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Cytotoxic Analysis of Old Drugs: New Drugs for Alzheimer's Disease

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by

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Abstract

Microglia are the resident immune cells of the CNS and constitute about 10% of all cells in the CNS. As the resident immune cells of the CNS, they are the first line of defense against infections and injury. As so, microglia have a vital role in the pathogenesis of Alzheimer's disease as either cytotoxic or neuroprotective. In accordance with these dual roles, microglia reside in the CNS in one of two phenotypes, M1 or M2. In Alzheimer's disease (AD), the M1 microglia exacerbate AD pathogenesis by releasing pro-inflammatory cytokines, chemokines and free radicals. In contrast, the M2 microglia are involved in releasing anti-inflammatory cytokines and chemokines and are involved in the clearance of Aβ. Interestingly enough, Aβ activates microglia to the M1 state, and due to the accumulation of Aβ among other factors, a positive cycle is formed in which microglia are continuously activated to the M1 state. As of yet, no drugs have been able to treat AD. But recently, drug repurposing has provided an alternative to finding AD treatments from drugs that have been used for other diseases, such as cancer. In this study, we analyze the cytotoxicity of prospective drugs to be repurposed, with the assumption that the more cytotoxic drugs exacerbate neuroinflammation.

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INTRODUCTION

Alzheimer's Disease

In 2017, it was estimated that around 50 million people worldwide have dementia, with Alzheimer's disease accounting for 60-70% (Dua et al., 2017). In the US, AD has become the sixth leading cause of death, with an estimated 5.8 million people living with Alzheimer's, a figure that is estimated to increase to 13.8 million cases by 2050 (Hebert, Weuve, Scherr, & Evans, 2013). The pathological hallmarks of AD are characterized by cognitive decline, neuronal loss and cellular dysfunction. For almost 30 years, the amyloid cascade hypothesis was the prevailing explanation for the pathology of AD. This hypothesis claims that the cause for neurodegeneration is Aβ, particularly extracellular amyloid plaques. In recent times, this hypothesis has been contested, with recent research supporting the ideas that inflammation may be what leads to neurodegeneration in AD.

Microglia

Microglia are the first line of defense in the CNS upon pathogen invasion or brain injury. Microglia constitute roughly 10% of the cell population in the CNS (Fakhoury, 2018). In healthy individuals, microglia reside in a resting state, and their function is to surveil the CNS for pathogens or markers of injury by monitoring for pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs) (Colonna & Butovsky, 2017; De strooper & Karran, 2016; Hansen, Hanson, & Sheng, 2018; Li, Du, Liu, Wen, & Du, 2012; Solito & Sastre, 2012). In addition to monitoring the CNS, microglia also play a role in the development of neural circuits by phagocytosing neurons and synaptic pruning (De strooper & Karran, 2016; Frost & Schafer, 2016; Hansen et al., 2018; Paolicelli et al., 2011; Salter & Beggs, 2014; Schafer et al., 2012). When an invading pathogen or brain injury is present, microglia are

able to recognize the insults because they have pattern recognition receptors in their membrane which can recognize PAMPs or DAMPs, resulting in microglial activation. When microglia are activated, they are polarized to one of two activated states that are phenotypically and morphologically different. The proinflammatory state, M1, is promoted by lipopolysaccharide (LPS), the pattern recognition receptors like Toll-like receptors (TLR) and interferon-gamma (IFNγ). In this activated state, microglia secrete proinflammatory cytokines and chemokines, which are thought to have beneficial effects at low concentrations, as well as having phagocytic activity (Fakhoury, 2018; Lynch, 2009). The other activation state is the M2 state and it is activated by IL-4 and IL-13, which releases anti-inflammatory cytokines, and promotes tissue repair, angiogenesis as well as extracellular matrix remodeling (Wang, Tan, Yu, & Tan, 2015). Due to the changing environment in the CNS, microglia are able to switch states in accordance with the microenvironment that it is present (Calsolaro & Edison, 2016; Varnum & Ikezu, 2012).

AD and microglia

As the resident immune cells of the CNS, microglia monitor the environment for pathogens and debris by recognizing pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). As such, microglia recognize Aβ as a DAMP and become activated into an M1-like state. At first this interaction is beneficial for the induction of phagocytosis, which includes the clearance of Aβ from the CNS (Solito & Sastre, 2012). Previously, microglia were shown to interact with amyloid plaques in the postmortem brains of AD patients(Calsolaro & Edison, 2016; B. Liu & Hong, 2003). In fact, microglia interact with Aβ through CD36 and Toll-like receptors, which activate microglia to an M1 state, resulting in the clearance of Aβ as well as the release of inflammatory cytokines, which can be cytotoxic (De strooper & Karran, 2016; Jana, Palencia, & Pahan, 2008; Reed-Geaghan, Savage,

Hise, & Landreth, 2009; Richard, Filali, Préfontaine, & Rivest, 2008). While activation of microglia is beneficial for the clearance of Aβ, it can also be detrimental because Aβ itself can also cause microglia to become overactivated, which results in the release of inflammatory cytokines, further exacerbating neurodegeneration (Fakhoury, 2018).

Drug Repurposing

Drug repurposing is an attractive alternative to finding cures for diseases like AD for various reasons. The process of discovering a drug, testing it, getting it approved and ready for the market is a long and expensive journey which can last about 15 years and cost up to $1billion dollars (Paranjpe, Taubes, & Sirota, 2019). Drug repurposing would make this a less time consuming and cheaper alternative because the drugs that would be tested have already been FDA approved, although pharmaceutical companies are less adamant about taking this alternative as it will not yield the highest profit for them (Paranjpe et al., 2019). Drug repurposing is an attractive alternative because neurodegeneration shares some similar pathways with other diseases. For example, it has been observed that there is an inverse relationship between cancer and AD in which cancer survivors have a decreased risk of AD and vice versa (Durães, Pinto, & Sousa, 2018).

In this study, we are treating microglial cultures with prospective drugs postulated to have beneficial effects in treating AD to determine if these drugs are cytotoxic. The safe drugs will then be used to treat a transgenic rat model of AD, to analyze whether the drugs have any therapeutical potential to treat AD.

Materials and Methods

Drugs: IBR2 (cat#: HY-103710; MedChemExpress, Monmouth Junction, NJ); Atractylenolide II (ATII) (cat#: HY-N0202; MedChemExpress, Monmouth Junction, NJ); (R)-ADX-47273 (ADX) (cat#: HY-13058; MedChemExpress, Monmouth Junction, NJ); Crenolanib (Cre) (cat#: HY-13223; MedChemExpress, Monmouth Junction, NJ)

Microglia HMC3 cell line: The human microglia clone 3 (HMC3 ATCC CRL-3304) cell line is derived from human fetal brain-derived primary microglial cultures. These cells are immortalized by simian virus 40 (SV-40) that expresses large T and small T antigens, of which immortalization is achieved through the inhibition of pRB and p53 by the large T antigen (Dello Russo et al., 2018; Janabi, Peudenier, Héron, Ng, & Tardieu, 1995; Jha, Banga, Palejwala, & Ozer, 1998). The HMC3 cell line is a model for primary cells since it retains its properties and are therefore ideal to study the role of microglia. The cells are positive for microglia markers IBA1 and CD14 but negative for the astrocyte marker GFAP. As well, markers of activated microglia such as MHCII and CD68 are negative in the resting HMC3 cells and are upregulated when the cells are activated by IFN-γ.

The cell cultures are maintained in EMEM media (cat#30-2003; ATCC, Manassas, VA; 500mL) supplemented with 10% non-heat inactivated FBS (cat#10437-028; Thermo Fisher, Waltham, MA; 50mL) and 1% penstrep (cat# 15140-122; Gibco, Waltham, MA; 5mL). The cells are split every three to four days.

Drug Treatment of HMC3 cultures: The cells were treated with one of four drugs: IBR2, ATII, ADX, or Cre for 24 hours. The concentrations were as follows: IBR2 5, 10, 20µM; ATII 10, 25, 50µM; ADX 5, 10, 20µM; and Cre 100nM, 1µM, 10µM. The solvent DMSO was used as a vehicle for the drugs, at a final concentration of 0.5%, which is not toxic to the cells. The

media was changed at least 30 minutes before treatment. Cell viability was then assessed with the MTT assay.

MTT assay: The MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was used to assess cell viability by measuring the cell's metabolic activity, specifically mitochondrial activity. Although the precise mechanism is not known, it is postulated that NADH, a reducing molecule, is involved and reduces MTT to formazan, a purple insoluble crystal, which accumulates inside the cell (Barile, 1997; Mosmann, 1983; Riss et al., 2004). The amount of formazan crystal formed is proportional to the number of cells in(Mosmann, 1983). After the 24 hour treatment, media was aspirated from each well. The cells were then incubated in EMEM media containing 10% FBS and 1% pen/strep with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After an hour incubation, the media was aspirated again and the cells/crystals are dissolved in 0.4N HCl dissolved in isopropanol, since the formazan crystals are insoluble in water, and placed on a shaker to fully dissolve (Barile, 1997; Riss et al., 2004). The MTT results were then quantified by spectrophotometry.

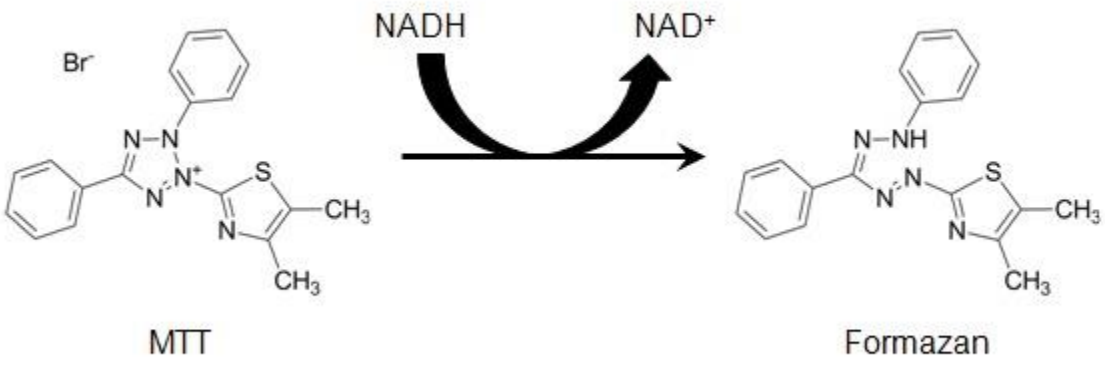

Figure 1. Structure of MTT and Formazan. (Riss et al., 2004)

Spectrophotometer: The PowerWave HT Microplate Spectrophotometer was used to measure absorbance at the end of the assay. The wavelengths used were 550nm and 620nm. To measure the percent of cells that survive, the difference between the values of 550-620nm for a sample is divided by the average of the difference between 550-620nm for the controls.

$$\frac{sample\ (550-620)nm}{control\ average\ (550-600)nm} * 100$$

Statistics: Statistical analysis was done using GraphPad Prism 7. Data is represented as mean ± standard error (SEM). Data was analyzed using a one-way ANOVA and Dunnett's multiple comparison test.

Results

HMC3 treatment with IBR2

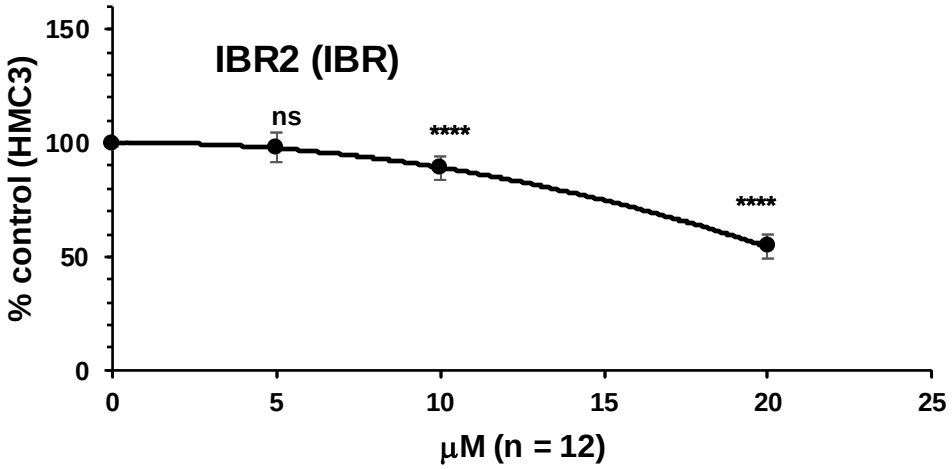

Figure 2. Microglia were treated with the Rad51 inhibitor, IBR2, at 5μM, 10μM and 20μM. At 10μM, only 88.96% of the microglia were viable compared to the control whereas the amount of viable microglia was reduced to 54.49% at 20μM. Data is shown as mean ±SEM; $^{****}p<0.0001$ vs control; ns= not significant.

IBR2 is an anti-cancer drug that inhibits the DNA damage repair. Specifically, it inhibits the Rad51 protein, which is essential for the repair of DNA damage through homologous recombination (Velic et al., 2015). DNA damage is a hallmark of aging as well of neurodegeneration, and as such, targeting the DNA damage repair pathway might be an attractive therapy (Madabhushi, Pan, & Tsai, 2014). As can be seen in the figure 1, treatment with 5μM IBR2 drops the viability of the HMC3 cultures by about 2%, and treatment with 10μM IBR2 drops the viability of the cultures by 12%. In contrast, at 20μM only 54.5% of the cells were viable.

HMC3 treatment with ATR

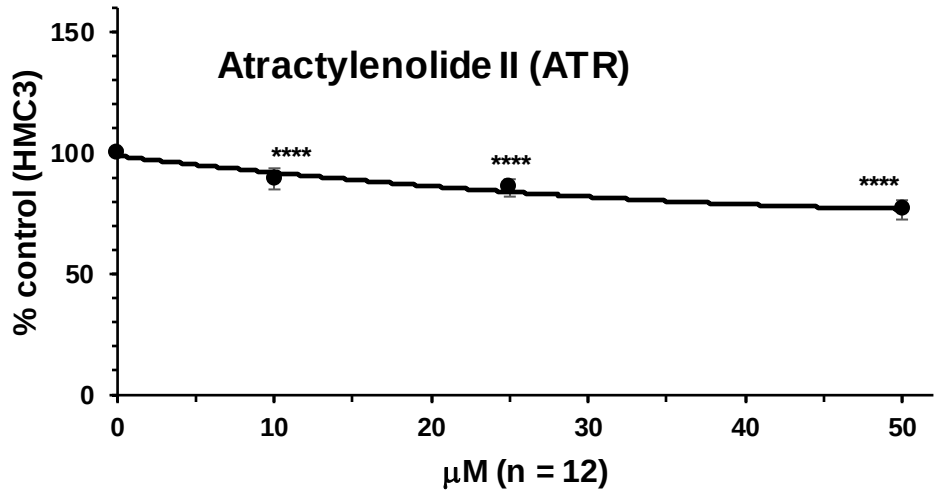

Figure 3. Microglia were treated with Atractylenolide II at 10μM, 25μM and 50μM. At 10μM, 89.35% of the microglia were viable, and at 25μM, it dropped to 85.61%. Increasing the concentration to 50μM dropped the viability to 76.53%. Data is shown as mean ±SEM; $^{****}p<0.0001$ vs control.

Atractylenolide II is a natural compound used in Eastern medicine and found in the rhizomes of the atracylode plant species. Reports have observed that atractylenolide II has the potential to inhibit the inflammatory mediator nitric oxide (NO) production in microglia (Hoang le et al.). Its orthologs, atractylenolide I and III, have been reported to have neuroprotective roles in Parkinson's disease and reduced production of inflammatory markers such as Il-6 and Il-1β (More & Choi, 2017; Yim, Gu, Park, Hwang, & Ma, 2018; Yun Hee, Wei, Younghoon, & You-Chang, 2019). The three concentrations with which the HMC3 cultures were treated showed that the cells are still moderately viable after treatment.

As the MTT assays revealed, the cell cultures are still viable even after treatment with high concentrations.

Treatment with ADX

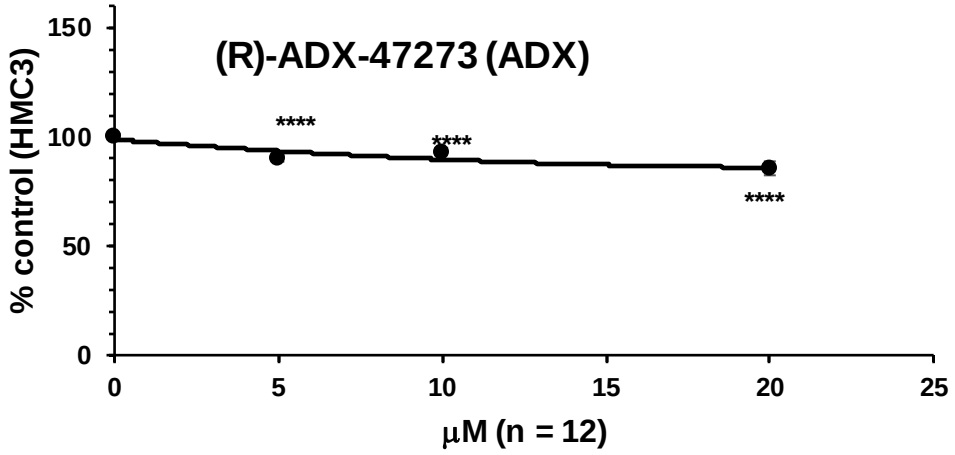

Figure 4. Microglia were treated with ADX at 5µM, 10µM and 20µM concentrations. Treatment of the microglia with ADX at 5µM had an 90.15% viability and increased at 10µM with a 92.16% viability. At 20µM, there was an 85.58% viability. Data is shown as mean ±SEM; $^{****}p<0.0001$ vs control.

ADX is a positive allosteric modulator for mGluR5, a G-coupled protein receptor. ADX was chosen as a possible drug for treatment because it is selective for mGluR5. Its orthologs, mGluR1, causes necrotic cell death although it is mostly expressed in neurons with only a negligible amount in microglia (Byrnes, Loane, & Faden, 2009; Loane et al., 2014). Previous studies have shown that ADX is able to attenuate NO and ROS at moderate to high concentrations (Byrnes, Loane, et al., 2009; Cleva & Olive, 2011). As shown in the figure above, treatment of ADX at 20µM resulted in a 15% drop in viability.

Treatment with CRE

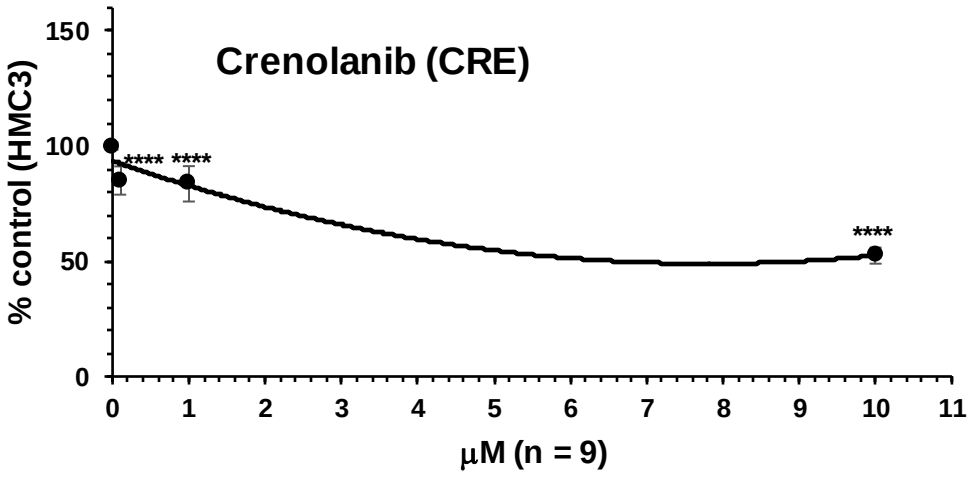

Figure 4. Microglia were treated with Crenolanib at 100nM, 1μM and 10μM concentrations. Treatment of microglia with Crenolanib at 100nM had a 85.06% viability, 83.62% viability at 1μM and dropped to 52.46% at 10μM. Data is shown as mean ±SEM; $^{****}p<0.0001$ vs control.

Crenolanib is an anti-cancer drug used for the treatment of cancers like gliomas. It inhibits the class III tyrosine receptors FLT3 and PDGFR-α/β, which are expressed in both microglia and macrophages, respectively (Deboy et al., 2010). FLT3 is involved in the activation of microglia as well as mediating inflammation by releasing inflammatory markers like IL-6 and CD86 (Deboy et al., 2010; Dehlin et al., 2013). As seen in the figure, Crenolanib is only slightly cytotoxic at low concentrations but at higher concentrations, only about 50% of the cells were still viable.

Discussion

This aim of this study was to identify drug candidates that were selected for possible therapeutic effects against Alzheimer's disease (AD). Importantly, the drugs should polarize the microglia from an M1 activated state, in which the microglia release neuroinflammatory factors, to an M2 state, in which anti-inflammatory cytokines are released.

Previous studies have observed that the persistent activation of the DNA repair pathway due to double strand breaks has the potential to revert post-mitotic neurons to undergo the cell cycle, resulting in either senescence or apoptosis (Herrup, Neve, Ackerman, & Copani, 2004; Tuxworth et al., 2019). According to Fielder et al., the re-entry of neurons into the cell cycle is common in Alzheimer's disease as well as other neurodegenerative diseases (Fielder, von Zglinicki, & Jurk, 2017; Pelegrí et al., 2008). Since neuronal disfunction and apoptosis/senescence can result from the over-activation of the DNA damage repair pathway, it would be interesting to see if treating with an inhibitor of the DNA damage repair would be of therapeutic value. It has been observed that treating neurons with an inhibitor of Mre11, Rad50 and Nbs1 (MRN), a complex involved in double strand break recognition, was neuroprotective (Tuxworth et al., 2019). The MRN complex detects double-stand breaks in the DNA and creates a 3' overhang that is shielded by the protein RPA, which then is replaced by Rad51 (Velic et al., 2015). In order for this to be an effective treatment, the other cells found in the brain, such as the microglia, should not be detrimentally affected by the inhibition of RAD51. In other words, treatment with IBR2 should not polarize the microglia to an activated M1-like state.

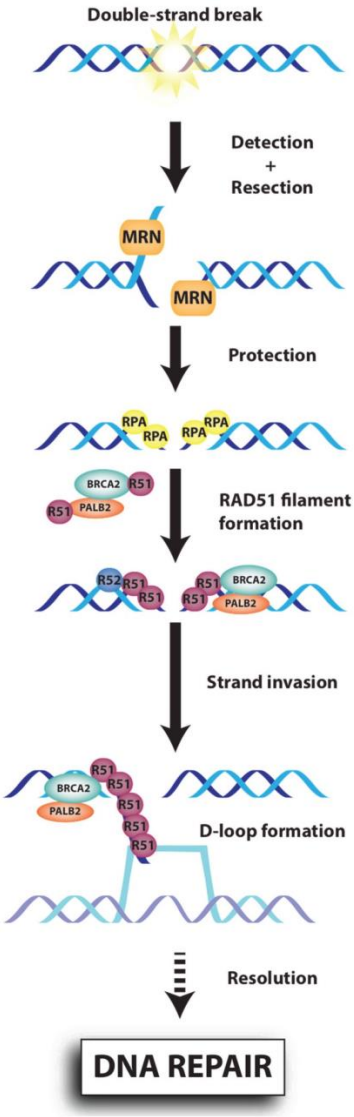

Figure 5. A representation of the DNA repair through homologous recombination. As shown, the MRN complex detects a double strand break and resections it to form a 3'overhang. The MRN complex is then replaced by RPA, which protects the overhangs. RPA is replaced by RAD51, which is the essential for strand invasion to occur (Bhat & Cortez, 2018; Velic et al., 2015).

A natural drug like ATII, whose orthologs are reported to reduce inflammatory markers, has the potential to reduce microglia production of NO. This is important for the survival of neurons in the brain. The ortholog atractylenolide I was shown to decrease the mRNA levels of

MCP-1 and MMP-9 in mice models of Parkinson's disease (More & Choi, 2017). MCP-1 is a chemokine that upon inflammation causes the migration of leukocytes such as monocytes and dendritic cells, whereas MMP-9 degrades the blood brain barrier (BBB), which would allow for the infiltration of leukocytes (Jayasooriya, Choi, & Kim, 2013; Thompson & Van Eldik, 2009). In a study by Hoang et al., ATII was found to be the third most potent compound that inhibits NO production in murine macrophage cell lines, while in a different study it was found to be one of the least cytotoxic compounds in the same cell line (Chen et al., 2016; Hoang le et al.).

An important aspect for these potential treatments to be effective is for their targets to be expressed in microglia and not be vital for the survival of other cells. An important neurotransmitter in the CNS is glutamate, which is involved in the regulation of cellular and synaptic activity, learning and memory, and cell death and survival amongst some other functions. Glutamate can bind to either ionotropic or metabotropic receptors, of which there are three groups. Of special interest is the group I receptor mGluR5, which is expressed in neurons and constitutively expressed in microglia cultured from rat brains (Byrnes, Stoica, et al., 2009). Previous studies have shown that activation of mGluR5 in culture attenuates microglial activation and the release of inflammatory factors, such as NO and ROS (Byrnes, Stoica, et al., 2009; Loane, Stoica, Pajoohesh-Ganji, Byrnes, & Faden, 2009). There are studies that report that using antagonists for this receptor is neuroprotective while others report that agonist for the receptor bring about neuroprotective functions; although it has been shown that the neuroprotective actions of the antagonist have nothing to do with targeting mGluR5 but are instead a result of interactions with the NMDA receptor (Lea, Movsesyan, & Faden, 2005). In fact, studies have shown that inhibiting the receptor in murine BV-2 microglial cells increased the production of reactive oxygen species (ROS), increased expression of inducible nitric oxide

synthase (iNOS) and the release of inflammatory cytokines and chemokines (Chantong, Kratschmar, Lister, & Odermatt, 2014). Moreover, it has been reported that binding of mGluR1, a group I receptor to its antagonist, is neuroprotective in vivo and in vitro. Because the group I receptors (mGluR1 and mGluR5) ligand binding region is highly conserved, allosteric modulators are preferred since they bind to less conserved regions thus making it more selective for the specific receptor (Homayoun & Moghaddam, 2010). In this study, we use the positive allosteric modulator (PAM) ADX-47273 to target mGluR5 because of its selectivity for the receptor and it is less likely to desensitize the receptor. As shown in figure 3, treatment with PAM was not cytotoxic, even at a high concentration of 20μM.

The last drug tested was the FLT-3 and PDGFR-α/β inhibitor crenolanib. Because crenolanib has more than one target, its effects range from beneficial to nonbeneficial depending on its concentration. This is because each of the receptors have different functions in relation to specific cells. In a previous study, FLT-3 inhibitors were found to provide protection against glutamate-induced apoptosis and necrosis, as well as decrease lipid peroxidation (Kang, Tiziani, Park, Kaul, & Paternostro, 2014). In an experiment studying multiple sclerosis, mice were treated with FlT-3 inhibitors and it was observed that there was a decrease in dendritic cells and improvement of encephalitis (Dehlin et al., 2013). In another study on multiple sclerosis, it was found that inhibiting FlT-3 also reduced the levels of pro-inflammatory cytokines in microglia stimulated with lipopolysaccharide (LPS) as well it was observed that the levels of FlT3 ligand correlated with the levels of total and phosphorylated tau, the microtubule-associated protein, which correspond with axonal degeneration and neurofibrillary tangles (Ahmed, Murakami, Hirose, & Nakashima, 2016; Deboy et al., 2010; Dehlin et al., 2013; Xin, Tan, Cao, Yu, & Tan, 2018). Crenolanib is also an inhibitor for platelet-derived growth factor receptor (PDGFR) α and

β, although it has been observed that microglia only express PDGFRβ (Roy & Pahan, 2013). PDGFRβ is activated by PDGF B and D, and it has been reported that activation of this receptor is involved in inflammation, macrophage migration and activation as well as the increase of TNFα (Miyata et al., 2014; Pei et al., 2017; Sil, Periyasamy, Thangaraj, Chivero, & Buch, 2018; Yang et al., 2016). Treatment of microglia with Crenolanib was the most cytotoxic drug tested as can be seen in the figures shown in the results section. Other studies have found that PDGFRβ signaling is neuroprotective in cells such as neurons and pericytes leading to problems such as neuronal death and blood-brain barrier (BBB) leakage (Ahmed et al., 2016; Giannoni et al., 2016; Y. Liu & Aguzzi, 2020).

Future Directions

The next steps in this project are to study the effects of these drugs in vivo. Ideally, the drugs chosen to be tested in rat models of AD should be safe and be able to cross the blood brain barrier. To do this, we will use the TgF344 transgenic rat model and will administer the drug through their food pellets. The advantage of using this rat model is that it displays a more complete repertoire of AD pathology compared to other rodent models such as mice. As well, compared to mice, rats are 4 to 5 million years closer to humans in evolution and have 6 tau isoforms that complement that 6 tau isoforms in humans (Cohen et al., 2013). These rats also have a higher amount of Aβ than mice, in fact it is within the clinico-pathological range of humans (Cohen et al., 2013). Then at 9 and 11 months of age, the rats will be subjected to the place avoidance test to analyze their cognition. Lastly, the rat brains will be dissected, with the left hemisphere used for immunohistochemistry and the right hemisphere used for biochemical analysis, such as Western blot.

Abbreviations

Alzheimer's disease= AD
Blood brain barrier= BBB
Damage-associated molecular pattern= DAMP
Interferon gamma= IFNγ
Inducible nitric oxide synthase= iNOS
Lipolisaccharide= LPS
Nitric oxide= NO
Positive allosteric modulator= PAM
Pathogen-associated molecular pattern= PAMP
Reactive oxygen species= ROS
Toll-like receptor= TLR

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