012). These results suggest that an intraperitoneal administration of elacridar could be a more efficient mode of administration of elacridar in a chronic dosage regimen for studies in preclinical models of cancer.

An oral dose of elacridar microemulsion at 10 mg/kg was administered to mice via oral gavage. The plasma concentrations reached a maximum at 1 hour post-dose, followed by a sharp decline in plasma concentrations (Figure 4.5.a). The plasma concentrations reached a maximum of $1.3 \pm 0.1 \mu g/ml$, at one hour post-dose. Brain concentrations show a much slower increase to maxima and reach a peak only at 8 hours post dose. The brain-to-plasma ratio shows an increase followed by a slow decline (Figure 4.5.b). The brain concentrations show a lag time, reaching a maximum of $0.3 \pm 0.1 \mu g/ml$, at 4 hours post-dose. The brain-to-plasma concentration ratios reach a maximum of $1.1 \pm 0.2$ at 4 hours and show a slight decline at 12 hours. The brain and plasma concentrations when normalized to the dose were higher than the dose normalized concentrations after an oral administration of conventional suspension at a dose of 100 mg/kg (Sane et al., 2012).

The half-life of elacridar when administered as an intraperitoneal injection was 3.2 hours, as compared to 10.5 hours when administered orally (Table 4.2). Interestingly, when elacridar was administered orally as a suspension, the half-life was also found to be ~4 hours for intraperitoneal administration and ~10 hours for oral administration (Sane et al., 2012).

The $AUC_{\text{plasma (0-inf)}}$ after an oral microemulsion dose was found to be $270.5 \text{ min}^*\mu g/ml$. This is lower as compared to the $AUC_{\text{plasma (0-inf)}}$ calculated after intraperitoneal
administration of the same dose (962.41 min*µg/ml) (Table 4.2). This could arise due to the differences in the pH and the area available for absorption in the GIT versus the peritoneal cavity.

The brain-to-plasma partition coefficient (Kp) was calculated by obtaining a ratio of AUC\text{brain} to AUC\text{plasma}. The Kp was very different for the two routes of administration. This could be an effect of the relative AUC\text{plasma} obtained post intraperitoneal and oral administration.

4.3.6 Intravenous administration of elacridar microemulsion

Elacridar microemulsion was administered to FVB wild-type mice at a dose of 2.5 mg/kg to study the distribution of elacridar when it is administered as the microemulsion formulation as an intravenous dose. The plasma concentrations show a mono-exponential decline (Figure 4.6.a). The brain concentrations at each time-point were found to be higher than the corresponding plasma concentrations. The brain-to-plasma concentration ratios are higher than one at all time-points, and show a peak at 30 mins (Figure 4.6.b).

The AUC\text{plasma}(0-inf) (90.9 ug*min/ml) was found to be lower than AUC\text{brain}(0-inf) (226.8 ug*min/ml) (Table 4.2). The Kp was calculated to be 2.5 indicating that when administered intravenously the elacridar microemulsion can partition into the brain easily. This could be a function of the components of the microemulsion formulation, such as the surfactant and the co-surfactant that reach the BBB after an intravenous dose. These components could change the permeability of elacridar into the brain. The half-life in plasma (2.16 hours) was longer than compared to the half-life in the brain, 1.5 hours.
We have observed this phenomenon in our previously published work (Sane et al., 2012) and have examined it in detail in another manuscript (Chapter III).

4.3.7 Bioavailability of elacridar microemulsion

The absolute bioavailability of elacridar microemulsion was calculated with respect to the area under the curve calculated after an intravenous injection of elacridar solution at 2.5 mg/kg from a previously published study (Sane et al., 2012). The absolute bioavailability was nearly 100% post intraperitoneal injection (Table 4.3). This could be due to the very large surface area available for absorption in the intraperitoneal cavity for the solubilized drug. The oral bioavailability post microemulsion dosing was calculated to be 47%, which is a two-fold increase from administration of a suspension. The microemulsion formulation was found to improve the oral and intraperitoneal bioavailability of elacridar. Another real advantage of using the microemulsion formulation is that the dose required to produce an effect can be reduced by several fold. This is important since elacridar could be potentially administered as an adjuvant to a chronic dosage regimen, and reduction in the daily dose administered could only help avoid potential adverse drug reactions.
4.4 Discussion:

The use of elacridar in preclinical models, as well as in clinical settings, for chronic administration is limited by its poor oral absorption and lack of injectable formulations. The poor solubility of elacridar in aqueous solvents has been limiting in formulating an injectable dosage form. The use of surfactants and lipids can enhance the solubility and permeability of poorly soluble drugs (Scott Swenson and Curatolo, 1992; Araya et al., 2005). This strategy has been used to improve the oral bioavailability of drugs such as antiviral compounds (marketed preparations of ritonavir, saquinavir), anesthetics (propofol), as well as chemotherapeutics (paclitaxel) (Morey et al., 2006; Talegaonkar et al., 2008; Wang et al., 2011). The objective of this study was to create a microemulsion formulation of elacridar that would improve its systemic bioavailability that also could be delivered on a chronic basis either orally or via injection into the peritoneal cavity (in preclinical studies). Many molecularly-targeted agents that are currently being developed for the treatment of glioblastoma are intended to be administered on a daily basis. Therefore, the long-term goal of the current study is to create a formulation that could be co-administered with these agents to improve their distribution across the BBB in the glioblastoma patient (Agarwal et al., 2011).

The in vitro studies herein suggest that elacridar retains its inhibitory potential in the microemulsion formulation. Cremophor EL, the surfactant used in the microemulsion, is known to be an inhibitor of P-gp (Regev et al., 2007). The microemulsion vehicle was used as a control to ensure that this surfactant at concentrations used in the formulation did not influence P-gp function. However, a higher concentration of the microemulsion
vehicle seems to cause a decrease in cellular accumulation of substrate drugs, possibly due to a micellar-trapping mechanism. This phenomenon may have some implications in its future use *in vivo*. A micellar-trapping mechanism of Cremophor EL has been shown to result in preferential binding of paclitaxel to the surfactant versus erythrocyte binding components at higher concentrations (Sparreboom et al., 1999).

The oral bioavailability of elacridar is dissolution-rate limited (Ward and Azzarano, 2004). In a previous study, we calculated that the absolute bioavailability of elacridar when administered as a suspension at 100 mg/kg dose to be about 22% (Sane et al., 2012). A phase I study carried out with elacridar and topotecan showed that the systemic exposure after oral administration shows high variability (Kuppens et al., 2007). This limited bioavailability of elacridar post administration of a suspension at such a high dose; coupled with high variability showed the need to develop a more bioavailable formulation of elacridar, particularly for chronic dosing. Microemulsion formulations of other poorly soluble drugs, such as cyclosporine (Neoral), have been particularly successful in increasing the oral bioavailability, and reducing the variability in the bioavailability of these drugs. A microemulsion formulation of cyclosporine prepared with cremophor (Gao Zhong-Gao, 1998) was used as a model composition to prepare a formulation of elacridar. When elacridar is administered as this microemulsion formulation at 10 mg/kg, the bioavailability increased two-fold to 47%. The plasma concentration time profile shows a peak at 1 hour postdose followed by a rapid decline (Figure 4.5.a). It is possible that this could be a result of precipitation of elacridar from the microemulsion system due to the changes in pH through the GIT. Microemulsion
systems are known to be sensitive to changes in the pH, and rapid changes in the pH could lead to instability in the system causing a precipitation of elacridar (Ovando-Medina et al., 2005). The slow absorption of the precipitated elacridar from the GIT may lead to flip-flop kinetics resulting in a longer than expected half-life of ~10 hours (seen after IV dosing (Sane et al., 2012)). The longer than expected half-life is consistent with that seen post oral administration of suspension of elacridar (Sane et al., 2012). After an intraperitoneal administration of elacridar microemulsion, the oral bioavailability was calculated to be nearly 100% (Table 3). In a previously published study, the bioavailability of elacridar after an intraperitoneal administration was only about 1% (Sane et al., 2012). The concentrations of elacridar achieved after an intraperitoneal administration of the microemulsion are much higher than those achieved after administration of a suspension (Sane et al., 2012). The plasma concentrations of elacridar after administration of the suspension could be lower due to poor dissolution of the suspension particles in the intraperitoneal fluid, leading to a slower absorption rate. When elacridar is administered as a microemulsion, it is already in a solubilized form, which allows it to be readily absorbed into the systemic circulation. This improves the area under the curve leading to enhanced bioavailability.

An intravenous administration of elacridar microemulsion was carried out to examine the brain distribution of elacridar. The brain concentrations were much higher than plasma concentrations at all measured time points. The brain-to-plasma ratios showed an increase to a maximum value followed by a decrease. This pattern of the brain-to-plasma ratios has been observed earlier for elacridar, and could arise due to an active efflux of elacridar.
from the brain (Sane et al., 2012). The $K_p$ observed post intravenous dose was much higher than intraperitoneal and oral administration of the microemulsion. The microemulsion components are a surfactant and co-surfactant mixture; these components are known to enhance permeability across membranes. Administration of the microemulsion intravenously introduces the microemulsion components directly into the systemic circulation, where they could possibly affect the permeability of several membranes, including the blood-brain-barrier. The increased permeability across membranes could increase the tissue partition coefficient of elacridar into the brain. Use of the elacridar microemulsion as an adjuvant to molecularly-targeted agents may help overcome the effect of transporters at the BBB, improve distribution to the brain, and thus may result in enhanced efficacy. This hypothesis can only be tested if a practical formulation of elacridar is available for chronic use, either in preclinical models or eventually in the patient.

There are several advantages to developing a microemulsion formulation of elacridar. The novel formulation helps overcome the poor physicochemical properties of elacridar, such as poor solubility and high lipophilicity. The improvement in bioavailability allows us to decrease the dose that is needs to be administered to achieve inhibition of efflux at the BBB. The microemulsion can also be administered easily, either orally or intraperitoneally, both modes of administration are typically suitable for chronic dosing in preclinical models of glioblastoma. Another advantage is the possible decrease in the amount of dose required to produce an effect. The studies described herein indicate that a microemulsion formulation of elacridar can be developed for use as an adjuvant to
therapy in the treatment of glioblastoma, particularly in preclinical models to improve
delivery of molecularly-targeted agents that are subject to active efflux at the BBB by P-
gp and BCRP and need to be administered on a chronic basis.
4.5 Footnotes

This work was supported by National Institutes of Health - National Cancer Institute [CA138437] (W.F.E.) and a Faculty Development grant at the University of Minnesota (W.F.E.). Financial support for Ramola Sane was provided by the Ronald J. Sawchuk Fellowship and Rowell Fellowship.
Figure 4.1: Phase diagram for Cremophor EL-Carbitol-Captex 355 system indicating o/w microemulsion region at Surfactant-Cosurfactant ratio (Smix) 2:1, 4:1 and 8:1
Figure 4.2.a: Effect of elacridar microemulsion (1uM) on accumulation of radiolabelled dasatinib in MDCKII wild type, MDCKII \textit{MDR1} transfected cells.

With elacridar solution (5uM) elacridar microemulsion (1 uM) and equivalent volume of microemulsion components (blank microemulsion) as control. Elacridar microemulsion was effective in increasing the accumulation of radiolabeled dasatinib in the \textit{MDR1}-transfected cell lines as compared to control.
Figure 4.2.b: Effect of elacridar microemulsion (1uM) on accumulation of radiolabelled dasatinib in MDCKII wild type, MDCKII Bcrp1 transfected cells, with elacridar solution (1uM) and microemulsion components as control. Elacridar microemulsion was effective in increasing the accumulation of radiolabeled dasatinib in the Bcrp1 transfected cell lines as compared to control.
Figure 4.3: Effect of increasing concentrations of elacridar microemulsion and blank microemulsion components on accumulation of radiolabelled dasatinib in MDCKII MDR1 cells and MDCKII Bcrp1 cells.

The cellular accumulation of dasatinib increases in both sets of transfected cells and then decreases. In the control groups, the cellular accumulation shows a decline with increase in the volume of microemulsion components.
Figure 4.4.a: Plasma and brain concentrations of elacridar after a 10 mg/kg intraperitoneal dose of elacridar microemulsion.

The plasma concentrations reach a maximal concentration at 1 hour post dose, while the brain concentrations reach a maximum at 4 hours post dose.
Figure 4.4.b: Brain-to-plasma concentration ratios of elacridar after a 10 mg/kg dose of elacridar microemulsion.

The brain to plasma ration is low at initial times and increases to greater than one at later time points.
Figure 4.5.a: Brain and plasma concentrations of elacridar after a 10 mg/kg oral dose of elacridar microemulsion.

The plasma concentrations reach a peak at 1 hour post dose. In the brain, the concentrations reach a peak only at 8 hours post dose.
Figure 4.5.b: The brain to plasma concentration ratios of elacridar after a 10 mg/kg oral dose of elacridar microemulsion.

The brain-to-plasma concentration ratios are greater than one at all time-points.
Figure 4.6.a: The brain and plasma concentrations of elacridar after an intravenous dose of 2mg/kg elacridar microemulsion.

The brain concentrations of elacridar are higher than plasma at all time-points.
Figure 4.6.b: The brain-to-plasma concentration ratios of elacridar after an intravenous dose of 2mg/kg elacridar microemulsion.

The brain-to-plasma concentration ratios are higher than one at all time-points up till 8 hours.
Table 4.1: Equilibrium solubility of elacridar at 37 °C in various components after 24 hours.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solubility (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>12.3 ± 5.8 x 10⁻⁵ mg/ml</td>
</tr>
<tr>
<td>Captex 355 (oil)</td>
<td>0.3 ± 0.001 mg/ml</td>
</tr>
<tr>
<td>Captex 300 (oil)</td>
<td>0.3 ± 0.001 mg/ml</td>
</tr>
<tr>
<td>Carbitol (co-surfactant)</td>
<td>1.31 ± 0.05 mg/ml</td>
</tr>
<tr>
<td>Solutol (surfactant)</td>
<td>0.85 ± 0.06 mg/ml</td>
</tr>
<tr>
<td>Solutol microemulsion system</td>
<td>0.84 ± 0.03 mg/ml</td>
</tr>
<tr>
<td>Cremophor EL (surfactant)</td>
<td>4.27 ± 0.31 mg/ml</td>
</tr>
<tr>
<td>Cremophor EL microemulsion system</td>
<td>4.2 ± 0.2 mg/ml</td>
</tr>
</tbody>
</table>
Table 4.2: Pharmacokinetic parameters of elacridar in plasma and brain when administered as a microemulsion formulation in FVB mice, as a 10 mg/kg dose intraperitoneally and orally and 2 mg/kg intravenously.

<table>
<thead>
<tr>
<th>Administration</th>
<th>Tmax (min)</th>
<th>Cmax (ug/ml)</th>
<th>Half-life (hr)</th>
<th>AUC0-tlast (ug*min/ml)</th>
<th>AUC0-inf (ug*min/ml)</th>
<th>Kp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraperitoneal administration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10 mg/kg)</td>
<td>plasma</td>
<td>60</td>
<td>6.8 ± 0.7</td>
<td>3.2</td>
<td>840.9 ± 45</td>
<td>962.4</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>240</td>
<td>2.2 ± 0.1</td>
<td>2.8</td>
<td>843.1 ± 27.6</td>
<td>916.2</td>
</tr>
<tr>
<td>Oral administration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10 mg/kg)</td>
<td>plasma</td>
<td>60</td>
<td>1.3 ± 0.1</td>
<td>10.4</td>
<td>163.1 ± 90</td>
<td>270.5</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>240</td>
<td>0.3 ± 0.1</td>
<td>4.1</td>
<td>116.1 ± 33</td>
<td>142.5</td>
</tr>
<tr>
<td>Intravenous administration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2.5 mg/kg)</td>
<td>plasma</td>
<td>30</td>
<td>0.4 ± 0.1</td>
<td>2.2</td>
<td>74.5 ± 4.2</td>
<td>90.9</td>
</tr>
<tr>
<td></td>
<td>brain</td>
<td>30</td>
<td>2.0 ± 0.2</td>
<td>1.5</td>
<td>217.8 ± 13.7</td>
<td>226.8</td>
</tr>
</tbody>
</table>
Table 4.3: Plasma pharmacokinetics of elacridar as microemulsion formulation and bioavailability of elacridar after intraperitoneal, oral and intravenous administration of elacridar (* Fabs is calculated with using AUCplasma after elacridar solution as a control.)

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Dose</th>
<th>CL/F (ml/min)</th>
<th>Vd/F (ml)</th>
<th>Fabs*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraperitoneal</td>
<td>10 mg/kg</td>
<td>0.31</td>
<td>88.69</td>
<td>1.35</td>
</tr>
<tr>
<td>Oral</td>
<td>10 mg/kg</td>
<td>1.13</td>
<td>1020.69</td>
<td>0.47</td>
</tr>
<tr>
<td>Intravenous</td>
<td>2.5 mg/kg</td>
<td>0.70</td>
<td>0.14</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER V:

ELACRIDAR AS A DRUG DELIVERY ADJUVANT IN THE
TREATMENT OF GLIOMA IN TUMOR-BEARING MICE
Erlotinib is an EGFR inhibitor that has been examined for use in treatment of glioma, however, several clinical trials have not succeeded in improving the overall survival rates in patients with glioma. The brain distribution of erlotinib is limited by its efflux due to P-gp and Bcrp at the BBB. This could be one of the possible reasons for its limited efficacy in targeting tumor cells behind an intact BBB. The objective of this study was to overcome the barrier to disposition of erlotinib by using elacridar, an inhibitor of P-gp and Bcrp at the BBB. Elacridar was administered intraperitoneally to FVBn mice as a microemulsion 30 min prior to erlotinib treatment. The brain concentrations of erlotinib were improved three-fold tumor bearing mice with co-administration of erlotinib.

Athymic Nu/Nu mice bearing glioma xenografts were co-administered 5 mg/kg elacridar microemulsion with 100 mg/kg erlotinib. The brain-to-plasma ratio showed a three-fold improvement in both tumor-bearing hemisphere and the contralateral hemisphere when elacridar was co-administered. Elacridar was co-administered as a microemulsion along with erlotinib in tumor-bearing mice; however, there was no improvement in overall survival rate with co-administration of elacridar. This study shows that while elacridar was effective in improving the brain distribution of erlotinib, the enhanced distribution of erlotinib is ineffective in improving the overall survival rates.
5.1 Introduction:

Erlotinib (Tarceva™) is a tyrosine kinase inhibitor that is used in the treatment of non-small-cell lung cancer (NSCLC) and pancreatic tumors (Starling et al., 2006; Cappuzzo et al., 2010). It is a molecularly-targeted agent that inhibits the epidermal growth factor receptor (EGFR) and a mutant form of EGFR, EGFR vIII. Several phase I and II studies have been carried out with erlotinib, either as a single agent or in combination with other drugs in treatment of glioblastoma (Prados et al., 2006; Reardon et al., 2010; Kesavabhotla et al., 2012) and the efficacy of erlotinib in treatment of GBM has been found to be limited as a monotherapy. One possible reason could be the poor distribution of erlotinib into the brain and the tumor tissue, especially the brain around tumor (Agarwal et al., 2011a).

Erlotinib is a dual substrate of the transporters P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) (Marchetti et al., 2008; Elmeliegy et al., 2011; de Vries et al., 2012). These two transporters together at the blood-brain barrier limit the distribution of xenobiotics into the brain (Loscher and Potschka, 2005). For many drugs with possible CNS activity, these transporters are an effective barrier to keep them from reaching their site of action, thus limiting their efficacy. Treatment of brain tumors with chemotherapy is difficult due to the presence of the BBB. Several tumor cells also express P-gp and BCRP at their cell surface (Lu and Shervington, 2008; Natarajan et al., 2012). This presents an additional barrier to delivery of drugs that are substrates for these transporters (Agarwal et al., 2011a). Elacridar has been used as a dual inhibitor of P-gp and Bcrp both in vitro and in vivo (Hyafil et al., 1993; Witherspoon et al., 1996; Allen et al., 1999). Co-
administration of elacridar has been found to increase the brain distribution of several drugs such as gefitinib, sorafenib, sunitinib, dasatinib as well as erlotinib in preclinical models (Agarwal et al., 2010; Lagas et al., 2010; Agarwal et al., 2011b; Agarwal et al., 2012; de Vries et al., 2012; Tang et al., 2012a; Tang et al., 2012b).

A recent study (Agarwal et al., 2012) shows that in U87 tumor-bearing rats, the distribution of erlotinib to the brain is limited by an intact BBB; furthermore, there are regional differences in the distribution of erlotinib between the tumor core, normal brain and brain surrounding the tumor core, and that distribution of erlotinib to normal brain is limited (Agarwal et al., 2012). The co-administration of elacridar with erlotinib was found to improve distribution of erlotinib to tumor core, tumor rim, as well as the normal brain. To improve the distribution of elacridar to the brain, especially in chronic multiple dose studies, we want to use a more bioavailable formulation of elacridar as an adjuvant. Our hypothesis was that co-administration of elacridar with erlotinib in a chronic dosage regimen can improve the distribution of erlotinib into the brain, and therefore improve efficacy. The purpose of this study was to examine the effect that chronic dosing of elacridar microemulsion as an adjuvant might have on the overall survival of tumor bearing mice that will receive erlotinib treatment. To this end, we utilized athymic Nu/Nu mice implanted with a patient-derived GBM12 tumor cell line (Sarkaria et al., 2006; Sarkaria et al., 2007). We examined the differences in the distribution of erlotinib in the presence and absence of elacridar in the tumor-bearing and contralateral (normal) hemispheres. We also studied the overall survival of GBM12-bearing mice that received a daily dose of erlotinib and elacridar.
The results from this study can help determine if improving the delivery of erlotinib to brain and brain tumors can influence the overall survival of tumor-bearing animals. Conversely, this would also help us determine if the delivery is an important limitation to the treatment of brain tumors, thereby helping design more appropriate preclinical studies of drug efficacy.

5.2 Materials and Methods:

5.2.1 Chemicals:

Elacridar (GF 120918) \([\text{N}-(4-(2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinoliny1)ethyl)phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide] \) of molecular weight 563.64 g/L was purchased from Toronto Research Chemicals (Ontario, Canada). Erlotinib was purchased from LC labs (Woburn, MA). Cremophor EL, Cremophor RH40, Solutol HS, Captex 355, Captex 300 were obtained from Abitech (Janesville, WI). Carbitol [2-(2-Ethoxyethoxy) ethanol] was purchased from Sigma-Adlrich (St. Louis, MO). All other chemicals used were reagent grade or HPLC grade from Sigma-Aldrich (St. Louis, MO).

5.2.2 Animals:

In vivo distribution studies were conducted in FVB wild-type mice (Taconic Farms, Germantown, NY). All animals were 8-10 weeks old at the time of the experiment. All mice were maintained under a 12 hour light/dark cycle and had unlimited access to food and water and were maintained under temperature controlled environment. All studies were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. Tumor distributional studies and survival studies carried out at Mayo Clinic,
Rochester were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic.

5.2.3 Influence of elacridar microemulsion on brain penetration of erlotinib

The elacridar microemulsion dosing solution was prepared as described above. Erlotinib dosing solution was prepared by suspending erlotinib in 1% Tween 80 to obtain a 2 mg/ml suspension. Elacridar microemulsion was made by preparing a 3 mg/ml solution of elacridar in Cremophor EL, Carbitol, and Captex 355 in the ratio 6:3:1 as in Chapter IV. This solution was diluted with saline prior to injection. Mice were administered a 5 mg/kg of elacridar microemulsion by injection into the intraperitoneal cavity 30 min prior to erlotinib administration. A 10 mg/kg dose of elacridar in solution (40% DMSO, 40% propylene glycol, 20% water) was administered to another group of mice, 30 min prior to elacridar administration (positive control). Mice were administered 10 mg/kg erlotinib by oral gavage. Animals were sacrificed at 1 hour by CO₂ euthanasia. Blood and brains were collected as described above. The concentration of elacridar and erlotinib were measured in plasma and brain by LCMS-MS.

5.2.4 Elacridar Influence on Erlotinib Efficacy

The efficacy of elacridar microemulsion in enhancing drug distribution to the brain and improving overall survival was examined in a xenograft model of glioblastoma. These studies were carried out at the Mayo Clinic, Rochester. This model has been previously used at the Mayo Clinic to examine efficacy of erlotinib (Sarkaria et al., 2006; Sarkaria et al., 2007).
A serially passaged xenograft (GBM 12) derived from a patient tumor was used. All xenograft therapy evaluations were conducted using an orthotopic tumor model and a protocol approved by the Mayo Institutional Animal Care and Use Committee. Tumors were induced as described earlier (Sarkaria et al., 2006; Sarkaria et al., 2007). Prior to treatment initiation, animals were randomized to treatment and placebo/control groups of 5 to 10 mice each. EGFR kinase inhibitor therapy was initiated 2 weeks before mice were expected to become moribund, as established through preliminary studies with each xenograft line. Erlotinib was administered by oral gavage (either 100 or 80 mg/kg daily, with methylcellulose used as the carrier) until mice became moribund, or until 4 weeks of treatment were completed (five times a week, maximum 20 administrations total). All mice used for therapy response evaluations were observed daily and euthanized at the time of a moribund condition.

5.2.5 Distribution of erlotinib in tumor and normal brain in a xenograft model of glioblastoma:

The effect of elacridar microemulsion in influencing distribution of erlotinib in tumor and normal brain was studied in tumor-bearing mice. Mice were administered elacridar microemulsion at 5 mg/kg IP 30 min prior to administration of erlotinib. 100 mg/kg erlotinib (1% methylcellulose as vehicle) was administered to the tumor-bearing mice. The control group received only erlotinib. The mice were sacrificed at 1 and 4 hours post dose. Blood was collected by cardiac puncture and plasma was separated by centrifugation. Brains were perfused with saline and then removed from skull and
separated into the tumor-bearing hemisphere and contralateral hemisphere. Both plasma and brain hemispheres were analyzed for concentrations of erlotinib by LCMS-MS.

5.2.6 Effect of elacridar adjuvant therapy on survival

Tumor-bearing mice (n=10) were administered elacridar (5mg/kg IP) and erlotinib (100 mg/kg) until morbid. The groups of mice that were used as control were administered only elacridar (5 mg/kg) or only erlotinib (100 mg/kg). Mice were treated with erlotinib, or elacridar or both elacridar and erlotinib, once a day, 5 times a week until moribund.

5.2.7 Analysis of elacridar and erlotinib by LCMS-MS:

The concentrations of elacridar and erlotinib in mouse plasma and brain were determined by HPLC coupled with mass spectrometry as described in Chapter IV. Brain samples were thawed and homogenized using a tissue homogenizer (Thermo Fisher Scientific) along with 5 % bovine serum albumin, three times the brain weight. 20 ng of internal standard, tyrphostin (AG1478) was added to 50 ul of plasma and 100 ul of brain homogenate along with 100 ul of pH11 buffer. Liquid-liquid extraction was carried out by adding 1ml of ethyl acetate and vortexing the mixture, followed by centrifugation at 7500 rpm for 15 min at 4 °C. 600 ul of the supernatant organic phase was dried under nitrogen, and reconstituted in 100 ul of mobile phase and transferred to autosampler vials. A 5 µl volume was injected into the HPLC using a temperature controlled auto sampler maintained at 10°C. A method to simultaneously detect elacridar and erlotinib was developed. The column used for chromatographic separation was purchased from Agilent Technologies (Santa Clara, CA) Eclipse XDB-C18 RRHT threaded column (4.6 mm id X 12.5 mm, 5 u). The mobile phase was composed of acetonitrile: 20mM ammonium
formate (with 0.1 % formic acid) (42:58 v/v) with a flow rate of 0.25 ml/min. Thermo Finnigan TSQ Quantum 1.5 detector (Thermo Fisher Scientific) was used to analyze the eluent. The instrument was equipped with an electrospray interface. The samples were ionized by the electrospray probe and analyzed in the positive ionization mode operating at a spray voltage of 4500 V for elacridar, erlotinib and the internal standard. The spectrometer was programmed to allow the [MH]+ ion of elacridar at m/z 564.6, m/z of 395.18 for erlotinib and 316.67 for the internal standard to pass through the first quadrupole (Q1) and into the collision cells (Q2). The collision energy was set at 39 V for elacridar and erlotinib, 9V for tryphostin. The product ions for elacridar (m/z 252.9), erlotinib (m/z 278.9 and 336.9) and internal standard (m/z 300.9) were monitored through Quadrupole 3 (Q3). The scan width and scan time for monitoring the two product ions was 1.5 m/z and 0.5 s resp. The assay was sensitive over a range of 2.5 ng/ml to 1000 ng/ml.

5.2.8 Statistical Analysis.

Comparison between groups were made using SigmaStat, version 3.1 (Systat Software, Inc., San Jose, CA). Statistical difference between two groups was tested by using the two-sample t-test, and significance was declared at p < 0.05. Multiple groups were compared by one-way analysis of variance with the Holm-Sidak post hoc test for multiple comparisons at a significance level of p < 0.05.
5.3 Results:

5.3.1 Influence of elacridar microemulsion on brain penetration of erlotinib in FVBn mice

Erlotinib is a dual substrate for P-gp and BCRP (Elmeliegy et al., 2011; de Vries et al., 2012). The presence of P-gp and BCRP at the BBB, limits the brain distribution of erlotinib. The aim of this study was to improve the brain distribution of erlotinib by co-administration of elacridar microemulsion. Erlotinib was administered as a 20 mg/kg oral dose to FVB wild type mice. The mice were pretreated with either intravenous elacridar in a conventional dosing solution (10 mg/kg, positive control) or an intraperitoneal injection of elacridar microemulsion (5mg/kg). The plasma and brain concentrations of elacridar and erlotinib were measured by LCMS/MS. The plasma concentrations of erlotinib at one-hour post dose were not significantly different across treatment groups (Figure 5.1.a). The mean plasma elacridar concentrations at 1.5-hour post elacridar dose achieved after an intravenous dose of elacridar solution were found to be 0.56± 0.06 µg/ml, compared to 3.2 ± 0.2 µg/ml after an intraperitoneal dose of the microemulsion. The mean brain concentration of erlotinib in the control group that did not receive an inhibitor was found to be 76 ± 7 ng/ml. The co-administration of elacridar as a solution (positive control), increased the mean brain concentrations to 366 ± 299 ng/ml and there was considerable inter-individual variability observed. The administration of the elacridar microemulsion increased the mean erlotinib brain concentrations three-fold to 253.3 ± 22 ng/ml (Figure 5.1.a).
The brain-to-plasma concentration ratios of erlotinib were calculated to normalize any effect due to difference in plasma concentrations of erlotinib across the groups. At 1 hour post-dose, the brain-to-plasma concentration ratio for the control group was calculated to be 0.07 ± 0.01, on co-administration of elacridar solution (positive control), the brain-to-plasma ratio improved to 0.37 ± 0.15 (Figure 5.1.b). The treatment group that received elacridar microemulsion also showed a statistically significant increase in brain-to-plasma ratio of erlotinib to 0.2 ± 0.04. These results demonstrate that the administration of elacridar as a microemulsion formulation can improve brain distribution of erlotinib and other substrates across the BBB.

5.3.2 Tumor distribution of erlotinib in presence of elacridar microemulsion

The distribution of erlotinib was studied in a tumor xenograft model. The aim of this experiment was to elucidate the effect of elacridar co-administration on the distribution of erlotinib between tumor and healthy brain tissue. Figure 5.2 represents the concentration of erlotinib in plasma and tumor-bearing and contralateral hemisphere. The concentration of elacridar in the plasma was consistent between the treatment groups. The concentration of erlotinib at 1-hour post dose in the control hemisphere was 1.4 ± 0.2 ug/g in the absence of elacridar, compared to 3.5 ± 0.8 ug/g observed with the co-administration of elacridar. This improvement in the distribution of erlotinib with co-administration of elacridar was observed also at 4-hour post dose. In the tumor bearing hemisphere, at one hour post dose, the erlotinib concentration was determined to be 0.9 ± 0.2 µg/g in the absence of elacridar and 2 ± 0.5 µg/g when elacridar is co-administered.

The concentration of erlotinib is lower in the tumor bearing hemisphere as compared to
the healthy brain in both treatment groups. This could possibly be a result of a leaky blood-brain barrier in the tumor core and perfusion of brain tissue following sacrifice. Co-administration of elacridar microemulsion was found to improve the brain-to-plasma concentration ratio by approximately three-fold in both tumor bearing hemisphere and healthy brain at both 1 and 4 hour post dose (Figures 5.3.a and 5.3.b). These results suggest that the use of elacridar microemulsion as an adjuvant in chemotherapy for glioblastoma enhance the limited drug distribution into the tumor tissue and in the healthy brain as well. Improved targeting of the chemotherapy to the tumor mass and also to tumor cells that reside in the “healthy” tissue, behind an intact BBB, could possibly enhance the efficacy of chemotherapy in treatment of glioma.

5.3.3 Survival studies:
Mice bearing GBM 12 xenografts were treated with erlotinib 100 mg/kg or a combination of elacridar (5 mg/kg) and erlotinib (100 mg/kg). The co-administration of elacridar did not significantly improve the overall survival rates of the tumor bearing mice as compared to mice that received only erlotinib (Figure 5.4). Therefore, the improvement in the brain distribution of erlotinib did not translate into an improvement in the overall survival of the tumor bearing mice. This could be due to several factors, one of them being that GBM 12 is a heterogeneous tumor cell line, that has growth driven by several different oncogenes, while erlotinib targets mainly EGFR. It is possible that there are a number of different pathways that promote growth in GBM12, and inhibition of EGFR alone does not result in a sufficient improvement in overall survival, regardless of improvements in delivery.
5.4 Discussion:

Erlotinib is an EGFR inhibitor that has been examined for use in the treatment of glioblastoma (Raizer et al., 2010a; Raizer et al., 2010b). Erlotinib is also a dual substrate for P-gp and BCRP, and active efflux from the brain limits the exposure of erlotinib to the brain tissue (Elmeliegy et al., 2011; de Vries et al., 2012). To overcome this barrier to drug delivery, elacridar could be administered as an adjuvant to therapy to inhibit efflux due to P-gp and Bcrp. The microemulsion formulation overcomes several difficulties in administration of elacridar. The co-administration of elacridar microemulsion via an intraperitoneal injection improved the brain distribution of erlotinib significantly. Intraperitoneal or oral route is more convenient to administer to tumor bearing mice as compared to a tail vein injection for chronic preclinical studies to examine efficacy enhancements. Also, we were able to decrease the dose of elacridar and still achieve a significant improvement in the brain distribution of erlotinib.

There are several advantages to developing a microemulsion formulation of elacridar. The novel formulation helps overcome the poor physicochemical properties of elacridar, such as poor solubility and high lipophilicity. The improvement in bioavailability allows us to decrease the dose that is needs to be administered to achieve inhibition of efflux from the BBB. The microemulsion can also be administered easily, either orally or intraperitoneally (Chapter IV). The microemulsion formulation of elacridar can be used as an adjuvant to therapy in the treatment of glioblastoma to improve delivery of chemotherapeutics that are to be administered on a chronic basis.
The co-administration of elacridar microemulsion with erlotinib in tumor bearing mice enhanced the concentration of erlotinib in both the tumor-bearing hemisphere as well in the healthy brain. There is an approximately three-fold increase in erlotinib concentrations in both tumor and healthy hemispheres. The brain-to-plasma ratio of erlotinib, a normalized distribution value correcting for any changes in plasma concentrations, also improved significantly in both hemispheres when elacridar microemulsion was co-administered.

Improvement in the brain distribution of drugs could potentially increase the efficacy of chemotherapeutic agents that are limited in their brain distribution. The results from this study showed that the use of an inhibitor of transporters as an adjuvant could be used to target tumor cells in the tumor hemisphere as well as tumor cells that reside behind an intact blood-brain barrier. The concentrations of erlotinib in the tumor bearing hemisphere was on average lower than the concentrations in the normal brain. There could be two possible reasons for this. The blood-brain brain is known to be ‘leaky’ or more permeable at tumor sites. Therefore, passive diffusion across the BBB could be a large component of mass transfer, and the effect of the efflux transporters could be muted. The brains were also perfused shortly after sacrificing the animals. Perfusion of the brains could have resulted in a diffusion of the drug molecules across the leaky BBB in the tumor into the perfusate, leading to a lower drug concentration as compared to hemisphere with an intact BBB.

The co-administration of elacridar microemulsion with erlotinib in tumor bearing mice did not improve the overall survival as compared to the group that received erlotinib.
alone. This result was disappointing since it seems that the three-fold improvement in brain concentration of erlotinib did not result in a corresponding increase in the overall survival of the tumor bearing mice. The lack of a significant change could be because the pharmacodynamic effect of erlotinib is non-linear, and the three-fold increase in drug concentration does not translate into a three-fold increment in the overall survival. A heterogeneous patient derived cell line was used for creating the xenograft, and this cell line could have multiple pathways that could promote growth. Therefore, administration of a single therapeutic drug could have limited efficacy in inhibiting tumor growth. Chemotherapy with a single kinase inhibitor in glioblastomas has been known to have limited efficacy (Sathornsumetee and Reardon, 2009). Erlotinib is mainly an EGFR inhibitor that has been shown to have some activity in inhibiting JAK2 kinase receptors (Li et al., 2007). This target heterogeneity and pharmacological specificity could be another reason for the lack of improvement in overall survival using erlotinib, even with elacridar as an adjuvant to help improve drug delivery.

One of the main advantages of utilizing the elacridar microemulsion formulation is the ease of administration to tumor bearing mice on a chronic basis. The microemulsion was administered at a dose of 5 mg/kg via intraperitoneal injections. Bioavailability of elacridar is nearly 100 percent when administered intraperitoneally (Chapter IV). Therefore, a 5 mg/kg dose was sufficient to produce an inhibitory effect at the BBB. A previously published study used elacridar orally at 100 mg/kg to improve brain delivery of sunitinib (Tang et al., 2012a; Tang et al., 2012b). This high dose of elacridar was able to improve the brain distribution of sunitinib. However, chronic use of such a high dose
cannot be carried out without a possibility of adverse reaction. The microemulsion formulation of elacridar can be useful in decreasing the required dose to produce an effect at the BBB. This is an important advantage as chronic treatment of tumor bearing mice would have otherwise required large amounts of elacridar.

The microemulsion of elacridar is effective in enhancing distribution of substrate drugs to the brain, as well as to the tumor, at a much lower dose than conventional dosage forms. The ultimate goal of improving survival in glioma may require an additional strategy, rather than simply improving distribution. The ability to target a number of growth pathways; inhibiting the tumor growth more efficiently by a combination of drugs, along with an adjuvant to improve CNS distribution, could help in improving the overall survival rate in glioma.
5.5 Footnotes

We would like to thank Dr. Jann Sarkaria, Brett Carlson and Cen Ling at the Mayo Clinic, Rochester, for their work on this chapter. This work was supported by National Institutes of Health - National Cancer Institute [CA138437] (W.F.E.) and a Faculty Development grant at the University of Minnesota (W.F.E.). Financial support for Ramola Sane was provided by the Ronald J. Sawchuk Fellowship and Rowell Fellowship.
Table 5.1: Plasma and brain concentrations of elacridar in tumor bearing Nu/Nu mice post an intraperitoneal injection of 5 mg/kg, at 1.5 and 4.5 hours post elacridar dose.

<table>
<thead>
<tr>
<th></th>
<th>1.5 hours</th>
<th>4.5 hours</th>
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<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td>0.26 ± 0.03 ug/ml</td>
<td>0.31 ± 0.07 ug/ml</td>
</tr>
<tr>
<td><strong>Left hemisphere</strong></td>
<td>1.6 ± 0.3 ug/g</td>
<td>1.2 ± 0.3 ug/g</td>
</tr>
<tr>
<td><strong>Right hemisphere</strong></td>
<td>0.9 ± 0.2 ug/g</td>
<td>0.8 ± 0.2 ug/g</td>
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Figure 5.1.a: The brain and plasma concentrations of erlotinib at 1 hour post a 20 mg/kg oral dose.

Pretreatment with elacridar solution (10 mg/kg intravenously) was used as a positive control. Elacridar microemulsion was administered intraperitoneally at 5 mg/kg. Administration of elacridar microemulsion improved the brain concentrations of erlotinib significantly (p<0.001).
Figure 5.1.b: Brain-to-plasma ratio of erlotinib at one hour post dose after pretreatment with elacridar solution (10 mg/kg intravenously) and elacridar microemulsion (5 mg/kg).

The brain-to-plasma ratio of erlotinib increased with co-administration of elacridar microemulsion (*p<0.05).
Figure 5.2: Plasma and brain concentrations of erlotinib in GBM12 xenograft model in mice.

The concentration of erlotinib in the brain is only a fraction of the erlotinib concentrations in the plasma. The co-administration of elacridar microemulsion increased the brain concentrations of erlotinib significantly at both 1 hour and 4 hours post-dose as compared to control.
Figure 5.3.a: Brain-to-plasma concentration ratios of erlotinib at 1 hour post-dose in tumor bearing hemisphere and control hemisphere in xenograft model of glioblastoma in mice.

Co-administration of elacridar microemulsion enhances the brain distribution of erlotinib in both hemispheres.
Figure 5.3.b: Brain-to-plasma concentration ratios of erlotinib at 4 hours post-dose in tumor bearing hemisphere and control hemisphere in xenograft model of glioblastoma in mice.

Co-administration of elacridar microemulsion enhances the brain distribution of erlotinib in both hemispheres.
Figure 5.4: Survival curve for xenograft (GBM12) bearing mice that received erlotinib (100 mg/kg/day, 5 times a week) and erlotinib with elacridar (5 mg/kg/day, 5 times a week).

No significant improvement was observed in mice that received elacridar as an adjuvant to therapy.
CHAPTER VI:

RECAPITULATION
The limited delivery of drugs to the central nervous system is a major obstacle to treatment of diseases of the brain and the spinal cord. The presence of the BBB effectively prevents drugs from reaching their site of action. This restricted delivery of drugs limits the efficacy of chemotherapeutics in treatment of aggressive and malignant diseases such as glioblastoma. The treatment of glioblastoma with temozolomide has resulted in statistically significant improvement in the overall survival rate for patients with GBM; however, the actual increase is only about 2 months. More recent clinical trials that have examined use of molecularly targeted agents either alone or in combination with other agents, have not been effective in the treatment of glioma. This lack of efficacy could result from a limited distribution of drugs to the target site in the tumors, as well as invasive tumor cells that reside behind an intact BBB. The invasive tumor cells escape surgical resection and give rise to recurrence of the tumor. In addition to this, tumor cells are known to express the efflux transporters P-gp and Bcrp at their cell surface which leads to development of resistance to drugs that are substrates for these drugs. Most molecularly-targeted drugs that are currently being examined for use in therapy for glioma are substrates for P-gp and BCRP, which limits their distribution into the central nervous system.

The main objective of this research project was to enhance the distribution of substrate molecules across the BBB by inhibiting the efflux transporters. To this end, we explored elacridar, an inhibitor of P-gp and Bcrp. However, we encountered difficulties in formulating a dosage form for elacridar that was suitable for chronic dosing. The ultimate goal of this project was to develop an effective formulation of elacridar that could be
administered as an adjuvant on a chronic basis that can be administered to glioma bearing animals to improve drug distribution to the brain. Enhancing the brain distribution of molecularly-targeted drugs could lead to better efficacy of these drugs by improving their access to target sites that are protected by the BBB.

The first step was to determine the exposure of elacridar in mice when administered via oral gavage or intraperitoneal administration in mice (Chapter II). The conventional formulation of elacridar as a suspension was seen to be associated with poor absolute bioavailability following both routes of administration. The bioavailability of elacridar post intraperitoneal administration was only about 1% and post oral administration was 22% when a dose of 100 mg/kg was administered. We also calculated the brain-to-plasma coefficient ratio (Kp) after oral, intraperitoneal and intravenous administration, to be 4.31, 0.4 and 0.82 respectively. This study showed that conventional dosing strategies show poor bioavailability and that there is a need to improve the bioavailability of elacridar. We also saw that the tissue partition coefficient of elacridar was different after different routes of administration, indicating saturation of active efflux process. We examine this phenomenon in detail in Chapter III.

In the next step of our research plan, we examined the interaction of elacridar with P-gp and BCRP at the mouse BBB using wild-type, P-gp knockout, Bcrp knockout and triple knockout mice. The mice were administered a single intravenous dose of elacridar, and brain concentration and Kp of elacridar was measured. The AUC_{brain} for elacridar was highest in triple knockout mice, followed by Bcrp knockout mice, P-gp knockout mice and wild-type mice respectively; indicating that elacridar is a substrate for P-gp and
BCRP and that its distribution of elacridar is limited by the presence of these transporters at the BBB. The Kp ratios for wild-type, P-gp knockout, Bcrp knockout and triple knockout mice were calculated to be 0.82, 3.5, 6.6 and 15 respectively (Chapter III). We also looked at the effect of increasing doses of elacridar on the brain-to-plasma coefficient ratio in all four genotypes and we found that the brain to plasma co-efficient ratios at one hour post-dose showed an increase to a maximum value and could be described by a simple Emax model. We can conclude from this study, that elacridar is a substrate for P-gp and Bcrp at the BBB, and its distribution into the brain is limited by the presence of these two transporters. The results also show that this active efflux can be overcome by increasing the dose of elacridar administered.

To overcome the limitations posed by the poor bioavailability of elacridar, we devised a strategy to develop a microemulsion formulation to improve its solubility (Chapter IV). The final optimized microemulsion formulation is composed of Cremophor EL, Carbitol, and Captex 355 in the ratio 6:3:1 and elacridar in a concentration of 3 mg/ml. The bioavailability of elacridar post oral gavage and intraperitoneal injection was calculated to be 47% and 100% respectively. This significant improvement in the bioavailability also allowed us to decrease the dose required to achieve the required plasma exposure. This is an advantage, since administration of lower doses in a chronic dosage regimen is less likely to give rise to adverse reactions.

In the final chapter (Chapter V), we looked at the effect of elacridar in the form of a microemulsion on the brain distribution of erlotinib. Elacridar microemulsion was effective in enhancing the brain distribution of erlotinib in FVBn mice. We also
examined the brain distribution of erlotinib in Athymic Nu/Nu mice that were implanted with glioma xenograft; with and without co-administration of elacridar microemulsion. The co-administration of elacridar was found to improve the brain penetration of erlotinib three-fold in both the tumor bearing hemisphere and the normal hemisphere. The tumor-bearing mice were also administered erlotinib with co-administration of elacridar, 5 days a week till moribund. The overall survival of mice that received erlotinib alone was not significantly different than mice that received erlotinib alone. This lack of improvement was disappointing; however, it gives us insights into the role of BBB in the treatment of glioma. The presence of BBB is not a limiting factor in the treatment of glioma with erlotinib. It is possible that the pharmacodynamic effect of erlotinib is non-linear; a three-fold improvement in the brain concentrations, do not result in a corresponding increase in the overall survival rate. Also, erlotinib inhibits a single target, EGFR. If the tumor has multiple pathways of growth, it is possible that inhibiting a single pathway, would not result in a significant effect.

In conclusion, the overall important findings of this work are outlined as follows; 1. The bioavailability of elacridar is limited due to its poor physicochemical properties 2. In addition to this, the brain distribution of elacridar is limited by the presence of P-gp and BCRP at the BBB. This is due to saturable efflux of elacridar by the transporters and can be overcome by increasing the dose of elacridar. 3. The bioavailability of elacridar can be improved by formulating it as a microemulsion 4. The co-administration of elacridar has been shown to improve the brain penetration of erlotinib in both tumor and normal brain;
however, there is no evidence that the overall survival of tumor-bearing shows a corresponding increase.

Future work will include testing a combination of molecularly targeted agents to inhibit multiple targets on tumor as well as invasive tumor cells using elacridar as an adjuvant to therapy. Taking into consideration the heterogeneity and invasiveness of this disease, treating it as a whole-brain disease is more likely to give successful results.
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