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Critical data-based re-evaluation of minocycline as a putative specific microglia inhibitor

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Main Points:
- Minocycline is not a specific microglia inhibitor
- Minocycline has *bona fide* effects on neuroinflammation *in vivo*
- Data generated with minocycline are difficult to interpret

Key Words: microglia, minocycline, lack of specificity, inhibitor

ABSTRACT

Minocycline, a second generation broad-spectrum antibiotic, has been frequently postulated to be a "microglia inhibitor". A considerable number of publications have used minocycline as a tool and concluded, after achieving a pharmacological effect, that the effect must be due to "inhibition" of microglia. It is, however, unclear how this "inhibition" is achieved at the molecular and cellular level. Here we weigh the evidence whether minocycline is indeed a *bona fide* microglia inhibitor and discuss how data generated with minocycline should be interpreted.

Minocycline, a second generation tetracycline antibiotic

Minocycline is a semi-synthetic, broad-spectrum tetracycline antibiotic. It was first synthesized from natural tetracyclines in 1966 (Redin 1966) and approved by the federal drug administration (FDA) for the US in 1971. The bacteriostatic activity of tetracycline antibiotics (incl. minocycline) results from binding the bacterial 30S ribosomal subunit, blocking the attachment of aminoacyl-tRNA to the ribosome and thus preventing the addition of new amino acids to the nascent peptide chain (Chopra and Roberts 2001). Minocycline has a long serum half life of 11-18 hours, which is 2-3 times longer than the typical water-soluble tetracyclines (Agwuh and MacGowan 2006). Minocycline is the most lipid-soluble of the tetracycline family, affording it the greatest CNS penetrance in the group (Brain-to-serum ratio in dogs: minocycline 2.8; doxycycline 1.3; tetracycline 0.3) (Barza et al. 1975). While this is an obvious advantage for CNS indications, such as Lyme disease, it also is the underlying cause of CNS side effects such as dizziness, vertigo, ataxia and tinnitus (Fanning et al. 1977). These vestibular side effects are more common in women than in men, occurring with a frequency of up to 7/10 (Fanning et al. 1977). Minocycline has other well described peripheral and central side and because of multiple potential safety issues, the FDA added minocycline to its Adverse Event Reporting System (ARES) in 2009 effects (Balestrero et al. 2001; Cohen 2004; Davies and Kersey 1989; Fraser et al. 2012; Golstein et al. 1997; Gordon et al. 1995; Gough et al. 1996; Hanada et al. 2016; Hardman et al. 1996; Healy et al. 2009; Lander 1989; Lefebvre et al. 2007; Matsuura et al. 1992; Porter and Harrison 2003; Schlienger et al. 2000; van Steensel 2004; Weller and Klockgether 1998). That said, minocycline remains a mainstay drug for anti-bacterial therapy and has been taken safely and efficiently by millions of people.

Anti-inflammatory effects of tetracycline-based antibiotics

Early evidence for non-antibiotic properties of tetracycline antibiotics (e.g. tetracycline, doxycycline, minocycline, for structures see Fig.1) surfaced in dermatology and rheumatoid arthritis (Clark 1995; Eady et al. 1993; Galland 1995; Greenwald et al. 1992; Kloppenburg et al. 1996; Miyachi et al. 1986). By early 2016 more than 2850 papers on the anti-inflammatory properties of tetracycline antibiotics were listed in PubMed. The reported mechanisms include inhibition of T-cell activation, inhibition of neutrophil transmigration, inhibition of proinflammatory cytokine release, inhibition of nitric oxide release and augmentation of anti-inflammatory cytokine release (Table 1) (Bernardino et al. 2009; Bostanci et al. 2011; Cazalis et al. 2009; Celerier et al. 1996; Eady et al. 1993; Garrido-Mesa et al. 2013; Greenwald et al. 1992; Jain et al. 2002; Kloppenburg et al. 1996; Kloppenburg et al. 1995; Koistinaho et al. 2004; Milano et al.

1997; Miyachi et al. 1986; Ochsendorf 2010; Pang et al. 2012; Sadarangani et al. 2015; Sapadin and Fleischmajer 2006; Shapira et al. 1996; Toussirot et al. 1997). The list of effects is far from complete and one has to keep in mind that not all properties have been investigated for all tetracyclines. However, there seems to be consensus that the second generation tetracyclines (e.g. doxycycline, minocycline) have improved anti-inflammatory properties (Garrido-Mesa et al. 2013; Leite et al. 2011). In fact, based on the intriguing anti-inflammatory properties of the second generation tetracyclines, efforts are underway to engineer tetracycline-based molecules without antibiotic, but improved anti-inflammatory properties (Cazalis et al., 2009; Monk et al., 2011; Tilakaratne and Soory, 2014).

Coincidently, tetracyclines are also being used to regulated gene transcription in transgenic animals. In the 1990 the Tet-Off and Tet-On systems have been developed utilizing the Tet repressor DNA binding protein (TetR) from the tetracycline resistance operon of Escherichia coli transposon Tn10 (Furth et al. 1994; Gossen and Bujard 1992; Kistner et al. 1996). This system is used to regulate the expression of a target gene that is under transcriptional control of a tetracycline-responsive promoter element (TRE). These systems have found wide adaption for the temporal regulation of gene expression or deletion (Mansuy and Bujard 2000; Sakai 2014; Zhu et al. 2002b). While originally coined the “tetracycline regulated expression system”, most experiments are performed with doxycycline as the Tet-On system responds poorly to tetracycline, but well to doxycycline (Baron and Bujard 2000). If these systems are used to investigate an animal model where the phenotype is associated with an inflammatory component, it might be difficult to separate the the Tet-On/Off effect from the anti-inflammatory properties of doxycycline (See Table 1).

As of early 2016, about 750 papers in PubMed refer to minocycline’s anti-inflammatory effects. Of the three major tetracyclines (i.e. tetracycline, doxycycline, minocycline) minocycline is the best investigated in regards to anti-inflammatory effects (Table 1) (Garrido-Mesa et al. 2013; Ochsendorf 2010). However, while the effects on inflammation and immune cells (e.g. monocytes, macrophages, T-cells, neutrophils) are well described, the actual molecular mechanism by which minocycline exerts these effects are far less understood. Many molecules in pro- and anti-inflammatory signal transduction cascades have been suggested, such NF-κB, LOX-1, LPS-induced TNF-α factor (LITAF), Nur77, p38 MAPK, PI3K/Ak, PKC, IRF-1 and the inflammasome (Dunston et al. 2011; Kauppinen et al. 2014; Nikodemova et al. 2007; Pang et al. 2012). However, while the evidence for anti-inflammatory activity of minocycline is overwhelming, the molecular target(s) of minocycline mediating these effects still remain elusive.

Minocycline employed as a putative microglia inhibitor

The keywords "microglia AND minocycline" return over 500 hits from PubMed in early 2016. There is a large body of data showing anti-inflammatory effects of minocycline on microglial (patho)physiology *in vitro* as well as *in vivo*. Similar to effects on peripheral immune cells, typical effects reported include reduction in cytokine, prostaglandin and nitric oxide release, reduced proliferation and reduced staining for "activation" markers such as CD11b, MHC-II or Iba-1 (El-Shimy et al. 2015; Hanlon et al. 2016; Hou et al. 2016; Nikodemova et al. 2007; Papa et al. 2016; Scholz et al. 2015; Silva Bastos et al. 2011; Tikka et al. 2001; Tikka and Koistinaho 2001) and reviewed in (Zemke and Majid 2004). Based on the available data for minocycline as a CNS penetrant anti-inflammatory these data are not surprising. However, several publications have called minocycline a "selective microglia inhibitor" and have drawn conclusions on microglia involvement in animal models of disease solely based on the effects of minocycline (Cui et al. 2008; Huang et al. 2014; Kobayashi et al. 2013; Ledeboer et al. 2005; Mika et al. 2007; Osikowicz et al. 2009; Raghavendra et al. 2003). It should be self-evident, that minocycline is not selective for microglia, but affects peripheral immune cells as well. Therefore, an unequivocal attribution of the pharmacological effects to microglia, especially in the *in vivo* experiments is not possible. Furthermore, it is unclear what is being "inhibited". Microglia "activation"? In turn, are only "activated" microglia effected my minocycline? What about microglia "surveying" the tissue? It is becoming clear that microglia exist in different phenotypes (Biber et al. 2014; Hanisch 2013). Consequently, which phenotype(s) are "inhibited" by minocycline with which outcome? The term "microglia inhibition" seems as vaguely-defined as the term "microglia activation".

Minocycline effects on astrocytes, oligodendrocytes and neurons

In addition to its broad effects on immune cells, minocycline has been shown to have multiple effects on astrocytes, oligodendrocytes and neurons *in vitro* as well as *in vivo*. For example, minocycline (and doxycycline) reduces the release of the pro-inflammatory cytokines TNF-α, IL-6, and IL-8 from cultured rhesus monkey astrocytes (Bernardino et al. 2009). Minocycline reduced the number of hippocampal $GFAP^+$ cells in LPS challenged mice and in the mutant SOD1 model of amyotrophic lateral sclerosis (Hou et al. 2016; Keller et al. 2011). Minocycline also protected oligodendrocytes and oligodendrocyte precursors against hypoxic and traumatic injury *in vitro* and *in vivo* (Scheuer et al. 2015; Schmitz et al. 2012; Stirling et al. 2004; Yune et al. 2007). There is a plethora of reports of neuroprotection and direct effects on neurons by tetracyclines including minocycline (reviewed in (Domercq and Matute 2004).

However, the effects of minocycline are not always positive. Several publications report neurotoxic effects of minocycline (Arnoux et al. 2014; Diguet et al. 2004a; Diguet et al. 2004b; Diguet et al. 2003; Tsuji et al. 2004; Yang et al. 2003). Of course, in the absence of proof positive, one has to acknowledge that the effects on astrocytes, oligodendrocytes and neurons, especially *in vivo* could be indirect. By the same token, however, the reported effects attributed to "microglia inhibition" could be indirect as well. For example, improved neuronal outcomes, would most likely be accompanied by less "activation" of surrounding microglia. Because of the uncertainty which cell(s) or networks are affected by minocycline, it only seems prudent to interpret minocycline *in vivo* data in regards to the cell type affected with the outmost caution.

Minocycline in clinical trials targeting microglia

Based on encouraging data on minocycline's broad anti-inflammatory effects, over 150 clinical trials have been registered at clinicaltrials.gov (early 2016) for the use of minocycline as standalone or adjunctive therapy in indications ranging from atrial fibrillation, to Angelman Syndrome and schizophrenia (US-National-Institutes-of-Health). In the last decade minocycline has received considerable attention for CNS applications reviewed (Garrido-Mesa et al. 2013; Zemke and Majid 2004). Of the currently registered trials, ten specifically refer to a "microglia activation" mechanism in diseases like hypertension, opioid-induced hyperalgesia, and schizophrenia. Registration in clinicaltrials.gov is voluntary and other pilot trials for minocycline have been reported for Parkinson's disease, Huntington's disease and Multiple sclerosis (Bonelli et al. 2004; Chen et al. 2011; Huntington Study Group 2010; Investigators 2006; Thomas et al. 2004; Zabad et al. 2007; Zhang et al. 2008). While many of the targeted diseases have a *bona fide* microglia/neuroinflammation component (Garden and Möller 2006; Möller 2010) it is not always obvious which effect of minocycline (anti-inflammatory, neuroprotective, etc.) the trials are aiming to leverage.

There is at least one CNS disease where minocycline is contraindicated. Based on positive effects in rodent models of amyotrophic lateral sclerosis (ALS) (Kriz et al. 2002; Van Den Bosch et al. 2002; Zhu et al. 2002a), and promising phase I/II clinical trials (Gordon et al. 2004; Pontieri et al. 2005), a randomized Phase III clinical trial was run in ALS patients. Unexpectedly, disease progression in ALS patients given minocycline was faster than the control group (Gordon et al. 2007). This effect, however, was not dose-dependent as patients on low and high doses fared equally unfavorable compared to controls (Gordon et al. 2007). This negative effect was later replicated in a mouse model of ALS where, in contrast to prior studies, minocycline treatment was started after disease onset (Keller et al. 2011).

Minocycline is not a selective microglia inhibitor

The anti-inflammatory activity of minocycline (and other tetracyclines) is well documented and undisputed. However, whether this activity can be solely attributed to a “microglia inhibitory effect” seems rather unlikely. The data presented here should make it clear that any activity attributed to minocycline has multiple potential cellular targets and a still unknown set of molecular targets. As such, data generated with minocycline need to be interpreted with caution. In the worst case, there might be experiments where the reported “improvements” in animal models are in fact due to the primary activity of the agent - i.e. antibacterial activity - in animals exposed to (unintentional) bacterial challenges. In the best case, the well documented, “anti-inflammatory” or “neuroprotective” activity of minocycline, coupled with its high CNS penetrance, might exerts the effect “somewhere” in the CNS. Regardless of the actual activity and target, this might be good news for patients in the long run. When minocycline, a drug with a well-documented record in the clinic, holds up in human trials, it could enable a quick entry into clinical practice for the benefit of patients. Nevertheless, the learnings from the ALS clinical trial should give the most endeavoring mind pause. In the preclinical area, data generated with minocycline, should not be misconstrued as a proof of microglial involvement. Instead, such preclinical data should be seen as document of minocycline’s undisputed plethora of anti-inflammatory, anti-apoptotic and neuroprotective properties.

Conflict of interest

TM, ED, RGWS, PDW were full time employees of Lundbeck Research USA.

FB, AB are full time employees of Janssen Pharmaceutica.

ZAH is a full time employee of Pfizer.

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Tables:

Table 1: Anti-inflammatory effects of selected tetracyclines. + indicates published data supporting anti-inflammatory effect of a given drug. CAVE: Absence of proof however, is not proof of absence of the effect. (Bernardino et al. 2009; Bostanci et al. 2011; Cazalis et al. 2009; Celerier et al. 1996; Eady et al. 1993; Garrido-Mesa et al. 2013; Greenwald et al. 1992; Jain et al. 2002; Kloppenburg et al. 1996; Kloppenburg et al. 1995; Koistinaho et al. 2004; Milano et al. 1997; Miyachi et al. 1986; Ochsendorf 2010; Pang et al. 2012; Sadarangani et al. 2015; Sapadin and Fleischmajer 2006; Shapira et al. 1996; Toussirot et al. 1997).

Figures

Figure 1: Chemical structures of tetracycline, doxycycline and minocycline.