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Identification of Small Molecule Compounds that Regulate Pdgfra Transcription in Oligodendrocyte Progenitor Cells

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Jelena Medved, PhD

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Abstract

Oligodendrocyte progenitor cells (OPCs) are precursor cells that give rise to oligodendrocytes. Oligodendrocytes are the cells that form myelin, an arrangement of membrane sheaths enwrapping axons necessary for the fast transmission of electrical impulses in the central nervous system (CNS). OPCs generate oligodendrocytes both during development and adulthood and also after the myelin damage caused by injury or demyelinating disease.

Generation of oligodendrocytes from OPCs is in part regulated by growth factors. The major survival factor and mitogen for OPCs is platelet derived growth factor (PDGF). PDGF and its receptor, PDGF receptor A (PDGFRA), are critical regulators of OPC proliferation and their differentiation into myelinating oligodendrocytes. PDGFRA gain-of-function can lead to excessive proliferation and is associated with a number of cancers, including glioma in the CNS. In terms of the role of PDGFRA in OPC differentiation, there is evidence that suggests that signaling through PDGFRA needs to be inhibited in order for OPC differentiation to occur.

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The goal of this study is to identify small molecule compounds that inhibit Pdgfra transcription in oligodendrocyte precursor cells. We hypothesized that inhibition of Pdgfra transcription by small molecule compounds would result in inhibition of OPC proliferation and stimulation of their differentiation into mature oligodendrocytes. Identification of such compounds may provide a novel direction in drug design for demyelinating disorders or specific types of cancers caused by aberrant Pdgfra expression.

We identified a group of compounds that downregulated Pdgfra transcription in Oli-neu cells, a cell line that represents mouse oligodendrocyte precursor cells, and that inhibited proliferation of PDGFRA-expressing Oli-neu cells and primary mouse OPCs but did not inhibit proliferation of primary mouse astrocytes and HEK 293 cells, that do not express PDGFRA, or glioblastoma-derived cell lines. However, these compounds did not promote differentiation of primary mouse OPCs.

Identification of Small Molecule Compounds that Regulate Pdgfra Transcription

in Oligodendrocyte Progenitor Cells

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Identification of Small Molecule Compounds that Regulate Pdgfra Transcription in Oligodendrocyte Progenitor Cells

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Introduction

Oligodendrocyte progenitor cells (OPCs, NG2 cells, polydendrocytes) represent a population of cells in the central nervous system (CNS) that is distinct from all other cell types (reviewed by Nishiyama, et al, 2009). OPCs are uniformly distributed throughout the gray and white matter and persist as the largest population of cycling cells in the adult mammalian CNS (Dawson et al, 2003). They are the major source of oligodendrocytes (Zhu et al, 2008), cells that generate myelin sheaths necessary for rapid conduction of electrical impulses in the CNS.

Generation of oligodendrocytes occurs both during development and adulthood in healthy rodent brain. However, OPCs can give rise to oligodendrocytes in pathological conditions as well. Upon myelin damage, OPCs proliferate and differentiate to compensate for myelin loss (reviewed by Miron et al, 2011). In fact, acute myelin lesions are efficiently repaired (Zawadzka et al, 2010). Chronic lesions however, such as those found in multiple sclerosis (MS) and its rodent models, are not remyelinated efficiently (Tripathi et al, 2010). It is not determined why OPCs are unable to repair myelin damage under these conditions, but one possibility is that OPC are inhibited from differentiation into myelinating oligodendrocytes (Kuhlman et al, 2008).

OPCs are commonly identified by the expression of platelet derived growth factor (PDGF) receptor A (PDGFRA). Signaling through PDGFRA is the key mechanism in regulation of OPC proliferation and differentiation. A large body of evidence indicates that PDGF is the major mitogen for OPCs (Noble et al, 1988; Richardson et al, 1988; Barres et al, 1992; Calver et al, 1998; Fruttiger et al, 1999; van Heyningen et al, 2001). PDGF presence is necessary for OPC proliferation *in vitro* and its withdrawal causes

rapid differentiation of OPCs. Studies have suggested the default pathway that OPCs undertake is differentiation. This pathway is normally inhibited by PDGFRA signaling in progenitor cells and signaling through PDGFRA needs to be repressed in order for OPCs to differentiate. Since PDGFRA is present in OPCs, but gets downregulated as OPCs differentiate, it is likely that PDGFRA and its downregulation are critical factors that control oligodendrocyte differentiation and that PDGFRA has to be downregulated in order for OPCs to differentiate (McKinnon et al, 2005; Zhu et al, 2014). However, molecular mechanisms that lead to PDGFRA repression and therefore trigger differentiation remain to be defined.

The goal of this study is to identify small molecule compounds that downregulate PDGFRA transcription. This may contribute in understanding the mechanism of PDGFRA expression and provide a novel direction in drug design for demyelinating disorders or specific types of cancers caused by aberrant PDGFRA expression.

To address this goal, we performed experiments outlined in the Specific Aims below.

Aim 1: Identify lead compounds that downregulate the transcription of PDGFRA.

Aim 2: Examine structure activity relationship.

Aim 3: Determine whether the compounds promote OPC differentiation.

Aim 4: Determine whether the compounds inhibit OPC proliferation.

Chapter 1 - Background

Brief history of glia

Early in the 19th century, Theodor Schwann recognized a cell as the fundamental unit of all living organisms. It was not until well into the 20th century, however, that neuroscientists agreed that nervous tissue, like all other organs, is made up of these fundamental units. The major reason was that the first generation of neurobiologists in the 19th century had difficulty resolving the unitary nature of nerve cells with the microscopes and cell staining techniques that were then available. As a result, some biologists of that era concluded that each nerve cell was connected to its neighbors forming a continuous network, or reticulum. The "reticular theory" of nerve cell communication, led by Italian neuropathologist Camillo Golgi, was eventually replaced with "neuron doctrine" by Spanish neuroanatomist Santiago Ramon y Cajal and British physiologist Charles Sherrington. The histological studies of Cajal, Golgi and many successors led to the further consensus that the cells of the nervous system can be divided into two broad categories: neurons and glia.

For the past 160 years, cells in the nervous system have been divided into neurons and glia (reviewed by Kettenmann and Verkhratsky, 2008). An idea on the brain connective tissue, the "nervenkitt", nerve cement or neuroglia was proposed by Rudolf Virchow in the mid 19th century. The term neuroglia and the concept behind them spread around the world. Soon after, many different forms of glial cells were described by Deiters, Henle, Retzius, Golgi and others (Deiters, 1865; Henle and Merkel, 1869; Retzius, 1894; Golgi, 1903). In the late 19th century, Michael von Lenhossek proposed the term

"astrocyte" (von Lenhossek, 1893) and slightly later, Kolliker (Kolliker, 1889) and Anderiezen (Andriezen, 1893) divided those into fibrous and protoplasmic astoryctes. Pio del Rio-Hortega recognized oligodendrocytes and microglia in the early 20th century and added them to the growing list of glial subtypes (del Rio-Hortega, 1919; 1921; 1932). It was not until mid 20th century that myelin, the term also introduced by Virchow, was recognized to be part of the Schwann cell (Geren, 1954).

OPCs as the fourth population of glia

The initial division of glia into three types, astrocytes, oligodendrocytes and microglia, accomplished in the mid 19th century, has recently been challenged. An entirely new class of glial cells, oligodendrocyte progenitor cells (OPCs), also known as polydendrocytes (Nishiyama et al, 2007), has been characterized and proposed as the 4th major population of glia (Nishiyama et al, 2009).

OPCs were discovered in the late 20th century and were thought to be precursor cells that most commonly generate oligodendrocytes, but can also generate astrocytes in some cases (Raff et al, 1989; Zhu et al, 2008; Zhu et al, 2011) and even neurons (Kondo and Raff, 2000; Rivers et al, 2008; Guo et al, 2009; Guo et al, 2010). However, OPCs are not solely progenitor cells. They can be considered a distinct type of glia for several reasons. First, they are uniformly distributed population of cells in gray and white matter of healthy adult rodent brain that persists into adulthood as the major pool of cycling cells and is as abundant as other cell types (Dawson et al, 2003). Second, the pattern of expressed markers distinguishes these cells from all the others. In addition, these is increasing evidence that suggests a population of OPCs persists in adult brain

and does not differentiate into oligodendrocytes, so it is likely that OPCs have a far more complex role in nervous system physiology that we are not yet aware of.

OPC development and fate choices

OPCs originate from discrete parts of the ventricular zone (VZ) but the region of VZ that will give rise to OPCs is different at different time points during development and temporarily progresses from the ventral towards the dorsal part of the VZ (Warf et al, 1991; Kessaris et al, 2006; reviewed by Richardson et al, 2006). Once committed to oligodendrocyte lineage, OPCs migrate away from the VZ through the developing CNS and into what will become the white matter of the CNS (Reynolds et al, 1988; Levine et al, 1988). OPCs continue to divide after they leave the VZ. Once they settle at their final destinations, OPCs will exit the cell cycle, express myelin genes and mature to fully differentiated oligodendrocytes (reviewed by Baumann and Pharm-Dinh, 2001). However, not all OPCs will differentiate. A population of OPCs persists in the rodent cortex throughout adulthood (Reynolds et al, 1997; Dawson et al, 2003).

When originally discovered, OPCs were considered bipotent glial precursors and were named O-2A progenitor cells because *in vitro*, they were capable of generating both oligodendrocytes and type-2 astrocytes (Raff et al, 1983). When cultured with PDGF producing type-1 astrocytes (Richardson et al, 1988) or simply PDGF (Noble et al, 1988), OPCs divide and exhibit features of progenitor cells over a period of several weeks (Noble and Murray, 1984; Raff et al, 1985; Dubois-Daicq, 1987), perhaps even indefinitely (Barres et al, 1994). If type-1 astrocytes are removed (Abney et al, 1981; Raff et al, 1985; Dubois-Dalcq et al, 1986) or PDGF is withdrawn (Noble and Murray, 1984; Temple and Raff, 1985; Behar et al, 1988), OPCs rapidly differentiate into

oligodendrocytes. OPCs also differentiate into oligodendrocytes if thyroid hormone has been added to the culture medium (Barres et al, 1994). To the contrary, addition of fetal bovine serum (FBS) triggers OPCs to obtain features of type-2 astrocytes (Raff et al, 1983; Raff et al, 1989). Some reports suggested that, depending on culture conditions, OPCs could differentiate into neurons as well (Kondo and Raff, 2000).

OPC fate choice *in vivo* has been controversial until the generation of transgenic mice that express cre recombinase under the control of OPC-specific genes. With this approach, it has been confirmed that that these cells are precursor cells that most commonly generate oligodendrocytes. However, their multipotency is limited *in vivo*. It was indeed true that OPCs can generate astrocytes in healthy rodent tissue as well, but this fate choice occurs only during prenatal development and only in specific regions of the forebrain (Zhu et al, 2008; Zhu et al, 2011). Although few studies showed that OPCs generate a small number of neurons *in vivo* (Rivers et al, 2008; Guo et al, 2009; Guo et al, 2010), the question of their neuronal fate is still debatable.

Markers that identify stages of OPC development

Starting from neural stem cell up to fully differentiated myelinating oligodendrocyte, oligodendrocyte lineage cell goes through several stages. Each stage can be identified by the expression of specific antigenic markers. Although these markers are commonly used to identify different stages of oligodendrocyte development by immunocyto- and histochemistry, it needs to be kept in mind that they are not solely markers, but also have important roles in the development of oligodendrocyte lineage cells. Transition from one stage to the next does not usually involve the loss of only one marker and acquisition of another one. So it is a unique pattern, rather than a single component, that

divides the path of oligodendrocyte differentiation into distinct phenotypic stages (Figure 1).

Oligodendrocyte transcription factor 1 (Olig1) and 2 (Olig2) and several members of the Sry-related HMG box (Sox) family are the earliest markers of oligodendrocyte lineage (Lu et al, 2000; Zhuo et al, 2000; Takebayashi et al, 2000; Stolt et al, 2002; Britsch et al, 2001; Kuhlbrod et al, 1998). Olig2 is also expressed by motor neuron precursors and is critical for generation of both OPCs and motor neurons (Lu et al., 2002; Takebayashi et al., 2002). Contrary to that, Olig1 is not essential for OPC production. Although its transcription starts less than a day after that of Olig2 (Zhou et al, 2000), it has been shown to play roles in oligodendrocyte development postnatally and is important for oligodendrocyte maturation and myelination in the CNS (Lu et al, 2002; Xin et al, 2005). Olig2 is a likely candidate that induces expression of Sox10 (Kuhlbrodt et al. 1998; Zhou et al, 2000; Stolt et al, 2002), which is likely the first transcription factor that marks cells committed to oligodendrocyte lineage (Kuhlbrod et al, 1998) and is critical for their differentiation into myelinating oligodendrocytes (Stolt et al, 2002). The expression of Olig1, Olig2 and Sox10 will persist throughout all stages of oligodendrocyte differentiation.

The most widely used markers for OPCs are PDGFRA and chondroitin sulphate proteoglycan 4 (CSPG4, NG2). Pdgfra transcript first appears at least one day upon initial specification of OPCs and is most likely induced by Sox family of transcription factors (Lu et al, 2000; Zhou et al, 2000; Tekki-Kessaris et al, 2001; Finzsch et al, 2008). NG2 expression follows that of PDGFRA, on cells that have exited the VZ (Nishyama et al, 1996; Zhu and Nishiyama; 2013). NG2 and PDGFRA will be coexpressed by OPCs as they migrate away from the VZ, but will be coordinately downregulated as the

progenitors differentiate (Nishiyama et al, 1996; Keirstead et al, 1998; Dawson et al, 2003).

As OPCs differentiate, they start expressing lipid antigens recognized by monoclonal antibodies O4 and O1. A population of O4 positive cells coexpresses NG2 and PDGFRA (Reynolds and Hardy, 1997). As OPCs transition to the stage of early (immature, premyelinating) oligodendrocyte, they lose the expression of NG2 and PDGFRA and the ability to proliferate and acquire the expression of galactocerebroside (GC, GalC) recognized by O1 antibody (Gard et al, 1990).

Myelin basic protein (MBP), myelin associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG) and myelin proteolipid protein (PLP) are classical markers expressed by mature oligodendrocytes and myelinated axons. MBP, MAG and PLP appear sequentially both *in vitro* (Dubois-Dalcq et al, 1986) and *in vivo* (Monge et al, 1986).

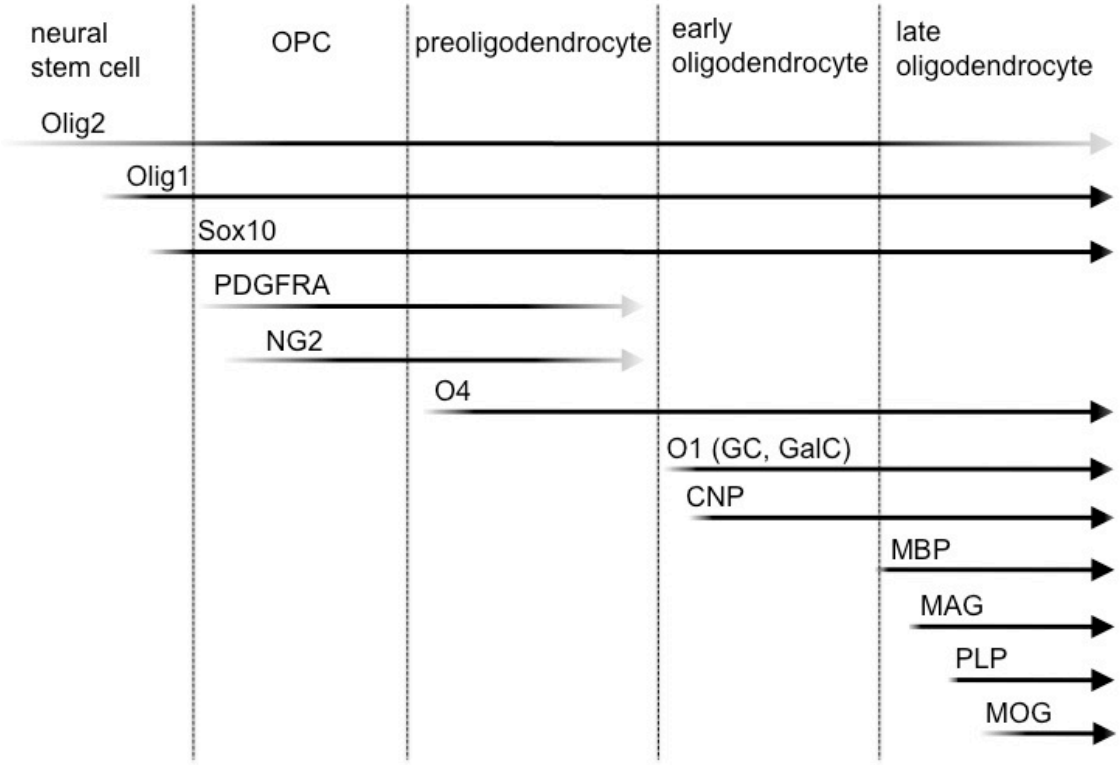

neural stem cell
OPC
preoligodendrocyte
early oligodendrocyte
late oligodendrocyte
Olig2
Olig1
Sox10
PDGFRA
NG2
O4
O1 (GC, GalC)
CNP
MBP
MAG
PLP
MOG

Figure 1. Markers that identify different stages of oligodendrocyte differentiation. Although not expressed exclusively by OPCs, but also by motoneuron precursors, Olig2 is the first marker of oligodendrocyte lineage cells. It is followed by Olig1 and Sox10, which expression will persist throughout an entire lineage. PDGFRA and NG2 are the most commonly used markers for OPCs that are downregulated as OPCs mature. As OPCs mature, they acquire the expression of O4 antigens, which is followed by O1 antigens and CNP. Late (mature) oligodendrocytes are identified by the expression of MBP, MAG, PLP and MOG, which appear in sequential manner.

Role of OPCs in remyelination

In response to a variety of insults to the CNS, OPCs become activated and undergo morphological changes, such as hypertrophy of the cell body and processes as well as increase in proliferation (Levine et al, 1994, Keirstead et al, 1998; Nishiyama et al, 1997; Di Bello et al., 1999; Levine and Reynolds, 1999). If the insult is directed towards oligodendrocytes resulting in their damage and death, OPCs will repopulate the oligodendrocyte-deprived areas and differentiate into oligodendrocytes in order to compensate for their loss (Tripathi et al, 2010; Zawadzka et al, 2010).

MS is an autoimmune disease of the CNS, characterized by inflammation, demyelination and astogliosis (Charcot, 1868; Frohman et al, 2006). Although axonal degeneration occurs in MS (Ferguson et al, 1997), oligodendrocytes and myelin are the principal targets of the inflammatory process. In the early stages of the disease, acute demyelination is associated with remyelination (Gledhill, 1973). However, long-standing, chronic lesions are not remyelinated efficiently (Keirstead and Blakemore, 1999). Although the source of remyelinating oligodendrocytes in MS remains unclear, studies have revealed the presence of OPCs in and around demyelinating lesions of MS (Wilson et al, 2006). Adult OPCs are shown to be the cells that generate oligodendrocytes following experimental demyelination in rodents in both acute (Zawadzka et al, 2010) and chronic (Tripathi et al, 2010) models of demyelination. In fact, acute demyelinating lesions are efficiently repaired (Zawadzka et al, 2010). Studies have implied that remyelination is not limited by an absence of OPCs in demyelinating lesions, but may be the result of OPC inability to differentiate into myelinating oligodendrocytes (Kuhlman et al, 2008). Current MS treatments focus on the immunomodulation of the inflammatory component of the disease and little progress has been made toward therapies that

promote the regenerative process of remyelination. However, this would be a highly effective complement to the immunomodulatory drugs.

Regulation of OPC proliferation and differentiation by PDGF

Oligodendrocyte differentiation in culture is regulated by various growth factors, such as platelet derived growth factor (PDGF), fibroblast growth factor (FGF), ciliary neutrophic factor (CNTF) CNTF and triiodothyronine (T3) (Barres et al, 1994; Noble et al, 1988; Raff et al, 1985; Raff et al, 1988). However, a large body of evidence shows that PDGF is the key regulator of OPC proliferation and differentiation both *in vitro* and during development (van Heyningen et al, 2001). Numerous studies have confirmed that PDGF is a major mitogen for OPCs (Noble and Murray, 1984; Noble et al, 1988; Richardson et al, 1988; Raff et al, 1988; Pringle et al, 1989, McKinnon et al, 1990; Raff et al, 1988; Pringle et al, 1989; Richardson et al, 1988; Noble et al, 1988). PDGF promotes survival (Barres et al, 1992), division and motility (Noble, 1988) of OPCs. At the same time, PDGF signaling has been shown to inhibit OPC differentiation *in vitro* and its withdrawal from the culture medium is sufficient to trigger OPC differentiation (Raff et al, 1985; Raff et al, 1988; van Heyningen et al, 2001). Early studies on the effect of PDGF on OPCs proposed that PDGF drives an “internal clock” that counts cell divisions and regulates the timing of OL development in culture (Raff et al, 1985; Raff et al, 1988). However some of the later studies suggested that in the presence of PDGF, OPCs can divide indefinitely (Barres et al, 1994). Although it is evident that PDGF has an important role in regulation of OPC differentiation, its role is more complex than a simple on/off switch and is still incompletely understood.

In vivo, PDGF is most likely produced by astrocytes (Raff et al, 1985; Richardson et al, 1988) and neurons (Yeh et al, 1991). Its presence in the rodent CNS throughout gliogenesis (Richardson et al, 1988; Pringle et al, 1989) suggests its role in the control of OPC differentiation and myelination *in vivo*. Its importance is further supported by the evidence that aberrant PDGF expression *in vivo* can altar OPC survival, proliferation and timing of their differentiation (Calver et al, 1998; Fruttiger et al, 1999).

A family of PDGFs includes 4 isoforms, named PDGFA, B, C and D. All isoforms are secreted as disulfide-linked bivalent ligands that can form both homomers and heteromers. PDGFs exert their effect through their receptors, PDGFRA and B. Ligand binding induces receptor dimerization, trans-phosphorylation and the activation of signal transduction cascades (reviewed by van der Geer et al, 1994).

Several other growth factors have also been shown to influence oligodendoroglial development *in vitro*, including insulin like growth factor 1 (McMorris et al, 1986; McMorris and Dubois-Dalcq, 1988), epithelial growth factor (EGF) (Sheng et al, 1989) and basic FGF (Eccleston and Silberg, 1985; Saneto and de Vellis, 1985, Besnard et al, 1989).

PDGFRA and its expression during development

The only PDGFR isoform expressed by OPCs is PDGFRA. Since in the CNS, PDGFRA is expressed predominately by OPCs (Pringle et al, 1992), it has become one of the most commonly used markers for OPCs (Pringle et al, 1989; McKinnon et al, 1990; Nishiyama et al, 1996; Dawson et al, 2003). PDGFRA is expressed by OPCs both *in*

vivo (Hart et al, 1989) and *in vitro* (Hart et al, 1989; McKinnon et al, 1990) and is rapidly downregulated in OPCs that start to differentiate (Hart, 1989; Hall, 1996).

PDGFRA is expressed early during development and is one of the earliest markers that label cells committed to oligodendrocyte lineage. In the mouse CNS, the first expression of PDGFRA is detected around E11, in a restricted region of ventral neuroepithelium (Spassky et al, 1998). In the rat CNS, the first PDGFRA transcripts appear around E13 and it are also restricted to a distinct region of vetral neuroepithelium (Pringle and Richardson, 1993; Pringle et al, 1996). The first PDGFRA-expressing cells appear as distinct focus, which then spreads out indicating OPC migration away from the VZ. PDGFRA expression persists in migrating OPCs, but also those that have settled to brain parenchyma. OPCs in the adult brain continue to express PDGFRA.

Similar to PDGF, PDGFRA is critical for OPC development and differentiation. PDGFRA disruption in OPCs results in their premature differentiation in culture, whereas OPCs from PDGFRA hemizygous mice show impaired proliferation and accelerated maturation (McKinnon et al, 2005). Together with the studies that indicate that PDGFRA ligand inhibits OPC differentiation (Raff et al, 1985; Noble et al, 1988; Raff et al, 1988; van Heyningen et al, 2001), McKinnon study suggests the default pathway that OPCs undertake is differentiation. This pathway is normally inhibited by PDGFRA signaling in progenitor cells and signaling through PDGFRA needs to be repressed in order for OPCs to differentiate. Since PDGFRA is present in OPCs, but gets downregulated as OPCs differentiate, it is likely that PDGFRA and its downregulation are critical factors that control oligodendrocyte differentiation and that PDGFRA has to be downregulated in order for OPCs to differentiate. However, molecular mechanisms that lead to

transcriptional repression of PDGFRA and therefore trigger differentiation remain to be defined.

PDGFRA regulation

The molecular mechanisms governing the transcription of PDGFRA are poorly understood. PDGFRA gene lacks a typical TATA box. A number of transcription factors were shown to regulate PDGFRA transcription in cells that do not belong to oligodendrocyte lineage, such as ubiquitously expressed NFkB (Kitami et al, 1995; Lindros et al, 1998), C/EBP (Fukuoka et al, 1999; Kitami et al, 1999), Sp1 and Sp3 (Khachigan et al, 1994; 1995; 1996; Bergeron et al, 2011), but only few transcription factors were shown to directly regulate PDGFRA transcription in OPCs.

FGF has been shown to positively regulate PDGFRA transcription, maintain high levels of PDGFRA expression and inhibit OPC differentiation (McKinnon et al, 1990) but the signaling pathway involved in this regulation is still unclear. Its role in oligodendrocyte development is likely complex and involves other signaling mechanisms and transcription factors since it has been shown that FGF can regulate PDGFRA transcription both positively (McKinnon et al, 1990) and negatively (Bonello et al, 2004).

SoxE family of transcription factors is critical for oligodendrocyte development (Stolt et al, 2002; 2003). PDGFRA expression starts with a delay relative to that of Sox9 and Sox10 and it is likely that these two transcription factors have a role in the regulation of PDGFRA transcription during OPC development. Sox9 has been shown to directly bind to PDGFRA gene and positively regulate its expression (Finzsch et al, 2008).

Transcription factor Nkx2.2 is expressed in OPCs in the developing mouse spinal cords and plays an essential role in the terminal differentiation of oligodendrocytes (Qi et al., 2001; Zhou et al., 2001). In addition, Nkx2.2 is upregulated in OPCs immediately before their differentiation, but rapidly downregulated upon their differentiation (Fu et al., 2002; Soula et al., 2001; Xu et al., 2000; Zhou et al., 2001). Its role in oligodendrocyte development might involve regulation of PDGFRA expression since Nkx2.2 has been shown to directly bind to the 5' region of PDGFRA and repress its transcription (Zhu et al, 2014).

Posttranscriptional regulation of genes by small noncoding RNAs has become an evident factor playing part in many physiological and pathological processes (reviewed by Stefani and Slack, 2008). Micro RNAs (miRNAs) are a class of small noncoding RNAs that are negative regulators of gene expression at the posttranscriptional level. They act either through inhibition of translation or degradation of their target RNAs and are able to regulate multiple targets simultaneously (reviewed by Flynt and Lai, 2008). Micro RNAs have been shown to play critical role in the development of neurons (Visvanathan et al, 2007; Cheng et al, 2009) and oligodendrocytes (Dugas et al, 2010; Zhao et al, 2010). One of these miRNAs, miR-219, is induced during OPC differentiation and targets negative regulators of differentiation. Its overexpression promoted, while its knockdown inhibited OPC differentiation (Dugas et al, 2010; Zhao et al, 2010). In addition, miR-219 has been shown to directly bind to Pdgfra mRNA and regulate its expression (Dugas et al, 2010).

Drug discovery

Discovery and design of new therapeutic chemicals and their development into medicines consists of several steps and each has its challenges. The initial difficulty in this process arises in the discovery of the lead compound. The lead is a prototype that has a number of attractive characteristics (desired biological activity) but may have other undesirable characteristics (toxicity, insolubility, metabolism).

Two types of approaches can be taken to identify the lead and they are based on random and nonrandom discovery. The first requirement for both approaches is to have ways of assaying compounds for desired biological activity. An assay (screen) is done in a biological system and can be done both *in vitro* and *in vivo*. *In vitro* assays are quicker and less expensive. High throughput screens (HTS) can be carried out robotically in 96, 384 or 1536 well plates with as little as submicrogram amounts of compound dissolved in submicroliter volume. With this approach, it is possible to screen 100,000 compounds in a day. HTS appears to have resulted in an increase in the number of hits, but has not been resolved yet whether this increase in the hit rate translates to a greater number of leads.

Nonrandom screen is performed when the lead has already been discovered. This is more focused screen aimed towards compounds that have resemblance to the lead compound discovered in random screen. Compounds used for nonrandom screens might have only vague structural resemblance to the lead. Even if the lead was shown to be only mildly active, screen can uncover lead derivatives that are more potent.

In the absence of known leads with desired activity, a random screen is a valuable approach. This type of screen involved a large number of compounds of various chemical structures. All compounds are screened in the bioassay without regard to their structures. Prior to 20th century, this was essentially the only approach because not many leads were known. Today, this is still an important approach particularly because it is now possible to screen huge numbers of compounds rapidly with HTS. This is also the lead discovery method of choice when nothing is known about the receptor target.

Neither random nor nonrandom screens involve rational approaches. However, rational approaches that involve designing a compound that has a particular biological activity have become an important route in lead discovery. Many diseases are caused by imbalance of particular chemicals in the body. Proteins, such as growth factors, receptors, enzymes, transcription factors or other members of signaling pathways, are common cause for the disruption of the homeostasis of a biological system. This imbalance can be corrected by agonism/antagonism of a receptor or activation/inhibition of an enzyme. However, for this kind of approach, it is critical to know which protein is responsible for this imbalance. This is sometimes difficult to achieve due to complex physiological responses that might be caused by disruption of balance.

Drug modifications

Upon discovery of the lead compound, its structure is modified by chemical synthesis to amplify the desired activity and to minimize or eliminate the unwanted properties. Even if the lead had only weak biological activity, its derivatives might be more potent. Structure modifications are the keys to activity and potency manipulations. Activity is the particular biological effect, while potency is the strength of that effect.

Interactions of drugs with their targets are very specific. Therefore, only a small part of the lead, called pharmacophore, may be involved in the interaction. Other residues, called auxophores, are also important because they may maintain the integrity of the drug, hold the pharmacophore in the correct position or stabilize the interaction between pharmacophore and its target. Some residues might have a negative effect and might be interfering or destabilizing the interaction. It is important to find out what residues are critical for drug action and what need to be removed and this can be determined by chemically excising different portions of the lead.

In addition to removal of residues that have negative effect on compounds potency, addition of certain residues and structural modifications of the lead can improve lead's physicochemical properties. Such properties include: size, shape, electronic distribution, lipid solubility, water solubility, chemical reactivity or hydrogen bonding. As a consequence, lead manipulations may cause changes in of the following effects. Structural – holding of other functionalities in a particular geometry, shape or hydrogen bonding. Target interactions – interaction with the target. Pharmacokinetics – absorption, transport or excretion of the compound. Metabolism – blocking or aiding metabolism. Since these changes and their effects can reflect on compounds potency in both positive and negative manner, multiple modifications may often be necessary to balance the effect.

Solubility of compound is one of the critical features that can greatly affect compound potency. For each drug to be active, it needs to interact with two environments – aqueous (extracellular space and cytoplasm) and lipophilic (membranes, such as cell membrane of blood brain barrier). Low lipophilicity is a great obstacle because it causes

poor permeability through membranes. Increased lipophilicity improves compound's permeability and therefore, its accessibility to the target. However, highly lipophilic compounds are not efficiently transported through the hydrophilic extracellular media and are easily metabolized, which limits their availability. Therefore, there needs to be a fine balance between drug's lipophylicity and hydrophilicity, this depends on compound structure and can be achieved by modifying the lead.

Adding or excising CH2 groups can greatly affect drug properties. For many compounds, lengthening of a saturated carbon side chain up to nine results in an increase in compound potency, while further lengthening causes sudden decrease in potency. This is associated with increased lipophilicity of the molecule until its lowered water solubility becomes its dominant feature. Chain branching decreases lipophilicity and lowers potency, but may also interfere with interaction of the pharmacophore and its target. Ionization and low pH lead to increased water solubility since under these conditions, compounds' residues are protonated and can participate in forming hydrogen bonds more easily.

Bioisosteres are substituents that have similar physicochemical and biological properties. This is an important lead modification that can fine-tune compound's metabolism, toxicity and potency. One of the common bioisosteric modifications is the replacement of hydrogen atom with fluorine at a site of metabolic oxidation in a lead may prevent such metabolism from taking place. Because the fluorine atom is similar in size to the hydrogen atom the overall topology of the molecule is not significantly altered, leaving the desired biological activity unaffected. However, with a blocked pathway for metabolism, the drug candidate may have a longer half-life.

Structure-activity relationships (SARs)

Structure-activity relationship (SAR) is relationship of the molecular structure of the compound and its biological activity. The analysis of SARs provides another way to reveal pharmacophore and auxophores.

The hallmark of SAR studies is the synthesis of as many analogs as possible of the lead and their testing to determine the effect of structure on activity or potency. Once enough analogs are made and tested, conclusions can be made regarding SAR. Ease of synthesis rather than logic rationale is often the guiding force behind the choice of analogs made.

Upon identifying the critical chemical groups, structure of the lead compound can be additionally manipulated in a rationale manner in order to further increase its potency. Biological properties of a compound are often a function of its physicochemical parameters, such as solubility, lipophylicicty, ionization, stereochemistry, etc. Therefore, it is possible to correlate compound structure to its biological effect. This can be used to direct the synthesis of lead derivatives.

PDGF signaling in malignancies

Tumors arising from glial cells (gliomas) are the most common forms of primary tumors of the CNS. They are among the most deadly types of cancers, but the malignancy grade is an essential factor in predicting patient's outcome. Grade I tumors are treatable. However, by the time it is diagnosed, tumor might have progressed to higher grade

when even with the most invasive procedures that combine surgery, radiation and chemotherapy, patient's prognosis is weak.

Hyperactivation of RTK signaling pathways is a frequent hallmark of malignant gliomas, EGF/EGFR being the most common. Aberrant PDGF signaling has also been associated with the development of different types of malignancies, including those occurring in the CNS (Hermanson et al, 1992; Di Rocco et al, 1998; Martinho et al, 2009; Ozawa et al, 2010; Heldin, 2013). Based on the expression profile, glioblastomas have been classified into several categories and mutations of EGFR and PDGFRA define the classical and proneural subtypes, respectively (Vehaak et al, 2010). Although PDGFRA mutations are not as common as those of EGFR, this still makes PDGFR the second most frequently mutated RTK gene in glioblastoma (The Cancer Genome Atlas Research Network, 2008). Amplification is the most commonly observed PDGFRA gene alteration, while point mutations, deletions and gene rearrangements are rare and occur more frequently in glioma samples that already have PDGFRA amplification (Martinho et al, 2009; Verhaak et al, 2010; Ozawa et al, 2010). In addition, the expression pattern of PDGF and PDGFRs suggest the presence of autocrine and paracrine stimulatory loops, which may contribute to tumor progression (Hermanson et al, 1992).

Growing knowledge on the contribution of PDGF signaling in cancer development and progression has led to different kinds PDGF signaling inhibitors that target either PDGFs of PDGFRs. Antibodies and soluble extracellular parts of the receptors can intervene with PDGF signaling by binding to PDGFs or PDGFRs, prevent their interaction or promote their degradation (Hawthorne et al, 2008; Shen et al, 2009). However, they are expensive and difficult to administer. Small molecule inhibitors of PDGFR are attractive

candidates for cancer therapy and are clinically explored (Morris and Abrey, 2010; Paulsson et al, 2011). One of the first and most promising drugs, imatinib, has been used in the treatment of several cancers (Iqbal and Iqbal, 2014). However, it was shown to be unsuccessful in the treatment of CNS malignancies (Reymond et al, 2008), which might be due to its limited penetration through the blood brain barrier (Takayama et al, 2002; Senior, 2003). In addition, imatinib, as well as other small molecule inhibitors, was shown to have a broader spectrum of targets, which may contribute to drug's side effects (reviewed by Heldin, 2013).

Small molecule inhibitors, which are currently used for treatment of cancers driven by aberrant RTK signaling or evaluated as potential therapies, are directed toward inhibiting kinase activity of respective RTKs and were shown to be non-specific. Targeting specific RTKs by regulating their expression instead of kinase activity might complement current therapies. In addition, design of novel small molecules that are able to cross blood brain barrier might provide significant contribution to the treatment of CNS malignancies. Since PDGFRA is one of the two most frequently mutated genes in gliomas and since PDGFRA signaling pathway plays an important role in tumor development, PDGFRA represents a good candidate for targeted therapy. Downregulating its expression at the mRNA level might lead to reduction in proliferation of PDGFRA-expressing cells, which may limit tumor progression and improve patient's survival.

Chapter 2 – Identification of small molecule compounds that downregulate the transcription of PDGFRA in mouse oligodendrocyte progenitor cell line

Introduction

PDGFRA signaling is the key regulator of OPC proliferation and differentiation. PDGFRA transcription is first detected in a distinct area of VZ early during development (at E11-E13 in the rodent CNS) and is one of the earliest markers that label cells of oligodendrocyte lineage. PDGFRA expression persists in OPCs that have migrated away from the subventricular zone and have settled in brain parenchyma. However, as OPCs differentiate, PDGFRA gets rapidly downregulated (Hart, 1989; Hall, 1996).

Change in transcription is generally the earliest event that occurs during differentiation. Regardless whether downregulation of Pdgfra transcription is the cause or consequence of differentiation, we used this change as an indicator of decrease in proliferation and increase in differentiation caused by small molecule compounds.

To identify compounds that downregulate Pdgfra transcription we used random screen. Compounds were purchased as stocks distributed in 96-well plates. They were tested with an assumption that each plate contained compounds of different and random structure. Compounds were tested on Oli-neu cells, which represent mouse oligodendrocyte precursor cell line. We performed three screens in parallel. Luciferase assay was used to indirectly evaluate changes in Pdgfra transcription. In addition to quantitative luciferase assay screen, we performed qualitative immunocytochemistry and morphology based screens to examine whether compounds promote Oli-neu cell differentiation or inhibit their proliferation. We next verified Pdgfra downregulation by

assaying the level of endogenous Pdgfra transcript upon treatment with the most promising candidate compounds.

Our results revealed a group of small molecule compounds, which structure is derived from the lead N-methyl-N-benzylguanidine, that downregulate luciferase activity of Pdgfra plasmid in a dose-response manner and inhibit transcription of endogenous Pdgfra.

Methods

Cell culture

Oli-neu cells, which represent mouse oligodendrocyte precursor cell line (Jung et al, 1995), were used for the primary screen as well as verification of hit compounds. Oli-neu cells were grown on poly-L-lysine-coated culture dishes. Cells were maintained in growth medium consisting of Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12) (Invitrogen) supplemented with 1% horse serum (Hyclone SH30074.03), N2 supplement (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin. Addition of 1 mM dibutyryl cyclic adenosine 3,5-monophospahte (dbcAMP) (Jung et al, 1995) or dexamethasone (DEX) (Joubert et al, 2010) has been shown to induce differentiation of Oli-neu cells towards mature oligodendrocytes. To induce differentiation of Oli-neu cells, dbcAMP (Sigma) and DEX (Sigma) were added to the culture medium, to 1 mM and 10 µM final concentration, respectively.

Plasmids

Plasmids were obtained by polymerase chain reaction (PCR), using DNA purified from the RP23-412J9 BAC clone as the template and primers listed in table 1. For the plasmids PR-111, PR-336, PR-1000 and PR-1595, KpnI and SacI restriction sites (underlined in the primer sequence) were incorporated into the forward and reverse primers, respectively, to facilitate the cloning of the resulting PCR fragment into the pGL4.10 vector (Promega). In order to generate PR-2229 plasmid, XhoI restriction site was incorporated into the reverse primer, while the internal SacI site, located 2229 nucleotides upstream from the TSS, was used for digestion of the PCR fragment at the 5' end. Plasmids were purified with Plasmid Midi Kit (QIAGEN) and the insert position and its sequence was confirmed by sequencing using vector backbone primers (vector backbone forward primer: GCTGTCCCCAGTGCAAGTGCAGG and vector backbone reverse primer: AGTGGGTAGAATGGCGCTGGGCC).

Transfections and luciferase assays

Transfections were carried out using Lipofectamine (Invitrogen) according to manufacturer's instructions. The day before transfection, cells were seeded in 24-well tissue culture plates. They were transiently co-transfected with 0.75 μg of the test plasmid and 0.05 μg of pGL4.73 vector (Promega) to control for differences in transfection efficiencies. As negative control, we used pGL4.10 vector that had no Pdgfra insert, while pGL3 promoter (Promega) was used as positive control. Cells were split the next day to 96-well plates in order to make triplicates. After allowing cells to adhere for 4-6h, they were induced to differentiate. Two days after induction of

differentiation, the cells were lysed and the luciferase activities were assessed using a DualGlo Luciferase Assay kit (Promega).

Plasmid name		Primer sequence	Restriction site
PDGFRA-111	Forward primer	CCGGTACCCAGAGAGCAAGGAGTCCTAGGG	Kpn I
	Reverse primer	CCGAGCTCCTCTCCCTCAAGCTCCAACAG	Sac I
PDGFRA-336	Forward primer	CCGGTACCCACCCCCAAATTGGGAAGTC	Kpn I
	Reverse primer	CCGAGCTCCTCTCCCTCAAGCTCCAACAG	Sac I
PDGFRA-1000	Forward primer	CCGGTACCTTGGTTCCTGGAGTGTCAGC	Kpn I
	Reverse primer	CCGAGCTCCTCTCCCTCAAGCTCCAACAG	Sac I
PDGFRA-1595	Forward primer	CCGGTACCGTGCAAGCCTGTTCGCAGAC	Kpn I
	Reverse primer	CCGAGCTCCTCTCCCTCAAGCTCCAACAG	Sac I
PDGFRA-2229	Forward primer	GACACCCTGGGTTGAGTGAC	Sac I (internal)
	Reverse primer	GGCTCGAGCTCTCCCTCAAGCTCCAACAG	Xho I

Table 1. Primers used to make plasmids containing 5' Pdgfra sequences of different lengths. Plasmid name indicates the position of the starting nucleotide and is relative to the transcription start site of the Pdgfra gene. All primers, except the forward primer used to make PR-2229, incorporate restriction enzyme digestion sites used to facilitate cloning. The position of these sites is underlined.

Small molecule compounds

A library of small molecule compounds for the primary screen was purchased from Chembridge as 96-well mother plates (plate identification: NT 1147 30941 through NT 1147 30957) with compounds dissolved in dimethyl sulfoxide (DMSO) as 5 mM stocks. Compounds were located in columns 2 through 11 of the 96-well plate, while columns 1 and 12 contained DMSO and were used as control. Upon identification of potential leads, 5 mg of each compound was purchased from Hit2Lead and dissolved in DMSO in order to make 50 mM stocks.

Luciferase assay screen

Transfections of Oli-neu cells were carried out using Lipofectamine (Invitrogen) according to manufacturer's instructions. The day before transfection, cells were seeded in 6-well tissue culture plates (1.5 million cells/well). They were transiently co-transfected with 3.5 µg of PDGFRA-luc plasmid and 0.5 µg of pGL4.73 (Promega) to control for differences in the transfection efficiency. Day after transfection, Oli-neu cells were split to flat bottom 96-well plates (10,000 cells/well). One well of the 6-well plate would usually yield sufficient number of cells for one 96-well plate. Cells were allowed to adhere for 4-6h when compounds were added at 50 µM concentration. Two days later, DualGlo Luciferase Assay (Promega) was performed.

Morphology and immunocytochemistry screen

Oli-neu cells were seeded in flat bottom, clear 96-well plates (10000 cells/well). They were let to adhere for 4-6h when compounds were added to the final concentration of 25 μM. Medium was not replaced during the incubation time and no fresh compound was added. After 4 days, cells were examined under the light microscope and any changes in cell morphology and/or changes in cell density were noted. Oli-neu cells were then fixed in wells using 95% ethanol 5% glacial acetic acid and immunostained using O1 antibody. Cells were observed with Zeiss Axiovert microscope.

RNA isolation, reverse transcription and quantitative PCR

Oli-neu cells were plated in 6-well plates and treated with selected small molecule compounds at 50 μM concentration for 2 days. Total RNA was collected from 500,000 cells using RNeasy Mini Kit (QIAGEN). An aliquot of the total RNA (1 μg) was then used as a template to synthesize cDNA using Superscript III (Invitrogen and according to manufacturers recommendations. Quantitative PCR (qPCR) was performed using SYBR Green PCR Master Mix (Life Technologies). For each reaction, we used 10 ng of cDNA and gene-specific primers listed in table 2. The PCRs were done using the following conditions: 2 min at 95°C followed by 39 cycles of denaturation (10s at 95°C), annealing and extension (30s at 60°C). The specificity of PCR products was confirmed by the analysis of the melting curve. Quantification of gene expression was first normalized to GAPDH and then expressed as a logarithm of the ratio of treated to control mRNA level. Each amplification reaction was performed three independent times.

Gene	Forward primer	Reverse primer
Gapdh	TGACAACTTTGGCATTGTGG	ATGCAGGGATGATGTTCTGG
Pdgfra	TCGAAGGCAGGCACATTTAC	TTGAGTCTCCGGATCTGTGG
Fgfr1	CTAACCGCAGAACTGGGATG	TGGACCAGGAGAGACTCCAC
Fgfr3	TGCACAAGGTCTCTCGCTTC	TCAGCAGGCAGCTCAAGTTC

Table 2. Primers used for quantification of mRNA in Oli-neu cells in control conditions and upon treatment with 50 μM selected compounds. All primers span adjacent exons and their specificity was confirmed by melting curve.

Results

Identification of the PDGFRA-luc plasmid for the screen

In order to identify the most suitable plasmid that we would use in the luciferase screening assay, we cloned various lengths of the mouse Pdgfra 5' flanking sequence into the pGL4.10 vector, which contains cDNA encoding firefly luciferase (FF) (Figure 2. A). We next transfected these plasmids into Oli-neu cells and induced the cells to differentiate towards oligodendrocytes using 1 mM dbcAMP and 10 μM DEX. Plasmid that contains cDNA for renilla luciferase (R) driven by SV40 promoter and enhancer (pGL4.73) was used as internal control. As negative control, we used pGL4.10 vector that had no Pdgfra insert, while pGL3 promoter (Promega), that contains firefly luciferase cDNA driven by SV40 promoter, was used as positive control. DualGlo Luciferase Assay was performed two days after induction of differentiation. Plasmid PR-1595, that contains 1595 nucleotides of the Pdgfra 5' flanking sequence, showed the highest luciferase activity and the most robust response to differentiation conditions decreasing its activity by ~35%. This plasmid was used for the screen and will be refer to as PDGFRA-luc (Figure 2. B).

To identify compounds that inhibit OPC proliferation and stimulate their differentiation, we performed 3 primary screens in parallel: luciferase assay, immunocytochemistry (ICC) and morphology screens. These screens were all done in Oli-neu cells.

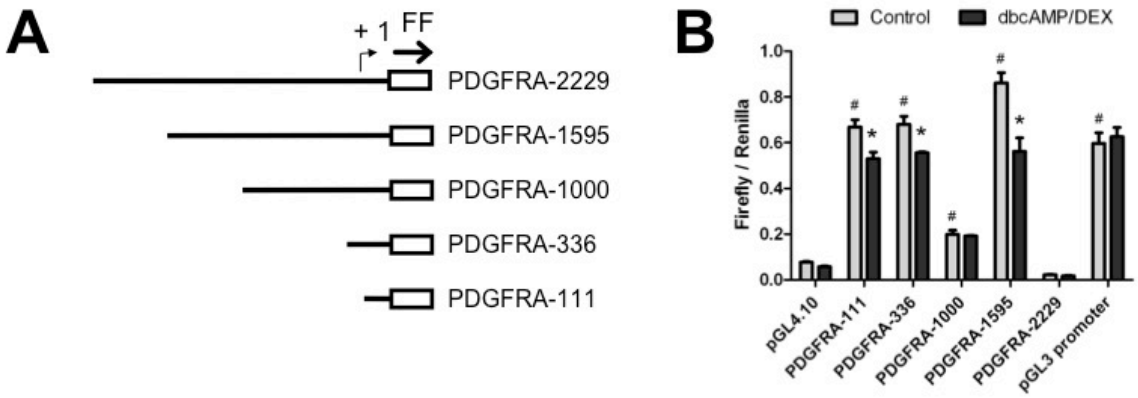

A
+ 1
FF
PDGFRA-2229
PDGFRA-1595
PDGFRA-1000
PDGFRA-336
PDGFRA-111
B
Control
dbcAMP/DEX
Firefly / Renilla
1.0
0.8
0.6
0.4
0.2
0.0
#
*
pGL4.10
PDGFRA-111
PDGFRA-336
PDGFRA-1000
PDGFRA-1595
PDGFRA-2229
pGL3 promoter

Figure 2. Identification of the PDGFRA-luc plasmid for the screen. Various lengths of the 5' sequence of mouse Pdgfra gene (black lines) were cloned into the pGL4.10 vector 5' to firefly cDNA (white box). The number in the plasmid name indicates the position of the most 5' nucleotide of the included insert relative to Pdgfra transcription start site (TSS), which is indicated as +1 (A). Upon transfection of Oli-neu cells and induction of their differentiation, PR-1595, which contains 1595 nucleotides of the Pdgfra 5' sequence, showed the highest luciferase activity under control conditions and the most robust response to differentiation conditions, decreasing its activity by 35% (B). This plasmid will be referred to as PDGFRA-luc. # $p < 0.001$ relative to pGL4.10 control, * $p < 0.001$ relative to control, two-way ANOVA, Bonferroni posttest. Error bars are standard deviations of the mean.

Luciferase assay screen

Since Pdgfra downregulation is associated with a decrease in OPC proliferation and their differentiation, we used changes in Pdgfra transcription to select for compounds that downregulate Pdgfra transcription indirectly, by luciferase assay.

To identify compounds that downregulate PDGFRA-luc activity, we transfected Oli-neu cells with PDGFRA-luc and pGL4.73, split them to 96-well plates and treated them with small molecule compounds for 2 days at 50 μM concentration before performing luciferase assay.

We tested approximately 1500 compounds in several batches so that 80 (1 plate), 160 (2 plates) or 320 (4 plates) compounds were tested at the same time. To eliminate day-to-day variation between experiments, each batch of samples was analyzed at the same time, but independently from other batches. Firefly luciferase was normalized to renilla, and averages and standard deviations of each batch were determined. To compare different batches of samples, normalized luciferase values were converted to standard scores (Z scores) using values for averages and standard deviations for each batch of samples.

Our results show that out of 1500 compounds tested, 931 downregulated PDGFRA-luc activity below the mean. Treatment with 87 compounds resulted in Z score value below -1 while 14 compounds had Z scores below -2. None of the compounds had Z score below -3. Contrary to that, 589 compounds upregulated PDGFRA-luc activity above the mean. Out of these, 125 compounds caused standardized PDGFRA-luc activity to be greater than 1, 60 compounds had Z scores above 60, while 33 compounds had Z

scores above 3 (Figure 3. A). For further evaluation, we selected compounds that resulted in Z score values of -1 and below.

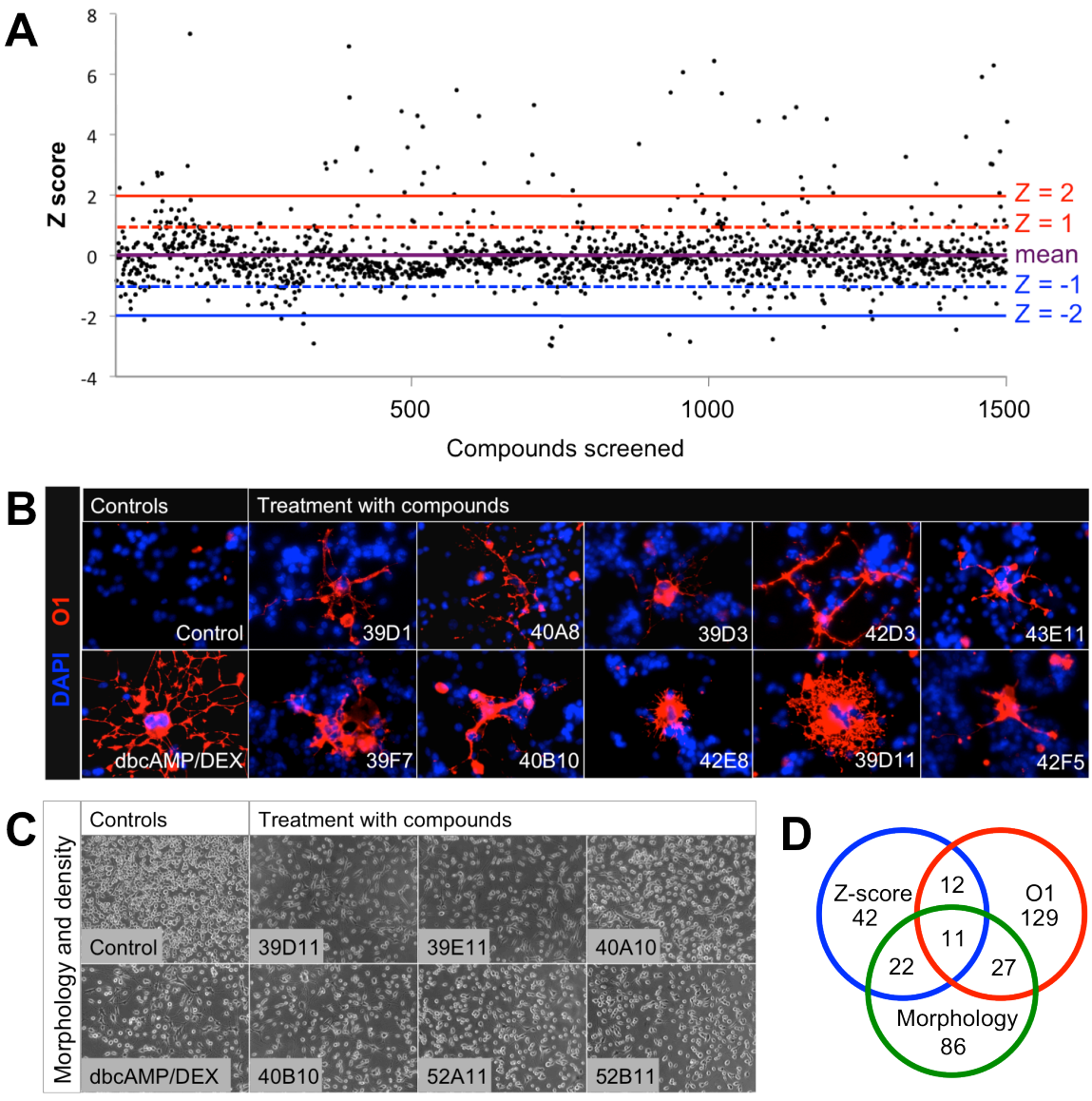

A
Z score
8
6
4
2
0
-2
-4
500
1000
1500
Compounds screened
Z = 2
Z = 1
mean
Z = -1
Z = -2
B
Controls
Treatment with compounds
O1
DAPI
Control
39D1
40A8
39D3
42D3
43E11
dbcAMP/DEX
39F7
40B10
42E8
39D11
42F5
C
Controls
Treatment with compounds
Morphology and density
Control
39D11
39E11
40A10
dbcAMP/DEX
40B10
52A11
52B11
D
Z-score
42
O1
129
Morphology
86
12
11
22
27

Figure 3. Screening for compounds that promote Oli-neu cell differentiation. For the luciferase assay based screen, Oli-neu cells were transfected with PDGFRA-luc and treated with small molecule compounds at 50 μM for 2 days. Each black dot represents standardized (Z score) PDGFRA-luc activity normalized to internal control. All compounds that downregulated PDGFRA-luc activity by more than Z = -1 (Z score values below the dashed blue line) were selected for further evaluation. Z score = (normalized PDGFRA-luc activity – mean)/SD (A). For morphology and ICC based screens, Oli-neu cells were seeded to 96-well plates and treated with small molecule compounds at 25 μM. After 4 days, they were examined for the changes in morphology and/or density (C). ICC for O1 revealed compounds that increased the expression of O1 and/or the percentage of O1+ cells (B). Venn diagram summarizes the results of the 3 screens: 87 compounds downregulated PDGFRA-luc activity (blue), 146 caused changes in Oli-neu cell morphology and/or density (green) and 179 caused an increase in the O1 expression and/or the percentage of O1+ cells (red) (D).

Morphology and immunocytochemistry screen

In addition to quantitative luciferase assay-based screen, we performed qualitative ICC- and morphology-based screens. Oli-neu cells were plated in 96-well plates and treated with 1500 small molecule compounds at 25 μM concentration. After 4 days, we examined cell morphology and noted any morphological changes that could indicate differentiation (elongation or increased branching of processes) or decrease in the proliferation rate (decrease in cell density). Each compound was scored (Figure 3. C). Oli-neu cells were then fixed in the same plates and immunostained for O1, a more mature marker of oligodendrocyte lineage. We noted and scored compounds that caused an increase in the O1 expression, assessed as brightness of the staining relative to control, and/or the percentage of O1 positive (O1+) cells (Figure 3. B). Out of 1500 compounds, 146 caused a change in Oli-neu cell morphology and/or decrease in their density, while 179 compounds caused an increase in the O1 expression and/or the percentage of O1+ cells. Results of the three screens were summarized (Figure 3. D) and all candidate compounds were scored and ranked.

It should be pointed out that since morphology and ICC-based screens were performed and analyzed independently from luciferase assay screen, some of the candidate compounds identified in these two screens turned out to be upregulators of PDGFRA-luc activity. We need to keep in mind that morphology and ICC-based screens were performed under different conditions. Most importantly, Oli-neu cells were treated with compounds over a longer period of time. Luciferase assay screen detects early changes in gene transcription and PDGFRA upregulation could have caused an increase in cell survival or initial increase in their proliferation. Primary OPCs were shown to have density-dependent control of proliferation (Zhang et al, 1996) and it is possible that a

similar mechanism exists in Oli-neu cells. Being an immortalized line, Oli-neu cells have a high rate of proliferation that persists over long period of time. However, with prolonged or irregular passaging that causes their overgrowth, there might be changes in the pattern of expression of several relevant markers, such as O1 (unpublished observations).

Verification of luciferase assay screen

From the rank list of all candidate compounds, we selected the top 20 to verify downregulation of PDGFRA-luc activity by performing dose-response assays. Similarly to the initial luciferase assay screen, Oli-neu cells were transfected with PDGFRA-luc and pGL4.73 and split to 96-well plates the day after transfection. We made serial dilutions of stock compounds and treated Oli-neu cells at concentrations ranging from 10 nM to 1 mM. Cells were treated for 2 days when luciferase assay was performed. All compounds were citotoxic at 1000 mM and many were citotoxic at 316 μM. Due to compound toxicity, assessed either by examining cell morphology or based on the low values for renilla luciferase activity measured in the luciferase assay, not all data points were included in the dose-response curves. We analyzed dose-response curves and determined half maximal inhibitory concentrations (IC50) using GraphPad Prism.

Out of 20 candidates tested, 7 were confirmed and downregulated PDGFRA-luc activity in a dose-response manner. Out of these 7 compounds, 3 had similar chemical structure (39D11, 40A10 and 40B10). They were derivatives of the lead N-methyl-N-benzylguanidine (guanidine compounds). The 4th guanidine compound (39E11) altered Oli-neu cell morphology and decreased their density, but it caused a modest dose dependent decrease in PDGFRA-luc activity. The remaining 4 compounds, that

downregulated PDGFRA-luc activity, were not structurally related to each other or to guanidine compounds (Figure 4). IC50 for these 8 compounds were shown in Table 3.

Compound	IC50 range (mM)
39D11	Very wide
40A10	0.01643 to 0.07563
40B10	0.02634 to 0.08656
39E11	Very wide
42E8	Very wide
54G2	0.001429 to 0.004847
44C8	$2.974*10^{-5}$ to 9162
45C11	$3.167*10^{-7}$ to 66571

Table 3. Half maximal inhibitory concentrations for compounds that were verified in the dose-response assay. Oli-neu cells were treated with indicated compounds at concentrations ranging from 10 nM to 1 mM. Luciferase assay was performed after 2 days of treatment. GraphPad Prism was used to create and analyze dose-response curves. However, due to compound toxicity assessed by examining cell morphology under light microscope and renilla luciferase activity, not all data points were included in the dose-response curves. Half maximal inhibitory concentrations (IC50) were deterimend using GraphPad Prism.

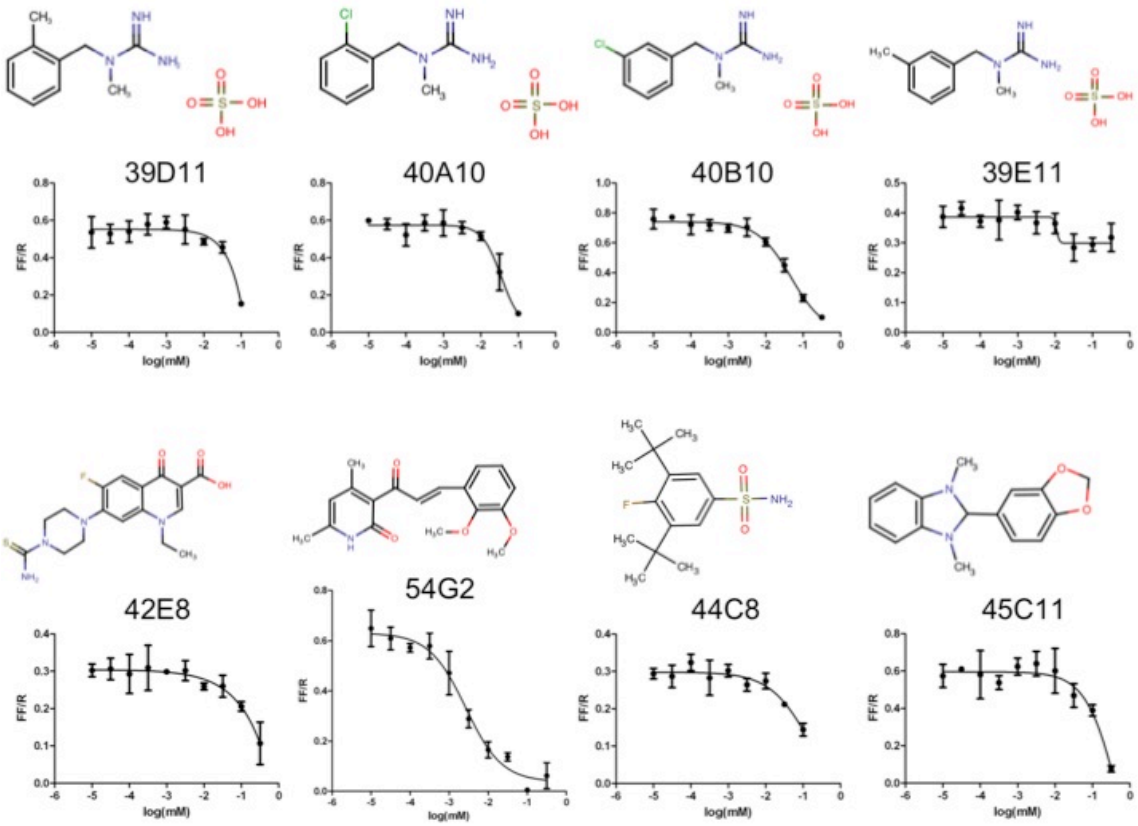

39D11
40A10
40B10
39E11
42E8
54G2
44C8
45C11
FF/R
log(mM)

Figure 4. Verification of the candidate compounds. Oli-neu cells were transfected with PDGFRA-luc and pGL4.73 and treated with top 20 candidate compounds selected from primary screens. Graphs show normalized luciferase activity as a response to different concentrations of compounds. Out of 20 candidates tested, 7 downregulated PDGFRA-luc activity in a dose-response manner. Out of these 7 compounds, 3 (39D11, 40A10 and 40B10) had similar chemical structure based off the lead N-methyl-N-benzylguanidine (top). Although the 4^{th} derivative of N-methyl-N-benzylguanidine (39E11) altered Oli-neu cell morphology in the morphology-based screen, it showed a modest dose response effect on PDGFRA-luc downregulation (top). Remaining 4 compounds, that also resulted in dose-dependent downregulation in PDGFRA-luc activity, were not structurally related to N-methyl-N-benzylguanidine or to one another (bottom).

Verification of endogenous Pdgfra transcription downregulation

Since PDGFRA-luc plasmid contains luciferase cDNA and only a portion of the mouse Pdgfra gene, downregulation of its activity upon treatment with guanidine compounds might not be an accurate reflection of the changes in endogenous Pdgfra transcription. Therefore, we examined changes in endogenous Pdgfra mRNA level upon treatment of Oli-neu cells with guanidine compounds. Oli-neu cells were seeded in 6-well plates and treated with 50 μM compounds for 2 days. As assessed by qPCR, Pdgfra mRNA level was ~15 fold lower compared to DMSO control. To the contrary, Pdgfra transcription was comparable to DMSO control when cells were treated with 42E8, a non-guanidine compound that caused dose-response in PDGFRA-luc activity. We also analyzed the changes in mRNA levels of two additional receptor tyrosine kinases (RTKs) expressed by OPCs. Changes in transcription for Fgfr1 and Fgfr3 were far less robust compared to those of Pdgfra. Compounds 39D11 and 39E11 caused a modest decrease in the level of Fgfr1 transcript, while treatment with 40A10 and 40B10 resulted in a marginal increase. Contrary to downregulation of Pdgfra transcription, Fgfr3 transcription was upregulated upon treatment with guanidine compounds. Fgfr3 transcript level increased by approximately 2.5 fold relative to DMSO control (Figure 5). These results suggest that downregulation of transcription by guanidine compounds is specific for Pdgfra, and it does not occur with other RTKs, such as Fgfr1 and Fgfr3.

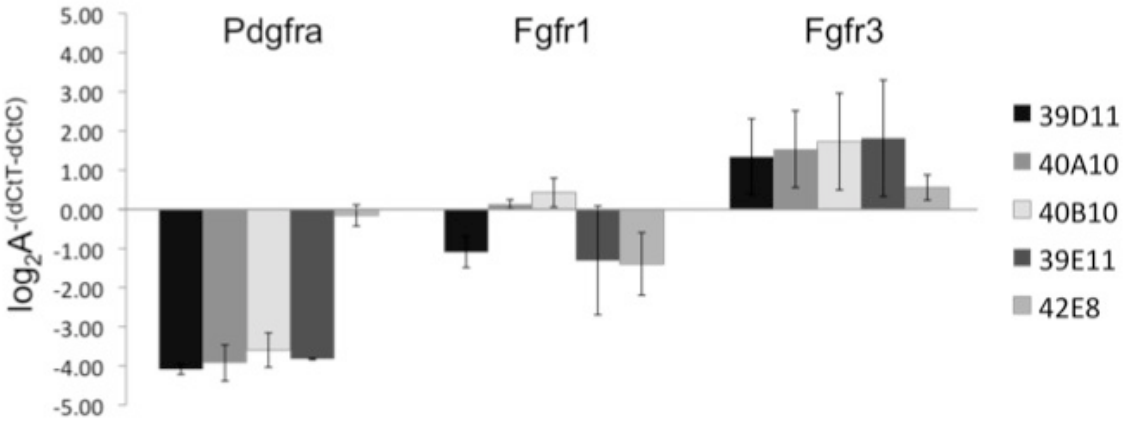

Pdgfra
Fgfr1
Fgfr3
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
log2A-(dCtT-dCtC)
39D11
40A10
40B10
39E11
42E8

Figure 5. Changes in endogenous transcription upon treatment with guanidine compounds. Oli-neu cells were treated with guanidine compounds at 50 μM for 2 days when RNA was collected and analyzed. Results of qPCR showed ~15 fold decrease in mRNA level of Pdgfra. To the contrary, the transcript level of Fgfr1 showed only marginal change, while that of Fgfr3 increased by ~2.5 fold. Each bar represents the average result obtained from 3 independent experiments. Error bars are standard errors of the mean. (A = amplification factor; dCtC = delta Ct of DMSO control; dCtT = delta Ct upon treatment with compounds)

Discussion

Results of the primary screens revealed candidate compounds that caused PDGFRA-luc downregulation in Oli-neu cells and may have promoted their differentiation. There were 11 compounds that met our criteria in all three primary screens: they downregulated PDGFA-luc activity, caused an increase in O1 expression and altered cell morphology. However, 61 compounds met only two out of three criteria. This implies that downregulation of PDGFRA-luc does not necessarily correlate with acquisition of mature phenotype. This makes sense because it is unlikely that PDGFRA-luc contains all the regulatory elements necessary for OPC-specific expression of Pdgfra gene. This is further supported by the results obtained during identification of the most suitable plasmid for the screen. Although treating Oli-neu cells with inducers of differentiation resulted in a decrease in PDGFRA-luc activity, this decrease was only ~35%.

Although luciferase assay has its flaws, such as incomplete regulatory sequences and introduction of foreign cDNA into the cell, it is fast and efficient and as such, it represents a very useful tool for screening. It would be more informative to run two luciferase assay screens in parallel, first using PDGFRA-luc and second utilizing regulatory elements of an early oligodendrocyte gene, such as Cnp, which gets upregulated as OPCs differentiate into mature oligodendrocytes. A great improvement to the technique would be to use stable instead of transient transfections, where the firefly luciferase cDNA is driven by regulatory elements of endogenous Pdgfra gene expressed by Oli-neu cells.

Oli-neu cells are immortalized cell line derived from primary mouse OPCs. Although it has been shown they are able to generate MAG-expressing cells (Jung et al, 1995), we were unable to push them down the oligodendrocyte differentiation pathway further than

O1-expressing cell. Contrary to that, a study by Pereira et al (2011) suggests that Oli-neu cells are father along the differentiation pathway compared to N20.1 cells, which represent immortalized oligodendroglial cell line (Verity et al, 1993; Newman et al, 1995; Pereira et al, 2011). Whether Oli-neu cells represent immature progenitor cell or early oligodendrocyte, this may contribute to low responsiveness of PDGFRA-luc to inducers of differentiation. With numerous transgenic mouse lines available, using primary OPCs from transgenic mice that have fluorescent reporter driven by endogenous genes of oligodendrocyte lineage might provide a better biological system to detect differentiating OPCs.

To select candidate compounds after the luciferase assay screen, we calculated averages and standard deviations of each batch of samples and selected compounds that caused PDGFRA-luc downregulation by at least 1 SD below the average. Using SD assumes normal distribution, which does not seem to be the case in our experiment. Compounds that caused robust upregulation of PDGFRA-luc activity skewed the distribution in their favor. Therefore, this criterion is arbitrarily determined in order to establish a cutoff.

Morphology based screen revealed compounds that resulted in a decrease in Oli-neu cell density relative to DMSO control or altered their morphology towards a more differentiated phenotype. Although the selected compounds might have a desired effect inhibiting Oli-neu cell proliferation of promoting their differentiation, we did not run consecutive experiments to verify their effect. Therefore, we need to keep in mind that the decrease in cell density might be due to partial cytotoxicity of compounds. Similarly, change in cell morphology might be caused by cytoskeleton reorganization that is not

directly related to differentiation. Therefore, these results need to be interpreted with caution and in perspective with results obtained from luciferase assay and ICC screens.

Only 7 out of 20 candidates tested were verified in a dose-response assay. Compounds based on N-methyl-N-benzylguanidine structure were further verified with respect to their effect on endogenous Pdgfra transcription. A robust downregulation observed in the level of Pdgfra transcript contrasts those of Fgfr1 and Fgfr3. This implies that the effect of compounds is specific for pathway that regulates Pdgfra transcription and not to those that regulate Fgfr1 or Fgfr3 transcription.

Chapter 3. Determine structure activity relationship for compounds that downregulate Pdgfra transcription in Oli-neu cells

Introduction

Drug discovery, especially the one based on random screen, usually does not yield in identifying the drug that meets all the criteria we have established. These criteria include, but are not limited to (1) desired biological activity, (2) high potency and (3) no toxicity. Instead, what is likely to be discovered is the compound that has the desired activity, but might have unwanted properties, such as low potency or some degree of toxicity. This compound is called “the lead”. After lead has been identified, it is important to determine the relationship between leads’ derivatives and their biological activity (structure-activity relationship, SAR). By examining multiple lead derivatives, it is possible to find out which residues are critical for maintaining drug properties and which can be removed or replaced in order to increase drug potency and diminish its toxicity. Determining SAR is often performed by synthesizing as many lead derivatives as possible and their testing for biological activity and potency. Structure modifications of the lead can be done rationally because compound properties are often based on its physicochemical properties, such as solubility and lipophylicity. However, it is usually the ease of synthesis that prevails when determining what structure modifications will be made. Once sufficient derivatives are tested, conclusions can be drawn regarding SAR.

In order to determine SAR for guanidine derivatives, we first performed structure-based screen on an entire Chembridge library, identified all guanidine compounds that were included in the library and related their structure to the biological effect they showed in

our primary screen. We next synthesized (courtesy of Michael VanHeyst and Dr. Dennis Wright) 12 additional guanidine derivatives and tested their effect on PDGFRA-luc activity and Pdgfra transcription.

Results of the SAR analysis suggest that the critical positions for modifications of the lead N-methyl-N-benzylguanidine, which are required for its biological effect, are carbons 2 or 3 of the benzyl ring. Addition of either methyl group or chlorine to carbons 2 or 3, as well as methoxy group to carbon 2, greatly increases the potency of the lead. Elongation of the carbon chain between the nitrogen atom and benzyl ring results in an increase in lead's potency even without the additional modifications.

Methods

Screening

Structure-based screening of the Chembridge library was done using Instant JChem 5.9.0, 2012 downloaded from ChemAxon website (http://www.chemaxon.com). Library was uploaded to Instant JChem and screened based on N-methyl-N-benzylguanidine structure.

Guanidine derivatives

Our collaborators, Michael VanHeyst and Dr. Dennis Wright, from the Department of Pharmaceutical Sciences synthesized 12 guanidine derivatives that were used in this study. Compounds were dissolved in DMSO and their purity was tested using Nuclear magnetic resonance (NMR) spectroscopy. We also examined and compared NMR

spectra of newly synthesized compounds to that of compounds purchased from Chembridge.

Luciferase assay

Luciferase assay was done as described in Chapter 3 (Methods). Briefly, Oli-neu cells were seeded to 6-well plate and transfected with PDGFRA-luc and pGL4.73. They were split to 96-well plate the following day and treated with guanidine compounds for 2 days at 3 μM and 30 μM.

RNA isolation, reverse transcription and quantitative PCR

RNA isolation, reverse transcription and qPCR were done as described in Chapter 3 (Methods). Briefly, Oli-neu cells were treated with guanidine compounds for 2 days at 50 μM. RNA was purified using RNeasy Mini Kit (QIAGEN). An aliquot of 1 μg RNA was used for cDNA synthesis using Superscript III (Invitrogen) and 10 ng cDNA was used for each qPCR using SYBR Green PCR Master Mix (Life Technologies). Quantification of gene expression was first normalized to GAPDH and then expressed as a logarithm of the ratio of treated to control mRNA level. Primers used for qPCR: Gapdh (forward 5'-TGACAACTTTGGCATTGTGG, reverse 5'-ATGCAGGGATGATGTTCTGG) Pdgfra (forward 5'- TCGAAGGCAGGCACATTTAC, reverse 5'-TTGAGTCTCCGGATCTGTGG).

Results

Structure-based screen for derivatives of N-methyl-N-benzylguanidine

Chembridge library contains approximately 100,000 compounds. However, only a small portion of approximately 1,500 compounds from the library was used for the primary screens in our study. Structure-based screen of Chembridge library revealed only 9 guanidine derivatives (Figure 6). These compounds were distributed between 2 mother plates and both plates happened to be selected for the primary screens. Out of these 9 compounds, 5 did not show the desired biological effect in any of the primary screens. On the other hand, 3 compounds (39D11, 40A10 and 40B10) met the criteria in all 3 primary screens. The remaining compound (39E11) did not cause decrease in PDGFRA-luc activity in the luciferase assay screen, but did cause decrease in Oli-neu cell density and change in their morphology.

Analysis of SAR suggests that N-methyl-N-benzylguanidine (compound 39C11) is necessary, but not sufficient to induce desired biological activity by itself. Methyl group or chlorine potentiates the effect of N-methyl-N-benzylguanidine. However, this occurs only if one of these residues is present on either carbon 2 (C2) or carbon 3 (C3) of the benzyl ring (Figure 6).

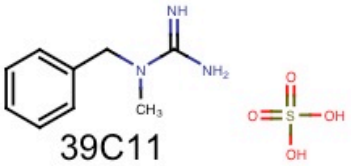
Guanidine compounds downregulate PDGFRA-luc activity

In order to further examine the relationship between the differences in structures of guanidine compounds and their activity, our collaborators Michael VanHeyst and Dr. Dennis Wright from the Department of Pharmaceutical Sciences generated 12 guanidine

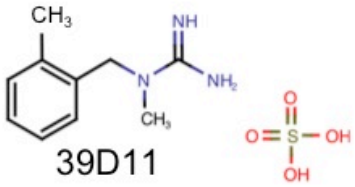
derivatives, arbitrarily named 001 through 012 (Figure 7. A). The effect of these compounds was tested in luciferase assay (Figure 7. B). Oli-neu cells transfected with PDGFRA-luc and pGL4.73 were treated with guanidine compounds at 2 concentrations that matched 2 data points of previously established dose-response curves (Figure 4). None of the compounds caused significant downregulation of PDGFRA-luc activity at 3 μM relative to control. Different potency of these compounds was more evident when Oli-neu cells were treated with higher concentration of compounds. At 30 μM, 002 and 008 were the most potent and caused significant and most robust downregulation of PDGFRA-luc, while 009, 010, 011 and 012 did not downregulate PDGFRA-luc activity. Compounds 003, 004, 005 and 007 caused a significant downregulation of PDGFRA-luc, although this effect was less robust compared to that of 002 and 008. Finally, treating Oli-neu cells with 006 resulted in a marginal decrease in PDGFRA-luc activity that did not reach significance. (Figure 7. B). Although structurally identical, 001 and 40A10, as well as 007 and 40B10, did not show the same potency. Therefore, our collaborators compared the purity of compounds 40B10 and 007 by NMR spectrometry. NMR spectrum showed that 40B10 contained traces of an unknown chemical, which might be responsible for the more robust effect of 40B10 relative to 007 (not shown).

SAR analysis revealed that replacing benzyl ring with pyridine abolished the biological effect of the compound. Compound 005 had stronger effect compared to 007. Therefore, the choice of the residue that will be added to C3 of the benzyl ring appears to be important in increasing compound's potency, methyl group being the better choice than chlorine. Compound 008 was one of the 2 derivatives that showed the most robust downregulation of PDGFRA-luc, which indicates that elongating the guanidine side chain increases potency. Finally, addition of methoxy group to C2 of the benzyl ring

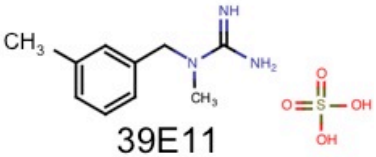
(compound 002) results in very robust biological effect. However, if methoxy group is placed on C3 of the same ring (compound 006), compound's potency is drastically decreased. Compounds 009, 010, 011 that have pyridine in place of benzyl, as well as 012 that has 2 methoxy groups on C3 and C4 did not alter PDGFRA-luc activity. This might be due to increased polarity of these compounds.



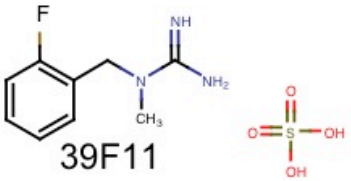
39C11



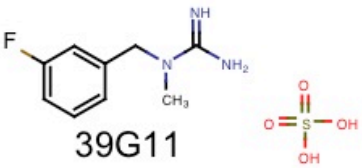
39D11



39E11

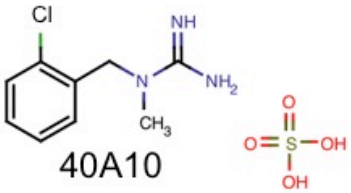


39F11



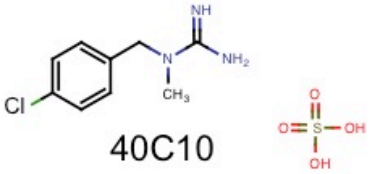
39G11

39H11



40A10

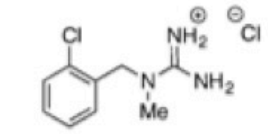
40B10



40C10

Figure 6. Structure-based screen for guanidine compounds. Chembridge library has approximately 100,000 small molecule compounds out of which only 9 are N-methyl-N-benzylguanidine derivatives. 39D11, 39E11, 40A10 and 40B10 were selected for verification after the primary screens, whereas remaining compounds did not alter either PDGFRA-luc activity, Oli-neu cell morphology or O1 expression. Selected 4 compounds had either methyl group or chlorine on C2 or C3 of the benzyl ring.

A



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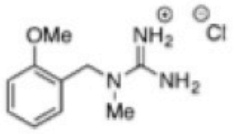

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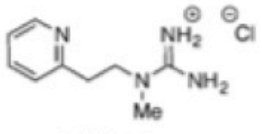
Molecular Weight: 229.71



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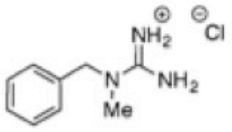

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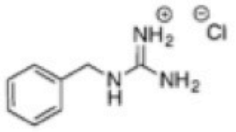

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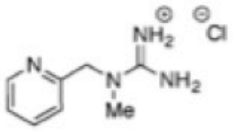

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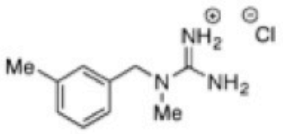

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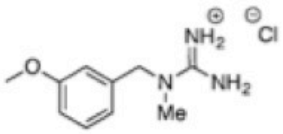

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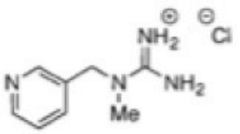
Molecular Weight: 229.71



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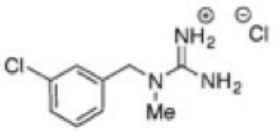

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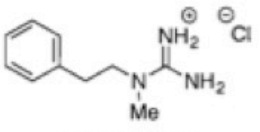
Molecular Weight: 234.12



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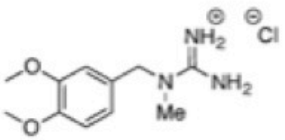

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Exact Mass: 259.11
Molecular Weight: 259.73

B

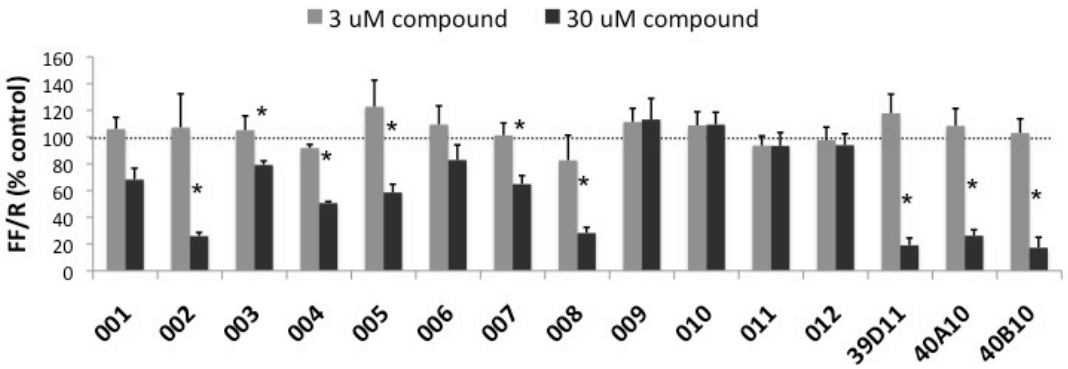


Figure 7. Structure and potency of guanidine derivatives. Our collaborators from the Department of Pharmaceutical Sciences synthesized 12 guanidine derivatives (A, courtesy of Micheal VanHeyst and Dr. Dennis Wright). Oli-neu cells were transfected with PDGFRA-luc and pGL4.73 and treated with newly generated compounds for 2 days at 3 or 30 μM when relative luciferase activity was measured. None of the compounds caused significant downregulation of PDGFRA-luc activity at 3 μM. At 30 μM, it was evident that compounds have different potencies. Treatment with compounds 002 and 008 resulted in the most robust downregulation of PDGFRA-luc, while compounds 009, 010, 011 and 012 did not alter PDGFRA-luc activity. Although structurally identical, 007 and 40B10 had different potencies (B). Each bar in panel B represents average PDGFRA-luc activity normalized to internal control and presented relative to DMSO control (100%). * $p < 0.05$ relative to control, one sample t-test.

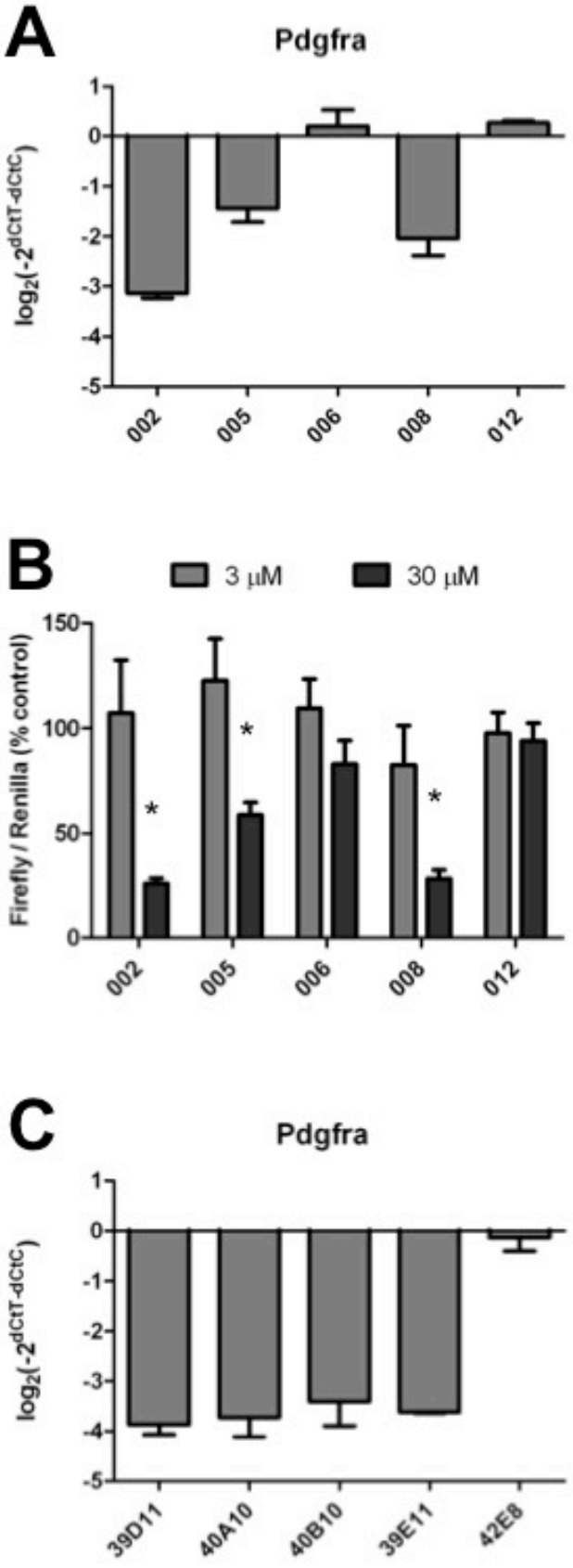
Guanidine compounds downregulate endogenous Pdgfra transcription

Results of the luciferase assay suggest that newly generated guanidine compounds downregulate Pdgfra transcription. The next question we asked was whether different potency of compounds would be reflected on Pdgfra transcript levels in the same manner that was observed in the luciferase assay. To answer this question, we selected 5 compounds (002, 005, 006, 008 and 012) that had different potency when previously tested in the luciferase assay. Compounds 002 and 008 were shown to be the most potent in the luciferase assay, while 005 caused significant but less robust effect. Compounds 002 and 006 were positional isomers that showed drastically different downregulation of PDGFRA-luc, while 012 did not alter PDGFRA-luc.

To determine whether endogenous Pdgfra transcription will follow the trend observed in the luciferase assay, Oli-neu cells were treated with selected compounds at 50 μM for 2 days when RNA was collected and analyzed by qPCR. As expected, changes in Pdgfra transcript levels (Figure 8. A) reflected those previously observed in the luciferase assay (Figure 8. B). Compounds 002 and 008 caused the most robust decrease in Pdgfra transcript levels, while 005 was less potent. Compounds 006 and 012 did not alter Pdgfra transcript level.

We also compared the potencies of Chembridge and compounds synthesized by our collaborators with respect to their effect on endogenous Pdgfra transcription. Similarly as in the luciferase assay (Figure 7), commercial compounds showed greater potency in the qPCR assay. Chembridge compounds caused a decrease in the Pdgfra transcript level between 10 and 16 fold, while compound 002, the most potent compound

generated in Dr. Dennis Wright's lab, decreased Pdgfra transcript level by less than 9 fold. Structurally identical 005 and 39E11 also had dramatically different potencies. Treatment with 005 resulted in ~3 fold decrease in Pdgfra transcript level, while that with 39E11 decreased Pdgfra transcript level by ~11 fold (Figure 8. A and C).


A
Pdgfra
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1
0
-1
-2
-3
-4
-5
002
005
006
008
012
B
3 μM
30 μM
Firefly / Renilla (% control)
150
100
50
0
*
*
*
002
005
006
008
012
C
Pdgfra
log2(-2dCtT-dCtC)
1
0
-1
-2
-3
-4
-5
39D11
40A10
40B10
39E11
42E8

Figure 8. Changes in endogenous transcription upon treatment with guanidine compounds. Oli-neu cells were treated with compounds 002, 005, 006, 008 and 012 (A) or guanidine compounds purchased from Chembridge (C) when RNA was collected and changes in Pdgfra transcription were analyzed by qPCR. Changes in Pdgfra mRNA levels (A) reflected those observed in the luciferase assay (B). Treatment with compounds 002 and 008 resulted in the most robust decrease in Pdgfra transcript level, while that with 005 was moderate. Compounds 006 and 012 did not alter Pdgfra transcription (A). Commercial compounds however were shown to be more potent compared to compounds generated in Dr. Dennis Wright's lab (C). In panels A and C, each bar represents the average result obtained from 3 independent experiments. Error bars are standard errors of the mean. dCtC = delta Ct of DMSO control; dCtT = delta Ct upon treatment with compounds. In panel B, each bar represents average PDGFRA-luc activity normalized to internal control and presented relative to DMSO control. The experiment was done in triplicates and error bars are standard deviations. * $p < 0.05$, one sample t-test.

Discussion

Structure based screen of Chembridge library followed by SAR analysis revealed that N-methyl-N-benzylguanidine is necessary, but not sufficient to induce desired biological activity. Addition of methyl group or chlorine potentiates the lead's effect, but the position of these 2 residues is critical. Improvement in compound's potency occurs only if either methyl group or chlorine is present on either C2 or C3 of the benzyl ring. It would be interesting to see whether compound's potency would be further improved if both C2 and C3 were occupied by either 2 methyl groups or 2 chlorines or a combination of methyl group and chlorine (Figure 9).

SAR analysis of 12 additional derivatives synthesized by our collaborators revealed several other pieces of information. (1) Compound 007 showed slightly more robust effect compared to that of compound 001 which suggests that addition of chlorine at C3 instead at C2 of the benzyl ring might be a better choice improving compound's potency. (2) We could not synthesize positional isomer for compound 005 that would have methyl group at C2 instead at C3 but the potency of these 2 isomers would be interesting to compare. (3) Since compound 004 showed greater potency relative to that of compound 003, this suggests that removal of methyl group from the nitrogen might further potentiate compound's effect. (4) Presence of methoxy group at C2 (compound 002) greatly improves compound's potency, but this does not happen if the same residue is present at C3 (compound 006). (5) Compound 008 is drastically more potent compared to 003. This suggests that elongating the chain between guanidine and benzyl ring by one carbon might be a critical modification. Such modification might exert its effect by increasing compound lipophylicity, stabilizing the compound-target interaction or

facilitating the interaction between the pharmacophore and the receptor. Based on SAR analysis of Chembridge and compounds generated by our collaborators, we propose further modifications that include a combination of selected residues and predict such modifications would increase the potency of guanidine compounds (Figure 9).

Replacement of hydrogen atom with fluorine may extend compound's half-life if such replacement was done at the site of metabolic oxidation. Because the fluorine atom is similar in size to the hydrogen atom, the overall topology of the molecule is not significantly altered, leaving the desired biological activity unaffected. Although structure based screen revealed 3 compounds that had such modifications, we did not perform an experiment to assess N-methyl-N-benzylguanidine stability and whether such modifications would improve it. However, this is something that needs to be kept in mind once the top candidates are tested for their biological effect *in vivo*.

The effect that guanidine compounds exerted on Pdgfra transcript level generally followed a similar trend to that observed in the luciferase assay. Compound 002 was the most potent in both assays, while 012 did not cause a change in Pdgfra transcription or PDGFRA-luc activity. Changes in transcription upon treatment with compounds 005 and 008 also reflected those observed in the luciferase assay. We were surprised to notice treatment with 006 did not alter Pdgfra transcript level although it did cause modest decrease in PDGFRA-luc activity that did not reach significance. In addition, for qPCR assay, cells were treated with compounds at 50 μM while the highest concentration used in the luciferase assay was 30 μM.

In both luciferase assay and qPCR assay, commercial compounds were shown to be more potent compared to those synthesized by our collaborators. Compounds 005 and 007 are structurally identical to 39E11 and 40B10, respectively. However, their effects on PDGFRA-luc and Pdgfra transcription are drastically different. We examined and compared purities of compounds 007 and 40B10 and identified traces of an unknown chemical, which might be responsible for the difference in the effect we have observed. It would be interesting to identify this chemical or separate it from N-methyl-N-(3-chlorinebenzyl) guanidine using column chromatography and analyze its effect independently from guanidine compound. Although we did not compare the purities of compounds 005 and 39E11, it is likely 39E11 contains compound other than N-methyl-N-(3-methylbenzyl) guanidine. Whether this is true as well as the nature of contaminating compound remains to be identified.

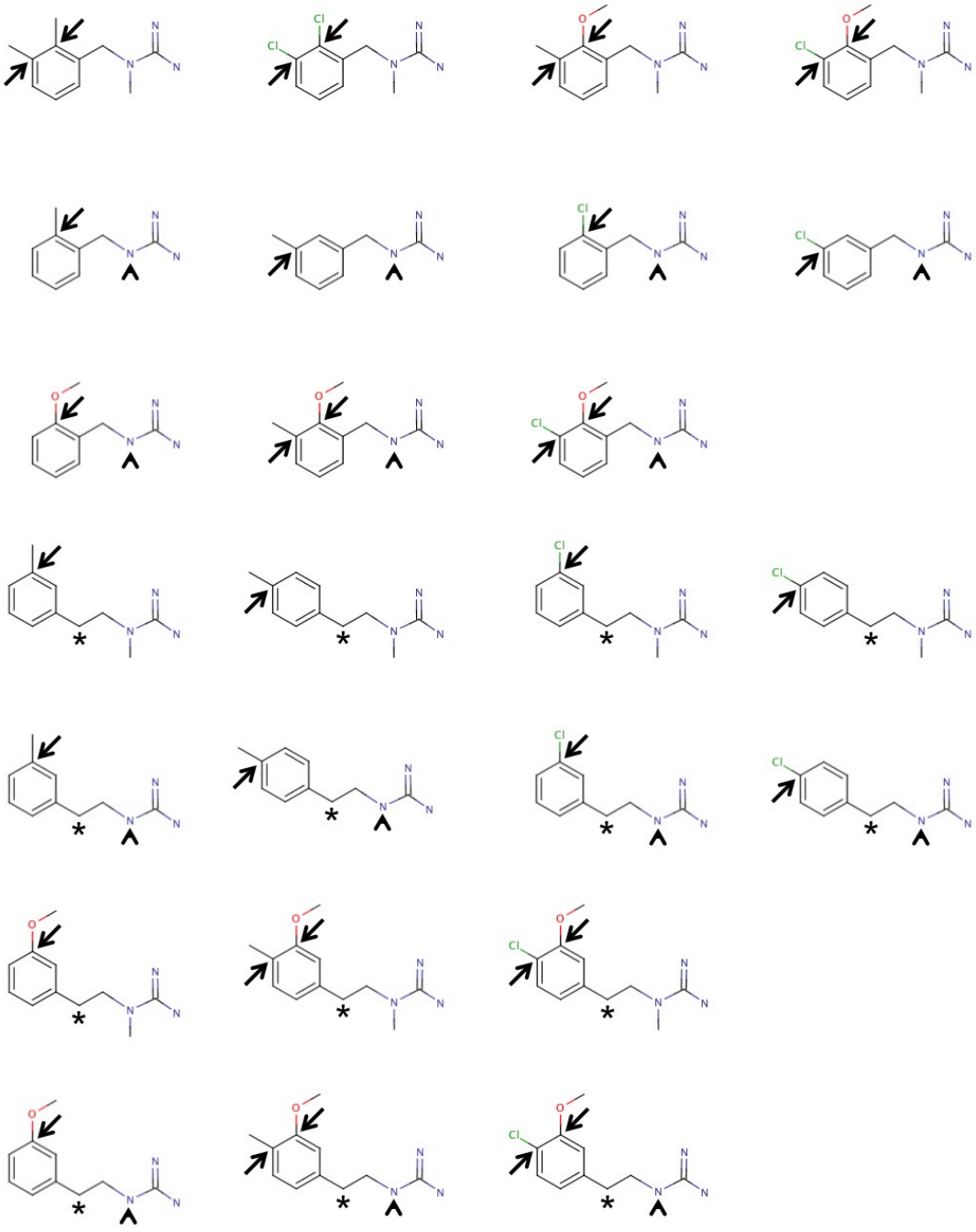

Figure 9. Predicted structural modifications of N-methyl-N-benzylguanidine that would result in its increased potency. SAR analysis of Chembridge and compounds synthesized by our collaborators revealed that the following modifications of N-methyl-N-benzylguanidine increase its potency: addition of methyl group or chlorine to C2 or C3 of the benzyl ring, addition of methoxy group to C2, removal of the methyl group from the nitrogen atom of the guanidine group and elongation of the carbon chain between the nitrogen atom of the guanidine group and benzyl ring. We predict that a combination of such modifications, generating structures shown in this figure, would result in a further increase in N-methyl-N-benzylguanidine potency. Such modifications are illustrated on this figure. Addition of a residue (chlorine, methyl or methoxy group) to the carbon atom of N-methyl-N-benzylguanidine is indicated by an arrow, removal of the methyl group from the nitrogen atom is indicated by an arrowhead, while asterisk marks elongation of the carbon chain.

Chapter 4 – Determine whether the compounds promote OPC differentiation.

Introduction

PDGF signaling is the major regulator of OPC proliferation and differentiation. Studies have implied that it has to be inhibited in order for OPC differentiation to occur (Noble et al, 1988; McKinnon et al, 2005). *In vitro*, this can be achieved by PDGF removal from the culture medium (Raff et al, 1985; Raff et al, 1988) or blockage with anti-PDGF antibodies (Richardson et al, 1988; Noble et al, 1988). *In vivo*, PDGF knockout results in decreased number of OPCs (Fruttiger at al, 1999). OPCs from PDGFRA knockout mice undergo accelerated maturation and the onset of differentiation appears to depend on the Pdgfra gene dose (McKinnon et al, 2005). PDGFRA is rapidly downregulated as OPCs differentiate (Hart, 1989; Hall, 1996) and we used the downregulation of its transcription as putative indicator of differentiation.

Here, we wanted to examine whether guanidine compounds, identified in the primary screen and verified as downregulators of Pdgfra transcription in Oli-neu cells, promote Oli-neu and OPC differentiation. To answer this question, we used Oli-neu cells and dissociated culture of primary rat OPCs and examined the changes in transcription of OPC- and oligodendrocyte-specific genes upon treatment with guanidine compounds. We also examined whether treatment of primary rat OPCs with guanidine compounds would result in an increase in the percentage of differentiating identified by O1 antibody. Our results show that, although compounds cause a decrease in the level of Pdgfra transcript, they are do not upregulate transcription of more mature markers of oligodendrocyte lineage or the percentage of O1+ primary rat OPCs.

Methods

Dissociated cultures of primary rat OPCs

Mixed glial cultures were prepared as descried previously (Yang et al, 2005). Briefly, cerebral cortices from P2-P4 CD rats (Charles River Laboratories) were dissected out, minced, triturated and plated in 75 cm^2 culture flasks coated with 30 µg/ml poly-L-lysine (Sigma). Cell cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco 11960) containing 10% FBS (Gibco 26140) at 37°C in humidified air with 5% CO2. After 6-8 days, loosely attached cells were shaken-off and preplated for 30 minutes in order to eliminate contaminating astrocytes and microglia from cells of oligodendrocyte lineage. The non-adherent cells were collected, resuspended in immunopanning buffer containing Dulbecco's phosphate-buffered saline (DPBS) (Gibco 14287), 0.02% bovine serum albumin (BSA) (Sigma A-4161), 5 µg /ml insulin (Sigma I-6634), and plated in petri dishes coated with monoclonal O1 antibody in order to separate mature oligodendrocytes from OPCs. Cells that did not adhere to the dish were collected and plated in petri dishes coated with O4 monoclonal antibody. Purified OPCs were resuspended in Sato's medium: DMEM (Gibco 11960), 2 mM L-glutamine (Gibco 25030), 1X penicillin/streptomycin (Gibco 15140), 100 µg/ml transferrin (Sigma T-1147), 100 µg/ml BSA (Sigma A-4161), 16 µg/ml putrescine (Sigma P-5780), 60 ng/ml progesterone (Sigma P8783), 40 ng/ml sodium selenite (Sigma S5261), 5 µg/ml N-acetyl cysteine (Sigma A8199), 1 mM sodium pyruvate (Gibco 11360), 1X trace elements B (Cellgro 99-175-CI), 10 ng/ml D-biotin (Sigma B4639), 5 µg/ml insulin (Sigma I-6634), 5 µM forskolin (Sigma F6886), 10 ng/ml CNTF (Peprotech 450-13), 1 ng/ml neutrophin 3

(NT3) (Peprotech 450-03). OPCs were then plated in poly-L-lysine coated tissue culture plates in the medium containing 20 ng/ml mouse PDGF (Peprotech 100-13A).

Immunocytochemistry

After positive selection of O4-expressing OPCs, cells were resuspended in Sato's medium and were plated in poly-L-lysine coated tissue culture plates in medium containing 20 ng/ml mouse PDGF (Peprotech 100-13A). They were allowed to proliferate for 2 days when they were trypsinized and split to coverslips coated with 100 µg/ml poly-L-lysine at the density of 5,000 cells/coverslip. They were kept in medium containing 5 ng/ml mouse PDGF (Peprotech 100-13A). For differentiation experiments, either T3 (Sigma T6397) or compounds were added to the final concentration of 400 ng/ml and 10 µM, respectively. They were treated for 2 days before fixing with 4% paraformaldehyde (PFA) and staining for O1.

RNA isolation, reverse transcription and quantitative PCR

RNA isolation, reverse transcription and qPCR for transcripts expressed by Oli-neu cells were done as described in Chapter 3 (Methods). Briefly, Oli-neu cells were treated with guanidine compounds for 2 days at 50 µM. RNA was purified using RNeasy Mini Kit (QIAGEN). An aliquot of 1 µg RNA was used for cDNA synthesis using Superscript III (Invitrogen) and 10 ng cDNA was used for each qPCR using SYBR Green PCR Master Mix (Life Technologies). Quantification of gene expression was first normalized to GAPDH and then expressed as a logarithm of the ratio of treated to control mRNA level.

Primary rat OPCs used for qPCR were obtained from P4 rat cortices by immunoselection using anti-O1 and goat anti-mouse PDGFRA antibodies. After selection of PDGFRA expressing cells, OPCs were resuspended in Sato's medium supplemented with 20 ng/ml mouse PDGF (Peprotech 100-13A), plated in poly-L-lysine coated tissue culture plates and allowed to proliferate for 3 days. Approximately 50% of medium was removed every day and replaced with 50% of fresh culture medium. On the 3rd day, OPCs were trypsinized and resuspended in Sato's medium that contained 5 ng/ml mouse PDGF. They were split to poly-L-lysine coated tissue culture dishes. To promote differentiation, either 400 ng/ml T3 or 10 μM 39D11 were added to the culture medium. Cells were treated for 2 days before RNA was extracted from approximately 250,000 cells using RNeasy Mini Kit (QIAGEN). An aliquot of 1 μg RNA was used for cDNA synthesis using Superscript III (Invitrogen) and 10 ng cDNA was used for each qPCR using SYBR Green PCR Master Mix (Life Technologies). The PCRs were done using the following conditions: 2 min at 95°C followed by 39 cycles of denaturation (10s at 95°C), annealing and extension (30s at 60°C). Quantification of gene expression was first normalized to GAPDH and then expressed as a logarithm of the ratio of treated to control mRNA level.

Gene	Forward primer	Reverse primer
Mouse Gapdh	TGACAACTTTGGCATTGTGG	ATGCAGGGATGATGTTCTGG
Mouse Pdgfra	TCGAAGGCAGGCACATTTAC	TTGAGTCTCCGGATCTGTGG
Mouse Cspg4	GGCCTTGTTGGTCAGATCTACAG	GCAGCAGGTCTACTCTGGTCAGA
Mouse Olig2	TTACAGACCGAGCCAACACC	GATGGGCGACTAGACACCAG
Mouse Sox10	TACCCTCACCTCCACAATGC	AGTCCGGATGGTCCTTTTTG
Mouse Cnp	GCCAGGTCTTTCTGGAGGAG	TTGTACAGTGCAGCACACCTG
Mouse Mbp	ACACACGAGAACTACCCATTATGG	TGTTCGAGGTGTCACAATGTTCTT
Mouse Gfap	GATCTATGAGGAGGAAGTTCGAGAA	CGTATTGAGTGCGAATCTCTCTCA
Rat Gapdh	TAGAGACAGCCGCATCTTCTTG	CGTTGATGGCAACAATGTCC
Rat Pdgfra	TTGGAGCTTGAGGGAGTGAAAC	AGACAGCTGAGGACCAGAAAGG
Rat Cspg4	TTACAAGTCCAGACGCCCAAC	GTTCTCCCCGAAGAAGGAGG
Rat Mbp	AAATCGGCTCACAAGGGATTC	AGGATTCGGGAAGGCTGAG

Table 4. Primers used for quantification of mRNA in Oli-neu cells and primary rat OPCs in control conditions and upon treatment with guanidine compounds. All primers span adjacent exons and their specificity was confirmed by melting curve.

Antibodies

Immunoselection of primary rat OPCs for immunocytochemistry was done with the following antibodies: secondary antibody used for coating petri dishes was goat anti-mouse IgM μ chain specific in 50mM Tris-HCl pH 9.5 (Jackson Immunoresearch 115-005-020) at 1 μg/ml in, while the primary antibodies were mouse anti-O1 and anti-O4 supernatant obtained from Dr. S. Pfeiffer (Farmington, CT) at 1:1 dilution in PBS with 0.2% BSA. For immunostaining, we used mouse anti-O1 (Dr. S. Pfeiffer) at 1:1 dilution in PBS with 5% normal goat serum (NGS) and Cy3 conjugated donkey anti-mouse IgM, μ chain specific (Jackson Immunoresearch 715-165-020). Immunoselection of primary rat OPCs for qPCR was done with the following antibodies: secondary antibody used for coating petri dishes was donkey anti-goat IgG (Jackson Immunoresearch 705-006-147), while the primary antibody was goat anti-mouse PDGFRA (R&D Systems). Antibodies were dissolved in 50 mM Tris-HCl pH 9.5 at 1 μg/ml.

Results

Guanidine compounds do not promote differentiation of primary rat OPCs

Results of the primary screen indicated that guanidine compounds cause an increase in O1 expression and change Oli-neu cell morphology towards a more mature phenotype. Therefore, we hypothesized that they will also promote differentiation of primary OPCs. To test this hypothesis, we used primary rat OPCs purified from neonatal rat cortices. OPCs were plated on coverslips in the medium that contained 5 ng/ml PDGF. They were treated with 10 μM compounds on the same day, while addition of 400 ng/ml T3 to the

culture medium was used as positive control. After 2 days, cells were fixed and stained for O1. Under differentiating conditions (medium supplemented with T3), the percentage of O1+ cells increased by approximately 3 fold. In addition, O1+ cells acquired different morphology. While O1+ cells under control conditions were small and had short processes, ones treated with T3 had longer and more branched processes as well as lamellipodia. Several guanidine compounds caused a change in OPC morphology, which resembled that observed when cells were treated with T3, but the length and branching of processes was less extensive compared to the T3 control (non-quantified observations). However, although several compounds caused an increase in the percentage of O1+ cells, this increase did not reach significance (Figure 10. B).

Changes in transcription occur before progenitor cells acquire differentiated phenotype. Although treatment with guanidine compounds did not cause a significant increase in the percentage of O1+ cells, we wanted to examine whether it would cause a change in Pdgfra, Ng2 or Mbp transcript levels (Figure 10. C). Although Pdgfra transcript level decreased upon treatment with 39D11, this decrease was marginal. Mbp transcript level also showed a slight decrease, while that of Ng2 increased. Taken together, these results do no support our hypothesis that guanidine compounds promote differentiation of primary rat OPCs.

Guanidine compounds do not upregulate transcription of more mature oligodendrocyte markers in Oli-neu cells

We used Oli-neu cells treated with 50 μM compounds for 2 days to analyze the endogenous transcription of several genes which expression changes during OPC differentiation. As OPC differentiate toward mature oligodendrocytes, Pdgfra and NG2

transcription decreases, while that of Cnp and Mbp increases. Olig2 and Sox10 are transcription factors that persist throughout an entire oligodendocyte lineage. Therefore, their transcript levels would not show a dramatic change. When induced to differentiate, Oli-neu cells were also shown to upregulate more mature markers of oligodendrocyte lineage, such as Cnp, Mbp and Plp (Joubert et al, 2010). Our qPCR results showed that Pdgfra transcript indeed follows the expected transcriptional changes when Oli-neu cells were treated with guanidine compounds. All commercially available guandine compounds caused a decrease in Pdgfra transcript level, while non-guanidine compound (42E8) did not cause a change in transcription (Figure 11. A). Similarly to Chembridge compounds, guanidine derivatives synthesized by our collaborators also caused a decrease in Pdgfra transcription and showed different potencies (Figure 11. B). However, Ng2 transcript level showed only marginal decrease when treated with Chembridge compounds. Contrary to Chembridge compounds, treatment with 002, 005, 006 and 008 caused a slight increase in Ng2 transcription. Changes in Sox10 transcript levels were similar to that of Ng2. Unexpectedly, Cnp transcription was decreased upon treatment with guanidine compounds while that of Mbp was marginally decreased upon treatment with Chembridge compounds and marginally increased upon treatment with 002, 005, 006 and 008. Surprisingly, Olig2 transcription was robustly inhibited when Oli-neu cells were treated with both sets of guanidine compounds (Figure 11. A and B).

When treated with FBS in culture, both primary OPCs and Oli-neu cells were shown to downregulate OPC-specific genes and upregulate astrocyte genes. Therefore, we asked whether guanidine compounds induce Oli-neu cell differentiation toward astrocytes, rather than oligodendrocytes. The trend we observed examining transcript levels of OPC and oligodendrocyte markers would support this hypothesis. Therefore, we looked at changes in transcription of glial fibrillary acidic protein (Gfap), which is a commonly used

marker for astrocytes (Bignami et al, 1972). Our qPCR results showed that compounds 002, 005, 006 and 008 caused a 2-fold increase in the Gfap transcript level (Figure 11. B). However, when Oli-neu cells were treated with commercial compounds, Gfap transcription was comparable to control (Figure 11. A). Taken together, these results suggest that although guanidine compounds downregulate Pdgfra transcription, they do not promote differentiation of OPCs to oligodendrocytes or astrocytes.

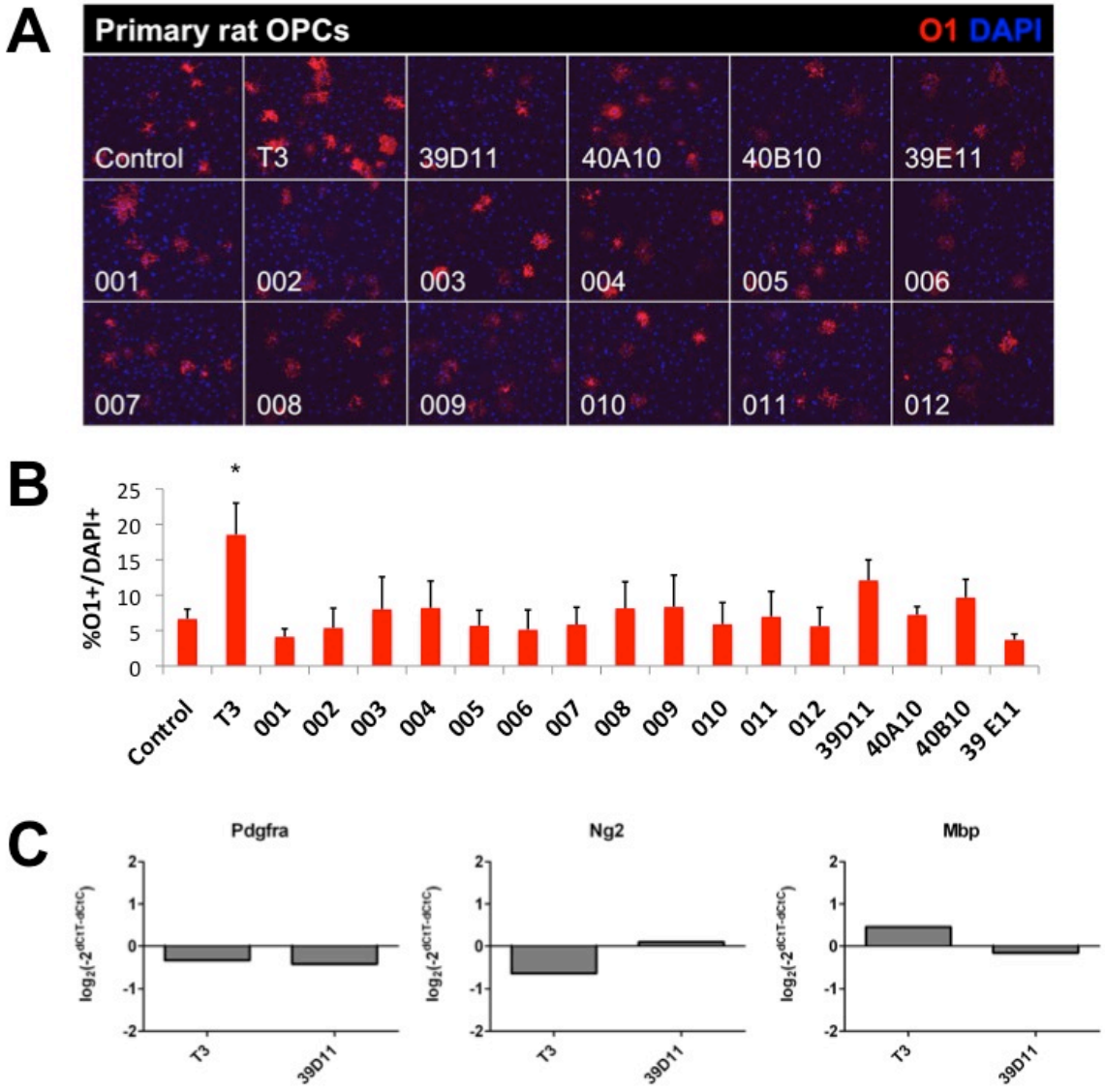
A
Primary rat OPCs
O1 DAPI
Control
T3
39D11
40A10
40B10
39E11
001
002
003
004
005
006
007
008
009
010
011
012
B
%O1+/DAPI+
*
25
20
15
10
5
0
Control
T3
001
002
003
004
005
006
007
008
009
010
011
012
39D11
40A10
40B10
39 E11
C
Pdgfra
Ng2
Mbp
T3
39D11

Figure 10. Differentiation of primary rat OPCs upon treatment with guanidine compounds. Purified rat OPCs were treated with 10 μM compounds for 2 days before they were immunostained for O1 (A). Quantification showed that addition of T3 resulted in a significant increase in the percentage of O1+ cells among all cells. Although several guanidine compounds caused a slight increase in the percentage of O1+ cells, this increase was modest and did not reach significance. (C) Primary rat OPCs, maintained in the medium that contained 5 ng/ml PDGF, were treated either with T3 or 39D11 at 400 ng/ml or 10 μM, respectively. After 2 days of treatment, RNA was collected and changes in transcription were analyzed by qPCR. dCtC = delta Ct of DMSO control; dCtT = delta Ct upon treatment with compounds. * $p<0.05$, two sample t-test relative to control.

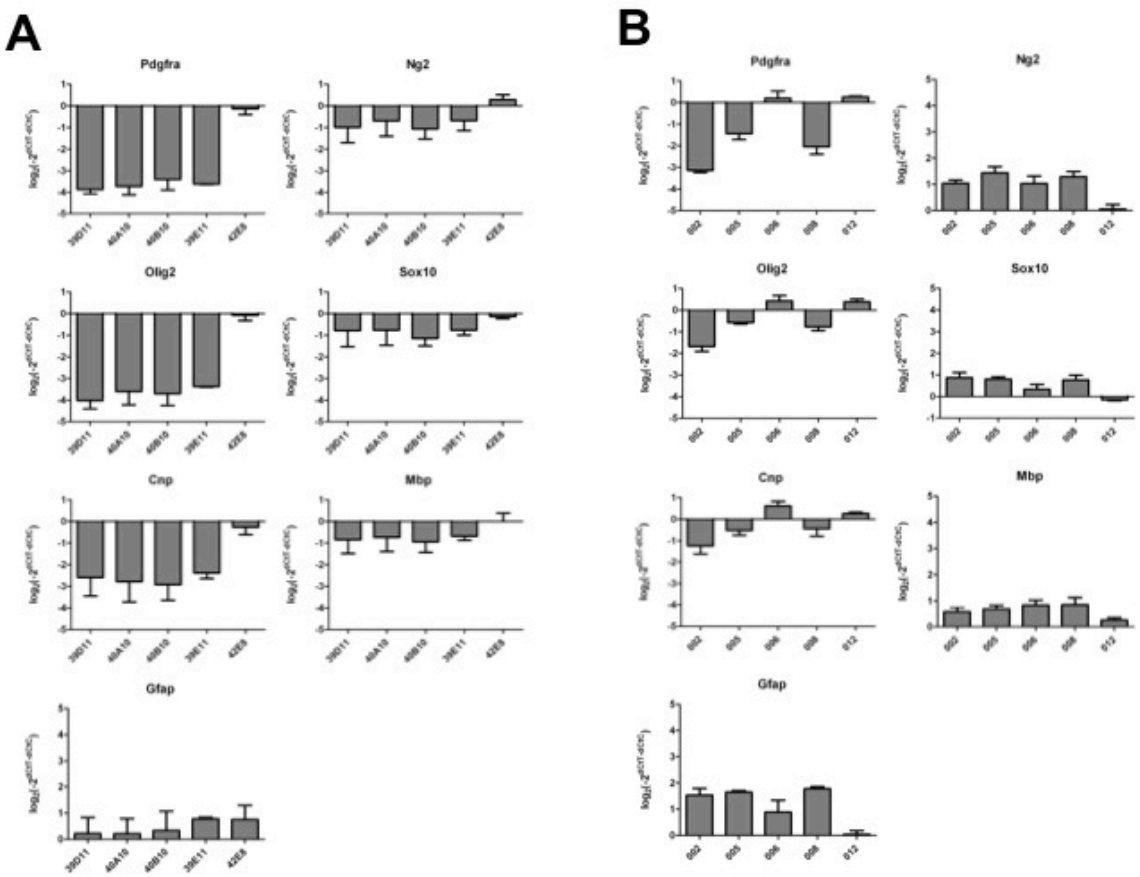
A
Pdgfra
Ng2
Olig2
Sox10
Cnp
Mbp
Gfap
39D11
46A10
46B10
39E11
42E8
B
Pdgfra
Ng2
Olig2
Sox10
Cnp
Mbp
Gfap
002
005
006
008
012

Figure 11. Changes in endogenous transcription upon treatment with guanidine compounds. Oli-neu cells were treated with 50 μM guanidine compounds, which were either purchased from Chembridge (A) or synthesized by our collaborators (B). After 2 days of treatment, RNA was collected and changes in transcription were analyzed by qPCR. Each bar represents the average result obtained from 3 independent experiments. Error bars are standard errors of the mean. dCtC = delta Ct of DMSO control; dCtT = delta Ct upon treatment with compounds.

Discussion

PDGFRA is rapidly downregulated as OPCs differentiate. Therefore, we hypothesized that downregulation of Pdgfra transcription caused by guanidine compounds would correlate with increase in Oli-neu and OPC differentiation.

Differentiation of OPCs was assessed by quantification of the percentage of O1+ cells, qualitative examination of cell morphology and qPCR. Although there was a slight decrease in the Pdgfra transcript level when cells were treated with 39D11, this decrease was less than 2-fold. Surprisingly, Mbp transcript level was decreased as well, but marginally. We did not observe a significant increase in the percentage of O1+ cells with any of the compounds. Several guanidine compounds seemed to have caused a change in cell morphology similar to that observed when OPCs were treated with T3 but of far less extent. All this suggests that treatment with guanidine compounds is not sufficient to induce differentiation of primary rat OPCs under the conditions we used. PDGF has been shown to be a potent mitogen for OPCs but also suggested as an inhibitor of their differentiation (Noble and Murray, 1984; Noble et al, 1988; Richardson et al, 1988; Raff et al, 1988; Pringle et al, 1989, McKinnon et al, 1990; Raff et al, 1988; Pringle et al, 1989; Richardson et al, 1988; Noble et al, 1988). When OPCs are treated with 10 ng/ml PDGF, they proliferate over a period of several days (Noble et al, 1988, Richardson et al, 1988). However, upon PDGF withdrawal, they rapidly differentiate (Raff et al, 1983; Noble, 1984; Temple, 1985). All this suggests that PDGF signaling needs to be finely balanced in order for differentiation to occur under given conditions. It is possible that lowering concentration of PDGF to 5 ng/ml was sufficient to induce differentiation of primary OPCs by itself and that treatment was 10 μM compounds was not sufficient to accelerate the differentiation process. Another possibility is that even 5

ng/ml PDGF caused an inhibition of OPC differentiation that could not be overridden by addition of 10 μM guanidine compounds.

Differentiation of Oli-neu cells along the oligodendrocyte lineage was assessed by examining changes in endogenous transcription upon treatment with guanidine compounds. Pdgfra transcript levels decreased upon treatment with guanidine compounds. Unexpectedly however, Cnp transcription is inhibited as well. Although some studies have implied that Olig2 gets downregulated in mature oligodendrocytes (Ligon et al, 2006), ~15 fold decrease in Olig2 transcript level upon treatment with guanidine compounds after only 2 days of treatment should not be attributed solely to differentiation. Decrease in the transcript level of all oligodendrocyte-specific markers observed upon treatment with Chembridge compounds might be explained by OPC fate switch to astrocytes. However, this does not seem to be the case here since Gfap transcript level is only marginally increased.

Taken together, our results suggest that although the transcript level of Pdgfra, one of the critical factors that regulates differentiation of OPCs, gets dramatically downregulated upon treatment with guanidine compounds, this event is not sufficient for differentiation to occur under given conditions. It is possible that guanidine compounds target a specific receptor or pathway, but there are factors that need to be regulated at the same time in order for successful differentiation to occur. This is supported by the fact that there is a discrepancy between the effects observed when cells were treated with Chembridge compounds compared to those when our own compounds were used. Sample 40B10 contains traces of an unknown chemical, which might be responsible for the inconsistent regulation of Ng2, Sox10 and Mbp transcripts. A combination of

guanidine derivatives, which downregulate Pdgfra, with other compounds, that cause an upregulation of Cnp and Mbp, might bring us closer to our goal.

Chapter 5 - Determine whether the compounds inhibit OPC proliferation.

Introduction

The roles of PDGF and PDGFRA in OPC proliferation and differentiation have been extensively studied. Numerous studies have shown that PDGF is the major mitogen for OPCs both *in vitro* and during development (Noble and Murray, 1984; Noble et al, 1988; Richardson et al, 1988; Raff et al, 1988; Pringle et al, 1989, McKinnon et al, 1990; Raff et al, 1988; Pringle et al, 1989; Richardson et al, 1988; Noble et al, 1988; van Heyningen et al, 2001). PDGF withdrawal from the culture medium or its block with anti-PDGF antibodies is sufficient to induce OPCs to exit cell cycle and differentiate (Raff et al, 1985; Raff et al, 1988; Richardson et al, 1988; Noble et al, 1988). PDGF exerts its effects though PDGFRA, which is the only PDGFR isoform expressed by these cells (Pringle et al, 1992; Nishiyama et al, 1996; Dawson et al, 2003). PDGFRA disruption in OPCs results in their premature differentiation in culture, whereas OPCs from PDGFRA knockout mice show impaired proliferation (McKinnon et al, 2005). Since PDGFRA is critical for OPC proliferation, we hypothesized that downregulating its transcription by guanidine compounds would result in decrease in OPC proliferation.

We tested the effect of guanidine compounds on the proliferation of PDGFRA-expressing Oli-neu cells and primary mouse OPCs, as well as HEK 293 cells and primary mouse astrocytes that do not express PDGFRA. We also examined the proliferation of three glioblastima-derived cell lines that show different levels of PDGFRA transcript (Lokker et al, 2002). Our results show that guanidine compounds robustly inhibited proliferation of Oli-neu cells and primary mouse OPCs, while they did not alter proliferation of HEK 293 cells and primary mouse astrocytes. Surprisingly, all

glioblastoma-derived cell lines showed a very mild decrease in proliferation and their response that did not correlate with the amount of PDGFRA transcript present. Contrary to Oli-neu cells and OPCs, guanidine compounds did not downregulate PDGFRA transcription in glioblastoma-derived cell lines.

Methods

Cell culture

Oli-neu cells were grown on poly-L-lysine-coated culture dishes. Cells were maintained in growth medium consisting of DMEM/F12 (Gibco 11330) supplemented with 1% horse serum (Hyclone SH30074.03), N2 supplement (Gibco 17502), 1X penicillin/streptomycin (Gibco 15140). Glioblastoma-derived cell lines A172, U251 and T98G, as well as HEK 293 cells were grown in DMEM (Gibco 11960) supplemented with 10% FBS (Gibco 26140), 2 mM L-glutamine (Gibco 25030), 1X penicillin/streptomycin (Gibco 15140). All cell cultures were maintained at 37°C in 5% CO_2 in a humid atmosphere.

Mixed glial cultures were prepared as descried previously (Yang et al, 2005). Briefly, cerebral cortices from P2-P4 FVB mice (Jackson Laboratory) were dissected out, minced, triturated and plated in 25 cm^2 culture flasks coated with 30 µg/ml poly-L-lysine (Sigma). Cell cultures were maintained in DMEM (Gibco 11960) containing 10% FBS (Gibco 26140) at 37°C in humidified air with 5% CO2. After 6-8 days, loosely attached OPCs and microglia were shaken-off. Remaining astrocytes were trypsinized, collected and plated on coverslips coated with 100 µg/ml poly-L-lysine at 5000 cells/coverslip.

Primary mouse OPCs were obtained from P2 mouse cortices from FVB mice by negative immunoselection using mouse anti-O1 and positive immunoselection using rat anti-mouse PDGFRA (BD Pharmingen). PDGFRA positive cells were resuspended in Sato's medium supplemented with 50 ng/ml mouse PDGF (Peprotech), plated in poly-L-lysine coated tissue culture dishes and allowed to proliferate for 3 days. Approximately 50% of medium was removed every day and replaced with 50% of fresh culture medium. On the 3rd day, OPCs were trypsinized and resuspended in Sato's medium that contained 20 ng/ml mouse PDGF. They were plated in coverslips coated with 100 ug/ml poly-L-lysine.

For proliferation experiments, cells were split to coverslips and treated with 50 μM guanidine compounds for 2 days. EdU (Life Technologies C10337) was added to the culture medium during the last 5h of incubation at the concentration of 10 μM. Cells were then fixed with 4% PFA and immunostained. Click-iT EdU Alexa Fluor 488 Imaging Kit (Life Technologies C10337) was used to label EdU+ cells.

Slice culture

Cortical organotypic slice cultures were prepared from P7-P8 NG2creBAC:ZEG double transgenic mice (Zhu et al, 2008) as described previously (Bahr et al, 1995; Zhu et al, 2011; Hill et al, 2013). Briefly, 300 μm coronal forebrain slices were cut with a tissue chopper and placed on Millicell culture inserts (Millipore PICM03050). Slices were maintained at 37°C in 5% CO_2 in a humid atmosphere. Slice medium contained 50% Minimal Essential Medium with Earle's Salts (MEM) (Gibco 11090), 25 mM HEPES

(Sigma H-4034) buffer at pH 7.22, 25% Hank's balanced salt solution (HBSS) (Gibco 14175), 25% horse serum (Hyclone SH30074.03), 0.4 mM ascorbic acid (Sigma A-0278), 1 mM L-glutamine (Gibco 25030) and 1 µg/ml insulin (Sigma I-6634).

For proliferation experiments, slices were treated with 1 µM or 10 µM guanidine compounds for 2 days in the medium that either had or did not have 50 ng/ml human PDGFAA (R&D Systems 221-AA). EdU was added to the slice medium during the last 5h of incubation at the concentration of 10 µM. Slices were then fixed and EdU+ were detected with Click-iT EdU Alexa Fluor 488 Imaging Kit (Life Technologies C10337).

Citotoxicity assay and flow cytometry

After 16h treatment with MMC or 50 µM guanidine compounds, Oli-neu cells were harvested. Apoptotic cells were detected using Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Life Technologies V13241) according to manufacturer's instructions with one modification. After incubating cell suspension with Alexa Fluor 488 Annexin V and propidium iodide, samples were washed once with 1 ml PBS in order to remove excess dye. Immediately following staining 10,000 cells were counted and analyzed with flow cytometry (BD FACSCalibur flow cytometer, Biotechnology-Bioservices Center, University of Connecticut, Storrs). Analysis was done using FlowJo software.

To compare the effect of guanidine compounds on proliferation and toxicity, Oli-neu cells were treated with 50 µM compounds for 16h with addition of EDU during the last 4h of treatment. Then they were harvested, resuspended in PBS and fixed by addition of PFA

to the final concentration of 2%. After washing once with PBS, pellets were resuspended in 50 ul Click-iT reaction cocktail (Life Technologies C10337). After 30 min, suspension was diluted to 500 ul with PBS. Cell pellets were collected, resuspended in 500 ul PBS and analyzed with flow cytometry (BD FACSCalibur flow cytometer, Biotechnology-Bioservices Center, University of Connecticut, Storrs). Analysis was done using FlowJo software.

Results

Guanidine compounds inhibit proliferation of Oli-neu cells

We previously demonstrated that 002, 005 and 008 caused a decrease in Pdgfra transcript level, 002 having the most robust effect, whereas 006 and 012 did not alter Pdgfra transcription (Chapter 5). Since PDGFRA gets downregulated with induction of differentiation, decrease in Pdgfra transcription might correlate with decrease in Oli-neu proliferation. In addition, we observed a striking difference in cell density when Oli-neu cells were treated with guanidine compounds (Figure 12. A). We therefore wanted to examine the possibility that the decrease in cell density is due to inhibition of proliferation. For this purpose, we treated Oli-neu cells with guanidine derivatives 002, 005, 006, 008 and 012 for 2 days at 50 μM concentration and assayed the percentage of proliferating cells during the last 5h of treatment (Figure 12. B and C). As expected, compounds 002, 005 and 008 caused a dramatic decrease in proliferation, assayed by the percentage of PDGFRA+/EDU+ among all PDGFRA+ cells. Surprisingly, although 006 did not inhibit Pdgfra transcription, treatment with 006 resulted in a significant decrease in the percentage of proliferating cells. When cells were treated with 012, there was a marginal decrease in proliferation that did not reach significance.

Quantitative PCR was performed in order to assess transcript levels of cyclin-dependent kinase inhibitors p21Cip1 (p21) p27Kip1 (p27), which were shown to be upregulated as OPCs are exiting the cell cycle (Casaccia-Bonefil et al, 1997; Durand et al, 1997; Ghiani et al, 1999). Our results showed that 002 caused the most robust increase in p21 and p27 transcript levels, while 005 and 008 caused somewhat moderate increase. Surprisingly, compound 006 resulted in less than 2-fold increase in transcript levels of p21 and p27 (Figure 12. D and E). As expected, 012 did not an effect. Taken together, these results suggest guanidine compounds inhibit proliferation of Oli-neu cells.

We also wanted to examine the potency of guanidine compounds with respect to their effect on proliferation of Oli-neu cells. For this purpose, Oli-neu cells were treated with 5 guanidine derivatives at several concentrations for 2 days with addition of EDU during the last 5h of treatment (Figure 12. F). Treatment with none of the compounds resulted in a significant decrease in the percentage of PDGFRA+EDU+ cells at 1 μM. As expected, compound 002 was the most potent, causing a robust decrease in the percentage of PDGFRA+EDU+ cells at concentration as low as 10 μM. Although treatment with 005 and 008 at 10 μM also inhibited proliferation and Oli-neu cells, this effect was less robust. Contrary to that, 006 was effective only at 50 μM, while 012 did not inhibit proliferation at any concentrations tested (Figure 12. F).

To determine whether decrease in cell proliferation is due to decreased expression of PDGFRA in general or increased internalization of PDGFRA from the surface of Oli-neu cells, we treated Oli-neu cells with guanidine compounds for 2 days at 50 μM and performed live staining for PDGFRA (Figure 13. A). We quantified the percentage of Oli-

neu cells that express PDGFRA on their surface at high level among all DAPI+ cells (Figure 13. B). Cells that express PDGFRA at high level were distinguished from those that express PDGFRA at low level by qualitative assessment of the intensity of immunostaining. The amount of receptor present on the surface of these cells varied within the cell population, even under control conditions. The percentage of cells expressing PDGFRA at high level, among all PDGFRA+ cells followed the general trend observed with proliferation experiments. However, the differences did not reach significance under any conditions. These results suggest that upon 2-day treatment of Oli-neu cells with guanidine compounds, majority of cells still express PDGFRA. Therefore, change in receptor expression and/or internalization are not likely causing the observed decrease in Oli-neu cell proliferation.

The decrease in cell density and proliferation of Oli-neu cells might be due to citotoxicity of guanidine compounds. In order to test this, we cultured Oli-neu cells in the presence of guanidine compounds for 16h. To detect cells that during this time underwent early/intermediate stages of apoptosis, we performed Annexin V staining in combination with propidium iodide (PI). Mitomycin C (MMC), an apoptotic inducer in variety of cancers, was used as positive control. The percentage of Annexin V+ cells was quantified by flow cytometry (Figure 14. A). Our results show that all guanidine compounds, as well as MMC, caused an increase in the percentage of Annexin V+ cells by at least 2 fold relative to control (Figure 14. B). We also examined proliferation of Oli-neu cells treated with guanidine compounds during the same time frame. The percentage of EDU+ cells was quantified by flow cytometry (Figure 14. C). After 16h treatment with compounds 005, 006 and 008, the percentage of EDU+ Oli-neu cells decreased by approximately 2 fold relative to control. As expected, compound 002 was the most potent, causing a decrease in the percentage of EDU+ cells close to 8 fold

(Figure 14. D). Taken together, these results imply that guanidine compounds are at least partially toxic. However, the effect of compound 002 cannot be attributed solely to toxicity since the inhibition of proliferation is greater compared to toxicity.

Guanidine compounds inhibit proliferation of dissociated primary mouse OPCs

The next question we asked was whether the inhibition of proliferation by guanidine compounds is cell-type specific. Oli-neu cells were derived from primary mouse OPCs by retroviral transduction with mutated analog of c-neu proto-oncogene. T-neu oncogene differs from its analog by a point mutation, which causes t-neu to be constitutively active and results in indefinite proliferation capacity of cells that carry this oncogene. Due to different profile, OPCs might not necessarily respond in the same manner to guanidine compounds.

We therefore wanted to examine whether change in OPC proliferation, as a response to treatment with guanidine compounds, would occur in the manner similar to that of Oli-neu cells. To answer this question, dissociated primary mouse OPCs were treated with 002, 005, 006, 008 and 012 at 50 μM for 1 day. Compounds were added to the cells together with 20 ng/ml mouse PDGFAA, a known survival factor and mitogen for OPCs, which is necessary for maintenance of progenitor phenotype in dissociated cultures. EDU was added during the last 5h of incubation when cells were fixed and stained for PDGFRA (Figure 15. A). Our results indicate that, although the response of OPCs followed a similar trend compared to that of Oli-neu cells, decrease in the percentage of proliferating cells was less robust (Figure 15. B). As expected, 002, 005 and 008 were the most potent, while 012 caused marginal decrease in the percentage of EDU+ cells.

Treatment of OPCs with 006 resulted in a moderate decrease in proliferation that did not reach significance.

OPCs are kept in dissociated culture in strictly defined medium and in an environment that differs to that *in vivo*. To provide conditions more similar to those *in vivo*, we used organotypic cortical cultures from NG2creBAC:ZEG double transgenic mice in which EGFP is expressed in OPCs and their progeny. To examine proliferation of mouse OPCs in slice cultures, we limited our treatments to compounds 002 and 006. These 2 compounds are positional isomers that differ only in the position of methoxy residue. However, they exert different potency in terms of Pdgfra transcription and proliferation of Oli-neu cells and OPCs. Cortices from NG2creBAC:ZEG mice were dissected and incubated for 7-9 days. Compounds were added during the last 2 days of incubation while EDU was added during the last 5h of treatment. Treatment with compound 002 resulted in a decrease in the percentage of EDU+ OPCs, which reached significance only in white matter and only at the higher concentration applied. Although compound 006 also caused a decrease in the percentage of proliferating OPCs, this decrease was not significant (Figure 15. C and D).

PDGF is the best known and most potent mitogen for OPCs. Addition of exogenous PDGF was shown to increase proliferation of OPCs in white matter, but not gray matter in organotypic slice cultures (Hill et al, 2013). We wanted to examine whether guanidine compounds would block the effect of exogenous PDGF. For this purpose, we added PDGF during the last 2 days of incubation to the final concentration of 50 ng/ml, as this was the amount that has been shown to cause a plateau in OPC proliferation in slice cultures. As expected, addition of PDGF resulted in an increase in proliferation in white matter, but not gray matter (Hill et al, 2013). However, when PDGF was applied together

with compounds 002 and 006, PDGF-induced proliferation was inhibited. Compound 002 was more potent and caused significant reduction in the percentage of EDU+ cells in white matter even at low concentration of concentration of 1 μM, while treatment with compound 006 resulted in inhibition of PDGF-induced proliferation only at higher concentration of 10 μM. At 10 μM, compound 002 inhibited proliferation of gray matter OPCs as well (Figure 15. C and D).

In our experiments with slice cultures, we also observed that OPCs were not the only cells which proliferation was inhibited by guanidine compounds. Other cell types, which we did not characterize, also incorporated EDU under control conditions and upon addition of exogenous PDGF. EDU incorporation was dramatically reduced upon treatment with guanidine compounds. This suggests that the effect our compounds exert is not specific for oligodendrocyte-lineage cells.

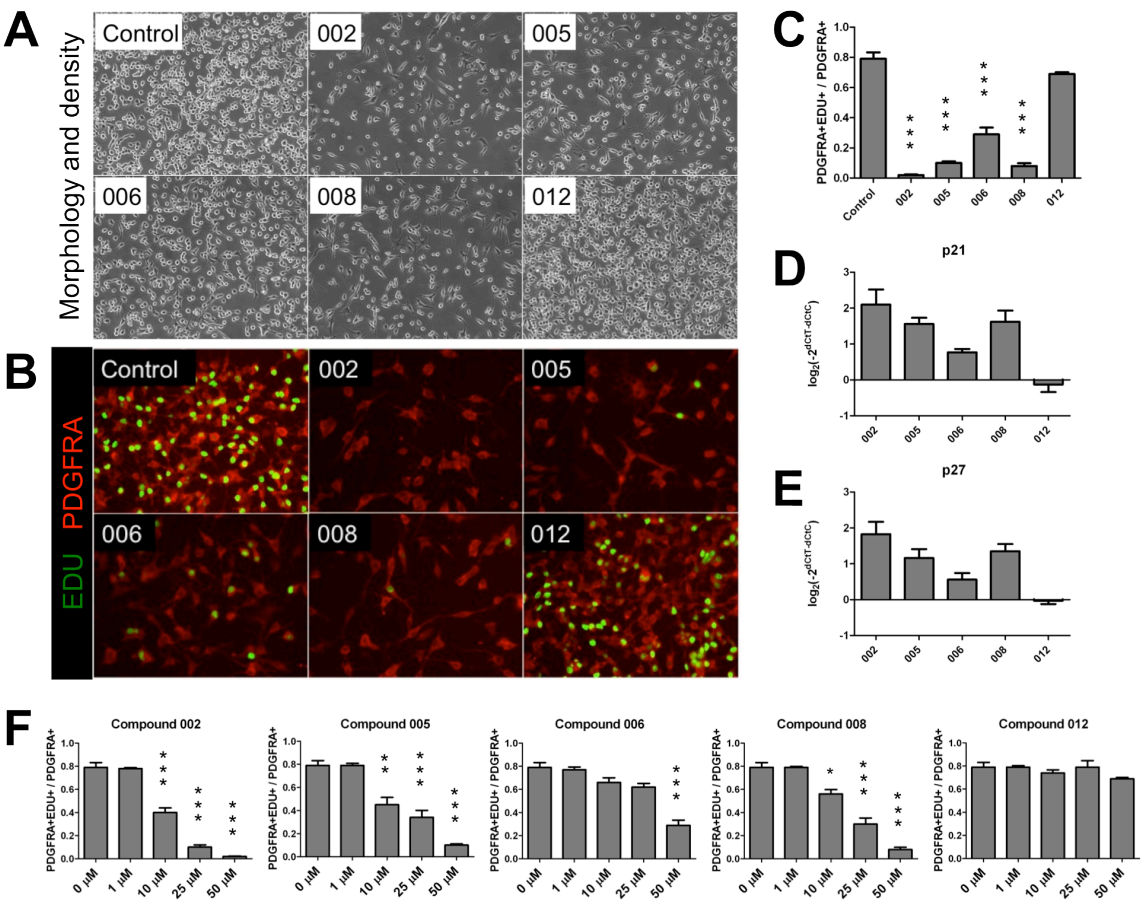
A
Morphology and density
Control
002
005
006
008
012
B
EDU PDGFRA
Control
002
005
006
008
012
C
PDGFRA+EDU+ / PDGFRA+
Control
002
005
006
008
012
D
p21
E
p27
F
Compound 002
Compound 005
Compound 006
Compound 008
Compound 012
PDGFRA+EDU+ / PDGFRA+
0 µM
1 µM
10 µM
25 µM
50 µM

Figure 12. Changes in Oli-neu cell density and proliferation upon treatment with guanidine compounds. Oli-neu cells were treated with guanidine compounds for 2 days at 50 μM with addition of EDU during the last 5h of treatment. We observed a striking difference in cell density (A). Oli-neu cells were fixed and stained for PDGFRA (B). EDU+PDGFRA+ cells among all PDGFRA+ cells were quantified (C). Upon 2-day treatment of Oli-neu cells with guanidine compounds, RNA was extracted. qPCR results show an increase in the transcript levels of p21 and p27 (D and E). (F) Quantification of PDGFRA+EDU+ among all PDGFRA+ Oli-neu cells after treatment with guanidine compounds at different concentrations. * $p < 0.05$ relative to control, ** $p < 0.01$ relative to control, *** $p < 0.001$ relative to control, one-way ANOVA with Bonferroni posthoc analysis. Error bars are standard errors of the mean.

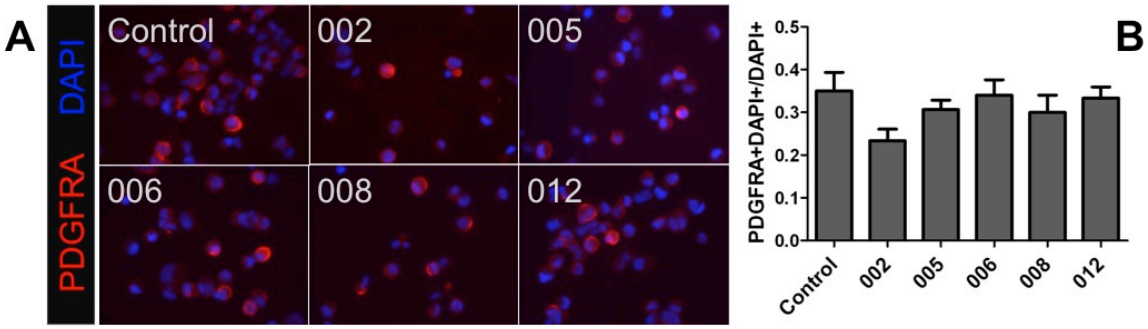
A
PDGFRA
DAPI
Control
002
005
006
008
012
B
PDGFRA+DAPI+/DAPI+
0.5
0.4
0.3
0.2
0.1
0.0
Control
002
005
006
008
012

Figure 13. Guanidine compounds do not cause decrease in PDGFRA expression or receptor internalization. Oli-neu cells were treated with guanidine compounds for 2 days at 50 μM when live staining for PDGFRA was performed (A). Quantification of the cells expressing PDGFRA at high level among all cells. Error bars are standard deviations of the mean, no statistical significance was found, one-way ANOVA with Bonferroni posthoc analysis.

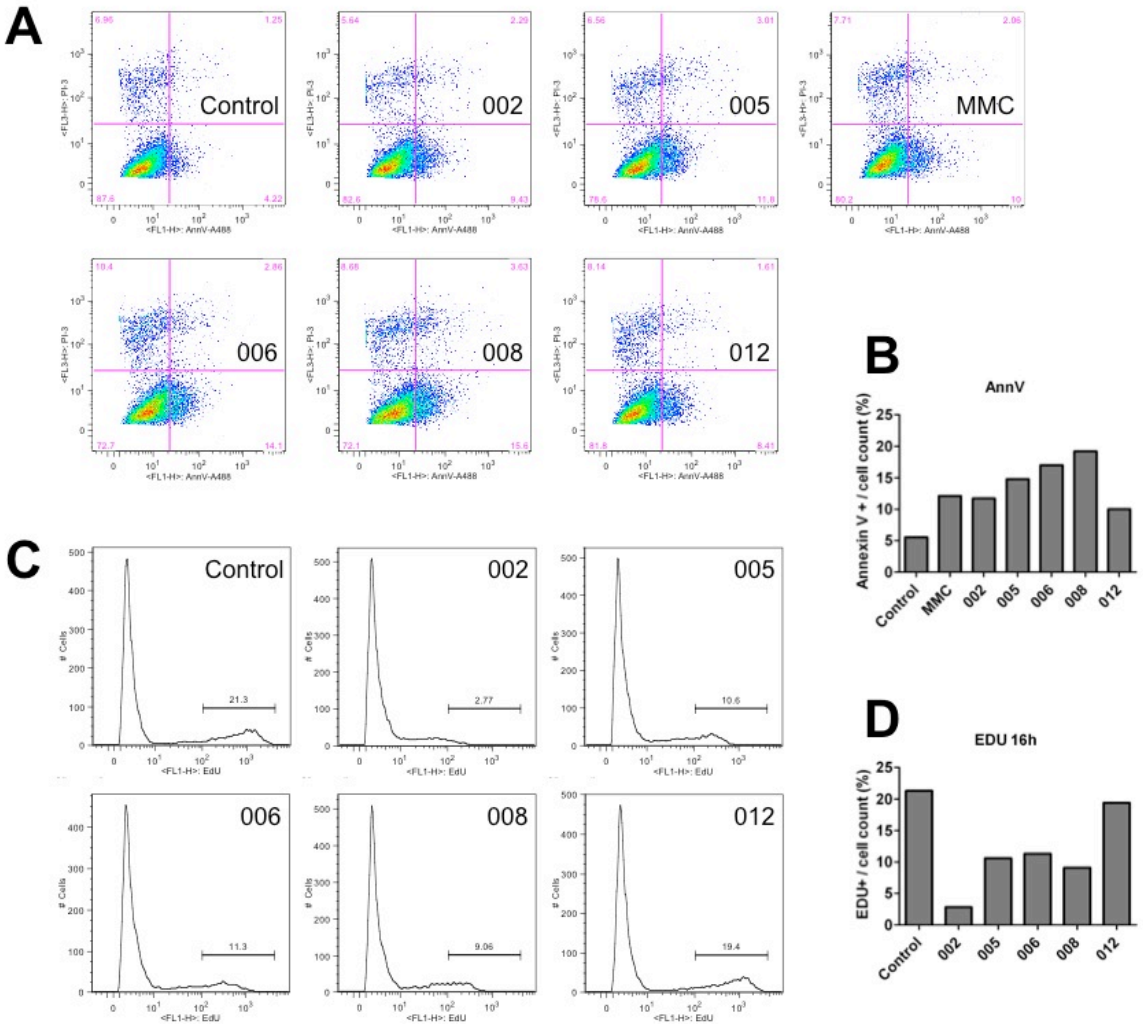
A
Control
002
005
MMC
006
008
012
B
AnnV
Annexin V + / cell count (%)
Control
MMC
002
005
006
008
012
C
Control
002
005
006
008
012
21.3
2.77
10.6
11.3
9.06
19.4
D
EDU 16h
EDU+ / cell count (%)
Control
002
005
006
008
012

Figure 14. Decrease in Oli-neu cells density upon treatment with guanidine compounds can be partially attributed to citotoxicity. Oli-neu cells were treated with guanidine compounds for 16h when they were stained for Annexin V and PI. Annexin V+ and PI+ cells were counted using flow cytometry (A). The percentage of apoptotic cells, identified by Annexin V labeling, was quantified. All compounds caused an increase in the percentage of apoptotic cells by at least 2 fold (B). We also examined changes in proliferation in Oli-neu cells treated with guanidine compounds for 16h. Oli-neu cells that incorporated EDU were counted using flow cytometry. Histograms are showing the number and percentage of EDU+ cells (C). Quantification (D) shows that compounds 005, 006 and 008 caused approximately 2-fold decrease, while treatment with 002 resulted in close to 8-fold decrease in the percentage of proliferating cells.

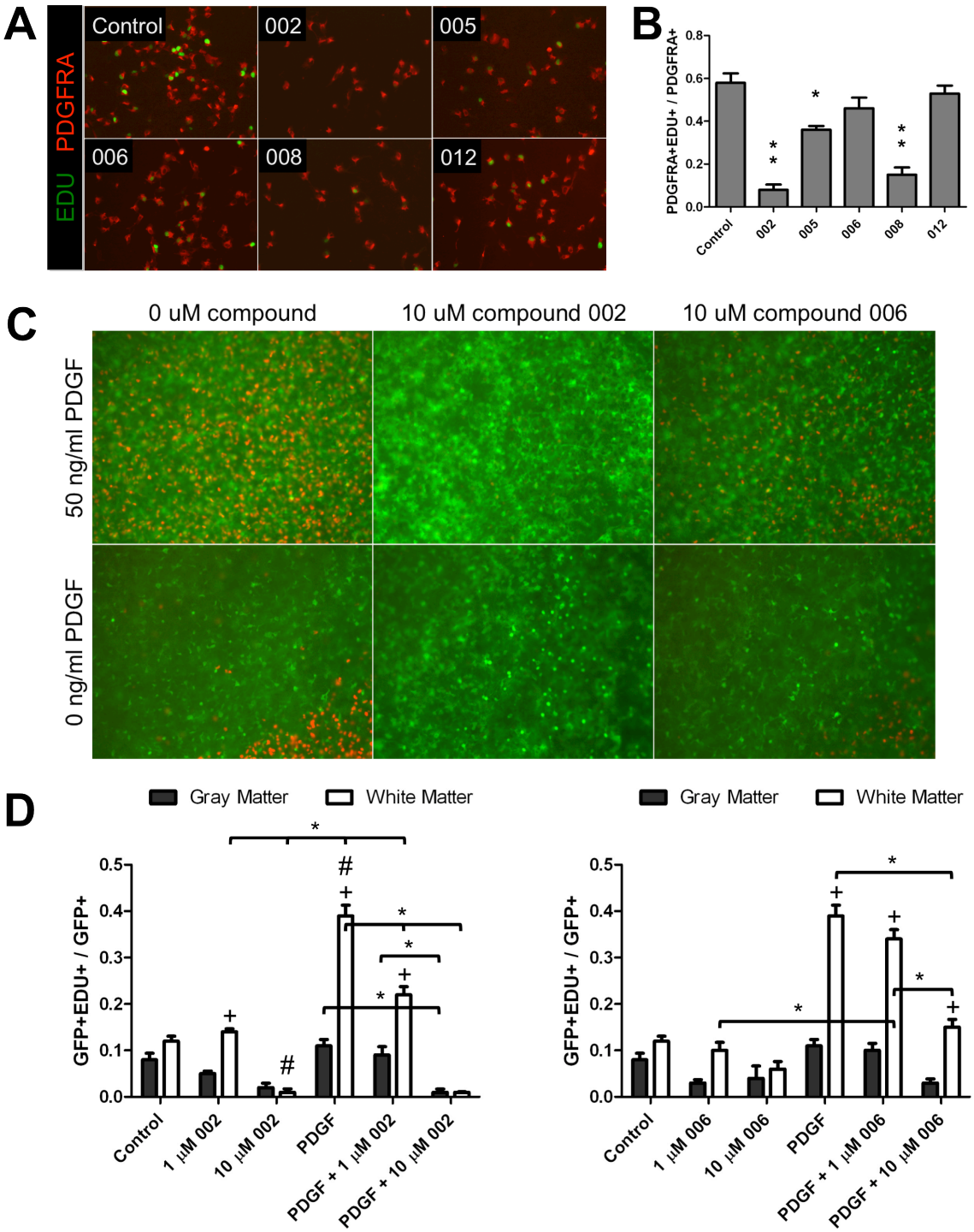
A
Control
002
005
006
008
012
EDU PDGFRA
B
PDGFRA+EDU+ / PDGFRA+
0.8
0.6
0.4
0.2
0.0
Control
002
005
006
008
012
C
0 uM compound
10 uM compound 002
10 uM compound 006
50 ng/ml PDGF
0 ng/ml PDGF
D
Gray Matter
White Matter
GFP+EDU+ / GFP+
0.5
0.4
0.3
0.2
0.1
0.0
Control
1 μM 002
10 μM 002
PDGF
PDGF + 1 μM 002
PDGF + 10 μM 002
Gray Matter
White Matter
GFP+EDU+ / GFP+
Control
1 μM 006
10 μM 006
PDGF
PDGF + 1 μM 006
PDGF + 10 μM 006

Figure 15. Changes in primary mouse OPC proliferation after treatment with guanidine compounds. After 1-day treatment of primary mouse OPCs with guanidine derivatives at 50 μM, OPCs were stained for PDGFRA and EDU (A). (B) Quantification of the PDGFRA+ cells that incorporated EDU during the last 5h of treatment among all PDGFRA+ cells. Compounds 002, 005 and 008 caused a significant decrease in the percentage of proliferating cells, 002 being the most potent. * $p < 0.01$ relative to control, ** $p < 0.001$ relative to control, one-way ANOVA with Bonferroni post-hoc analysis. Error bars are standard errors of the mean. (C) Organotypic cortical slice cultures obtained from NG2creBAC:ZEG double transgenic mice were exposed to guanidine compounds for 2 days with and without addition of exogenous PDGF. EDU was added during the last 5h of treatment when slices were fixed and stained for EDU (red). (D) Quantification of the GFP+ cells that incorporated EDU during the last 5h of treatment among all GFP+ cells. + $p < 0.001$ relative to gray matter, # $p < 0.001$ relative to respective control, * $p < 0.001$, two-way ANOVA with Bonferroni post-hoc analysis.

Guanidine compounds do not inhibit proliferation of glioblastoma-derived cell lines, primary astrocytes and HEK cells

To further address the question whether compounds-induced inhibition of proliferation is specific for PDGFRA-expressing cells, we tested the effects of guanidine compounds on several other cell types. All treatments were done as previously. Cells were treated with 50 μM compounds for 2 days with the addition of EDU during the last 5h of treatment. Then, they were fixed and stained for EDU and the percentage of EDU+ cells among all cells, identified by DAPI, was quantified.

Astrocytes and HEK 293 cells do not express PDGFRA. Therefore, we hypothesized that guanidine compounds would not inhibit proliferation of these two cell types. As expected, treatment with none of the compounds resulted in a decrease in the percentage of EDU+ cells (Figure 16. A).

Glioblastoma-derived cell lines A172, U251 and T98G differ in transcript levels of PDGFRA and PDGFRB. Compared to the remaining two cell lines, U251 has the highest level of PDGFRA, while A172 has the highest level of PDGFRB transcript. T98G has moderate levels of both PDGFRA and PDGFRB. Therefore, U251 most closely resembles OPCs (Lokker et al, 2002). We hypothesized that, upon treatment with guanidine compounds, U251 would show the most robust decrease in the percentage of EDU+ cells, T98G would show modest decrease, while A172 would have little or no response. Quantification of the percentage of EDU+ cells showed that all 3 glioblastoma-derived cell lines respond in a manner that resembles that of Oli-neu cells. However, contrary to that of Oli-neu cells, the observed decrease in proliferation was modest for all cell lines. None of the compounds significantly reduced proliferation of U251 and T98G.

Unexpectedly, compounds 002 and 005 caused a significant decrease in the proliferation of A172. However, the decrease in proliferation was less robust compared to the one observed with Oli-neu cells or primary mouse OPCs (Figure 16. B). We also wanted to examine whether guanidine compounds would alter PDGFRA transcription in glioblastoma-derived cell lines. Our qPCR results showed that the change in PDGFRA or PDGFRB transcript levels upon treatment with guanidine compounds was marginal and it did not reach 2 fold (Figure 16. C).

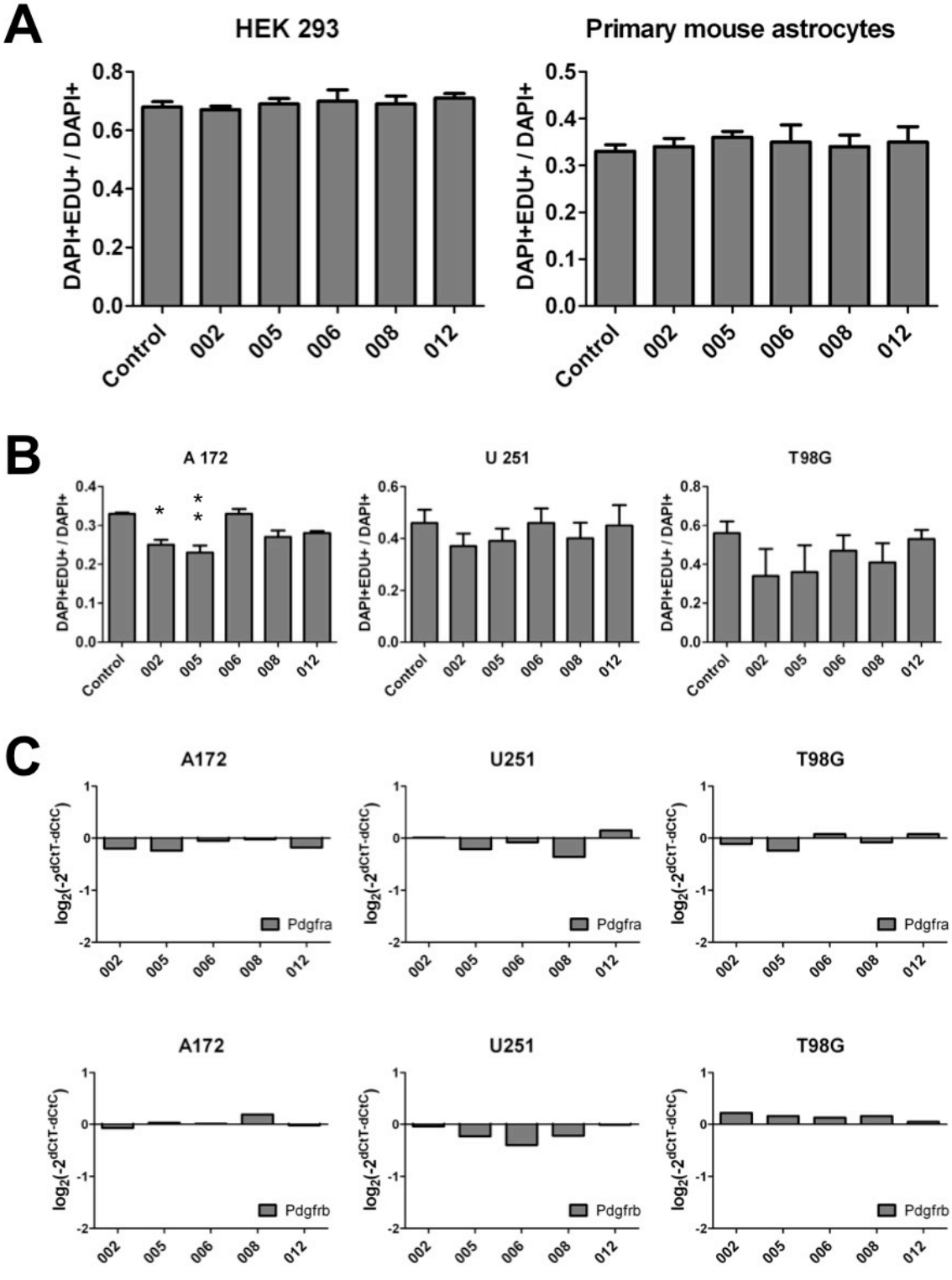
A
HEK 293
Primary mouse astrocytes
DAPI+EDU+ / DAPI+
Control
002
005
006
008
012
B
A 172
U 251
T98G
C
A172
U251
T98G
Pdgfra
Pdgfrb

Figure 16. Guanidine compounds inhibit proliferation of A172, but do not inhibit proliferation of U251, T98G, HEK 293 cells and primary mouse astrocytes. Cells were treated with guanidine compounds for 2 days when the percentage of EDU+ cells among all cells was assessed. Bar graphs show quantification of cells that incorporated EDU during the last 5h of treatment. Primary mouse astrocytes and HEK 293 cells did not show a change in proliferation (A). The three glioblastoma-derived cell lines showed proliferation changes similar to those of Oli-neu cells and OPCs. However, the decrease in proliferation was not significant in any circumstances, except when cell line A172 was treated with compounds 002 and 005 (B). * $p < 0.05$ compared to control, ** $p < 0.01$ compared to control, one way ANOVA with Bonferroni post hoc analysis. (C) We analyzed the changes in Pdgfra and Pdgfrb transcript levels after glioblastoma-derived cell lines had been treated with guanidine compounds for 2 days. Changes in transcription in all three cell lines were marginal and less than 2-fold under all conditions examined. dCtC = delta Ct of DMSO control; dCtT = delta Ct upon treatment with compounds.

Discussion

Since signaling through PDGFRA is critical for OPC proliferation, we hypothesized that downregulation of Pdgfra transcription would inhibit proliferation of PDGFRA-expressing cells. As expected, the percentage of proliferating Oli-neu cells and primary mouse OPCs was robustly reduced upon treatment with guanidine compounds. Decrease in cell proliferation might be due to toxicity since treatment with guanidine compounds resulted in an increase in the percentage of apoptotic Oli-neu cells by approximately 2 fold. This might be the case for compounds 005 and 008 where decrease in proliferation under the same conditions was also 2 fold. However, decrease in proliferation cannot be attributed solely to toxicity for compound 002. Although 16h treatment with 002 resulted in 2.1-fold increase in the percentage of apoptotic cells, decrease in proliferation observed under the same conditions was 7.7 fold.

Decrease in OPC proliferation is also evident in organotypic slice cultures. Only high dose of more potent compound 002 was able to inhibit proliferation under basal conditions. However, when compounds were added together with PDGF, they inhibited PDGF-induced proliferation of OPCs. Low dose of compound 002 and high dose of compound 006 were required for this effect. Upon 2-day treatment of Oli-neu cells, PDGFRA protein was shown to be present on the cell surface. We did not examine the expression of PDGFRA on the surface of OPCs in the slice culture. However, since slice cultures were treated with compounds for 2 days, it is possible that primary OPCs showed continues expression of PDGFRA at this time as well. This suggests that PDGFRA is not the primary target for guandince compounds. This is further supported by our observation that proliferation of GFP- cells, which are unlikely of oligodendrocyte lineage, was also inhibited by guanidine compounds.

Both Oli-neu cells and OPCs express PDGFRA, while HEK 293 cells and astrocytes do not, so it is possible that PDGFRA downregulation is at least in part responsible for the observed decrease in proliferation. This appears to contradict the result obtained from a similar experiment that utilized glioblastoma-derived cell lines. However, we need to keep in mind that glioblastoma-derived cell lines used in our study might not necessarily be derived from oligodendrocyte lineage cells. It is possible that they express other growth factor receptors and that signaling through these pathways overrides the effect of compounds. Another possibility is that target molecule for compounds that regulates Pdgfra transcription is not expressed by glioblastoma-derived cell lines. This is supported by the fact that treatment with guanidine compounds did not result in a decrease in PDGFRA mRNA level in glioblastoma-derived cell lines.

Chapter 6 – Summary, discussion and conclusions

Summary

OPCs are the major source of oligodendrocytes during embryonic development and adulthood, but also in pathological conditions. Since OPCs are the largest pool of cycling cells that are readily available for recruitment upon myelin injury, they represent an attractive target for therapies for demyelinating disorders. Signaling through PDGFRA is the key mechanism that regulates OPC proliferation and their differentiation into mature oligodendrocytes. Aberrant PDGFRA expression has been implied in the occurrence of CNS malignancies and inhibition of PDGFRA signaling could be a valuable addition to the current treatments of certain CNS cancers. Small molecules are putative candidates for the therapeutic interventions because they can be designed to be highly specific for their targets to be able to cross blood brain barrier. Identification of such compounds might provide a novel direction in the drug design for nervous system disorders. Since PDGFRA is critical for regulation of OPC proliferation and differentiation, identification of compounds that target regulators of its expression and/or signaling could provide a significant contribution to treatments of demyelinating disorders and CNS malignancies.

The purpose of this study was to identify small molecule compounds that downregulate Pdgfra transcription in OPCs. We hypothesized that the inhibition of transcription of this particular receptor would result in inhibition of OPC proliferation and stimulation of their differentiation into mature oligodendrocytes.

In the primary screen, we indeed identified 7 compounds that downregulated PDGFRA-luc activity in a dose-response manner. Guanidine compounds (39D11, 40A10, 40B10

and 39E11) also downregulated endogenous Pdgfra transcription, assessed by qPCR, while structurally non-related compound 42E8 did not. Downregulation of transcription was specific for Pdgfra and not for other RTKs expressed by OPCs. Guanidine compounds also inhibited proliferation of Oli-neu cells and primary mouse OPCs, assessed by EDU labeling, but did not inhibit proliferation of primary mouse astrocytes, HEK 293 cells and glioblastoma-derived cell lines. They did not, however, promote differentiation of primary rat OPCs into mature oligodendrocytes.

Discussion

Luciferase assay is a powerful tool widely used for high-throughput screening purposes. However, studies using luciferase assay are most commonly done on stably transfected cell lines where firefly luciferase is driven by gene's endogenous regulatory sequences. Using only a portion of Pdgfra gene to drive reporter expression was a disadvantage in our primary screen, which could be overridden by stable transfection of Oli-neu cells.

To identify compounds that promote differentiation, a valuable addition to the technique would be to use a second plasmid for the primary screen, which contains regulatory elements of an early oligodendrocyte gene driving reporter transcription. Cnp would be a good choice for this purpose, since it gets upregulated when Oli-neu cells are treated with differentiation inducers (Joubert et al, 2010). Differential regulation of OPC-specific and early oligodnedrocyte gene in differentiating Oli-neu cells would prevent selection of false positives.

Oli-neu cells are immortalized cell line and, in our hands, they were less responsive to differentiation inducers than primary OPCs. At the time when screening was preformed, we could not obtain sufficient numbers of viable OPCs and therefore chose to use Oli-

neu cells instead. Using primary OPCs from one of the numerous transgenic mouse lines available that have florescent reporter driven by regulatory sequences of OPC- and oligodendrocyte-specific genes might provide better biological system to detect differentiating OPCs.

SAR analysis of guanidine compounds revealed residues that are responsible for compounds' potency. Two structural modifications that resulted in the highest potency of guanidine compounds are: presence of methoxy group at carbon 2 of the benzyl ring and elongation of the carbon chain linking guanidine group and benzyl ring. Such modifications might exert their effect by increasing compound lipophylicity, stabilizing compound-target interaction or facilitating the interaction between the pharmacophore and the receptor. A combination of these and other modifications might further improve compound potency (Figure 9). Once the top candidates are ready to be tested for their biological effect *in vivo*, replacement of hydrogen atoms with fluorine might be a valuable modification. Presence of fluorine may extend compound's half-life without affecting the biological activity if such replacement is done at the site of metabolic oxidation.

We did not perform thorough analysis of a relationship between compound structure and toxicity. Although our top candidate's biological effect greatly surpasses its toxicity, guanidine compounds were found to be at least partially cytotoxic for Oli-neu cells (Figure 14). Further analysis needs to be conducted to identify residues that are responsible for compounds' toxicity and which structural modifications can be made to decrease it.

Guanidine compounds dramatically inhibited Pdgfra transcription and proliferation of Oli-neu cells. They did not, however, promote their differentiation into oligodendrocytes. Together with the data obtained from experiments performed on primary OPCs, these results suggest that downregulation of Pdgfra transcription and inhibition of proliferation is not sufficient for OPC differentiation to occur under the conditions we used. This is consistent with studies that suggest cell cycle withdrawal is necessary, but not sufficient to induce OPC maturation and that control of oligodendrocyte differentiation involves other factors that operate between cell cycle exit and differentiation (Casaccia-Bonefil et al, 1999). Identification of compounds that target such factors will contribute in succeeding in our goal to use small molecule compounds as additional therapy for demyelinating disorders.

Together with the correlation between downregulation of Pdgfra transcription and inhibition of Oli-neu cell proliferation, lack of compounds' effect on astrocytes and HEK 293 cells, which do not express PDGFRA, implies that Pdgfra is a direct/indirect target of guanidine compounds. This is further supported by the fact that neither PDGFRA transcription nor proliferation of two glioblastoma-derived cell lines was inhibited by guanidine compounds. However, since compounds 002 and 005 inhibited proliferation of A172 but did not downregulate transcription of PDGFRA or PDGFRB in these cells, this leads to speculation that PDGF receptors are not the only targets for guanidine compounds. This is consistent with the biological effect of compound 006, which did not alter Pdgfra transcription, but did inhibit proliferation of Oli-neu cells. Future research, aimed toward identifying direct target/s of guanidine compounds, will contribute to the growing knowledge of PDGFRA regulation and its role in OPC differentiation.

It was encouraging to discover that the effect of guanidine compounds is somewhat cell-type specific, since proliferation of Oli-neu cells and primary OPCs was inhibited by guanidine compounds, but that of astrocytes and HEK 293 cells was not. However, we also observed a decrease in the percentage of EDU+ cells that were not GFP+ in our slice culture system, which implies guanidine compounds act on a broader spectrum of cell types. Although proliferation of glioblastoma-derived cell lines was not efficiently inhibited, this does not necessarily mean that cells derived from other types of cancers would respond similarly to the three cell lines examined. It would be interesting to find out whether cells obtained from biopsies of patients with gliomas would be more responsive. In addition, characterization of cell types that these compounds are specific for might provide us with a direction toward other types of cancers that can be potentially treatable.

To this date, the effect that N-methyl-N-benzyl guanidine or its derivatives have on proliferation of varios cell types has never been examined. In fact, to my best knowledge, none of the guanidine compounds identified in this study, have been examined in other types of bioassays. However, several other guanidine derviatvies have been tested in 2 other studies. These compounds were not, however, derivatives of our lead compound, N-methyl-N-benzyl guanidine, but of another lead, N-benzyl guanidine, which does not have methyl group on the nitrogen athom of the guanidine. Four compounds were candidates in the high throughput screening study related to obesity and diabetes, aimed towards identifying small molecule compounds that would alieveiate these disorders. These compounds were: N-(2-chloro-benzyl)guanidine, N-(2-methoxy-benzyl)guanidine, N-(3-methoxy-benzyl)guanidine and N-(4-methoxy-benzyl)guanidine. They were identified as top candidates from the screening. However, eventually, they were shown to be inactive. In a separate study, aimed towards

identifying inhibitors for substrates of cytochrome p450 in a high throughput screen, a different derivative of N-benzyl guanidine, N-(3-iodo-benzyl)guanidine, was identified and shown to be active. This compound has iodine as a substituent on the benzyl ring. Results of these studies are encouraging as they support our conclusions that presence of chlorine and methoxy groub on C2 and C3 of the benzyl ring might be an important modification. In addition, we should also consider using iodine as substituent as well.

Aberrant PDGF and PDGFR expression has been detected in various types of malignancies, including those occurring in the CNS (Hermanson et al, 1992; Di Rocco et al, 1998; Martinho et al, 2009; Ozawa et al, 2010; Heldin, 2013). PDGFRA is the second most frequently mutated RTK gene in glioblastoma (The Cancer Genome Atlas Research Network, 2008). Amplification is the most commonly observed PDGFRA gene alteration, while point mutations, deletions and gene rearrangements are rare and occur more frequently in glioma samples that already have PDGFRA amplification (Martinho et al, 2009; Verhaak et al, 2010; Ozawa et al, 2010).

Awareness of the PDGF signaling contribution to cancer growth has led to development of different types of inhibitors that are now under preclinical and clinical evaluation (Pietras et al, 2003; Heldin, 2013). Antibodies and soluble extracellular parts of the receptors can intervene with PDGF signaling by binding to PDGFs or PDGFRs, prevent their interaction or promote their degradation (Hawthorne et al, 2008; Shen et al, 2009). Although reasonably specific, these types of inhibitors are expensive and difficult to administer. Using small molecule compounds for cancer therapy has numerous advantages – they are potent, less expensive and easier to administer. Numerous studies have explored the effects of developing compounds and several compounds

were shown to be very promising in cancer treatment. However, none of these compounds is specific and they were all shown to inhibit other kinases (Morris and Abrey, 2010; Paulsson et al, 2011; Heldin, 2013). Imatinib is one of the first and most promising candidates used in the treatment of multiple cancers, such as chronic myelogenous leukemia and gastrointestinal stromal tumors (Iqbal and Iqbal, 2014). However, in addition to PDGFRA, imatinib inhibits two other RTKs, Abl and Kit, as well as serine/threonine kinase Raf (Heldin, 2013). The lack of specificity of imatinib and other small molecule compounds contributes to their side effects. In addition, imatinib was shown to have mild potency in clinical trials in glioma patients (Reymond et al, 2008), which might be attributable to its poor penetration through the blood brain barrier (Takayama et al, 2002; Senior, 2003).

In addition to the lack of imatinib's specificity, its action, as well as that of other compounds, is directed towards inhibiting PDGFRA kinase activity and has not been successful in treatments of gliomas. Targeting PDGFRA activity in its core, through regulation of its transcription, might prove to be more effective. Guanidine compounds discovered in our study were shown to be specific for Pdgfra transcription and not that of other tested RTKs, Fgfr1 and Fgfr3, and therefore represent promising candidates for future cancer therapies. In addition, their lower molecular weight might contribute to easier delivery to the CNS. Future research needs to be conducted to assess guanidine compounds' transportation and metabolism.

Concluding remarks

Glial cell dysfunctions are involved in the occurrence and development of many nervous system disorders. The dynamic of the disease progress is such that it often prevents the development of the diagnostic and therapeutic strategies applicable at each time point and in every location. One of the most important initiatives in the therapy today is development of treatments that are personalized and tailored to the biology and stage of the disease in the individual patients. Small molecules are putative candidates for therapeutic interventions in the brain because they can be designed to be highly selective, are able to cross blood brain barrier and can be conveniently modified to adjust to different conditions, such as different patients or pathological processes. Identification of such molecules may provide a novel direction in the drug design for devastating nervous system disorders, which can complement current treatments. PDGFRA signaling pathway is an attractive target for small molecules. Pdgfra transcription and proliferation of PDGFRA-expressing cells were found to be inhibited by compounds discovered in our study. How specific these compounds are and whether they can be used to inhibit PDGFRA signaling *in vivo* remains to be established.

Chapter 8 – References

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