

# 1 Neuroprotective effects of tibolone 2 during astrocytic metabolic 3 inflammation: a network based 4 approach

## 5 Abstract:

### 6 1.1. Introduction

#### 7 Astrocyte-Neuron Metabolic Relationships

8 Astrocytes are the most abundant cells in the human brain and play important roles in  
9 the central nervous system (CNS) [1]. They are highly associated to several homeostatic fun-  
10 ctions such as glutamate, ion, and water homeostasis, energy storage in the form of glycogen,  
11 synapse formation and remodeling, defense against oxidative stress, scar formation, tissue  
12 repair and modulation of synaptic activity via the release of gliotransmitters [2]. Astrocytes  
13 metabolize glucose in anaerobic way to produce lactate, which is released to neurons through  
14 monocarboxylate transporters [3]. Lactate is used in neurons as an energy substrate after  
15 its conversion to pyruvate and subsequently to ATP via oxidative phosphorylation [4]. As-  
16 trocytes play an important role in glutamate mediated synaptic activity [5]; according to the  
17 astrocyte–neuron lactate shuttle model, astrocytes respond to glutamate induced activation  
18 by increasing their rate of glucose uptake and the release of lactate into the extracellular spa-  
19 ce, increasing the lactate available to be used by neurons to supply their energetic needs [6].  
20 Glutamate is uptaked by astrocytes through the glutamate aspartate transporter and glial  
21 glutamate transporter-1, inducing events that involves the activation of  $\text{Na}^+\text{--K}^+\text{--ATPase}$   
22 and maintaining extracellular glutamate at homeostatic levels [7]. Part of incorporated glu-  
23 tamate is converted to glutamine through glutamine synthetase, which is only associated to  
24 glial cells and released to neurons using electroneutral systems-N transporters coupled to  
25  $\text{Na}^+$  and  $\text{H}^+$  [8]. In neurons glutaminase enzyme converts glutamine back into glutamate  
26 which can be used again for neurotransmission or metabolized into the neuronal Krebs cycle  
27 [9]. Astrocytes release many other substances related to synaptic transmission [10]. However  
28 D-serine, a neurotransmitter that act as a coagonist with glutamate at NMDA receptors is

one of the most important [5]. Due only glial cells can synthesize serine, all available D-serine at synapsis is associated to be primarily produced and secreted by astrocytes [8]. D-serine is synthesized in astrocytes by serine racemase from L-serine [11]. Additionally to these energy and synaptic support associated functions, astrocytes also play an important role in the reduced glutathione (GSH) metabolism of the brain [12]. GSH is the major cellular antioxidant and plays an important neuroprotective role [13]. Cellular GSH levels are closely correlated with cell survival under adverse conditions [14]. GSH is synthesized from glutamate, cysteine, and glycine and release directly from Astrocytes through GSH transporters ion-independent, net transport is concentration-gradient dependent [15].

This strong metabolic cooperation between astrocytes and neurons allows to predict that even an small astrocytic dysfunction might cause and/or contribute neurodegenerative processes.

## Inflammation

Inflammation is a biological response to injuries, metabolic disorders or infections and its dysregulation induce many complex diseases. In brain, inflammatory response acts as a defense mechanism against any threat to homeostatic state inducing changes in glucose metabolism and release of proinflammatory factors. Inflammation responses in CNS are mediated by glial cells that acquire reactive phenotypes to participate in repair mechanisms. Astrocytes, as glial cells are highly sensitive cells to inflammatory mediators, they respond to inflammation through a complex reaction named astrogliosis. During astroglyosis, glial cells generally associated to several beneficial activities in the CNS, also act as a source of inflammatory mediators and as generators of reactive oxidant species (ROS) that have the potential to damage neurons. Astrogliosis is characterized by a low regulation of mitochondrial dynamics that result in mitochondrial failure. Mitochondrial failure induces the deregulation of  $\text{Ca}^{2+}$  homeostasis and increased ROS generation, both of which are linked to neurotoxicity. At metabolic level, inflammatory process has been associated to an increase of free saturated fatty acid in comparison with healthy conditions in some brain tissues. The increase of free saturated fatty acid induce metabolic inflammation, a response associated with the induction of diverse intracellular stresses, such as mitochondrial oxidative stress, ER stress, and autophagy defects. Lipid excess in metabolic inflammation activates hypothalamic  $\text{IKK}\beta$  and  $\text{NF-}\kappa\beta$  signaling pathways, which ultimately impairs hypothalamic leptin and insulin signaling and further triggers the synthesis and release of increased amounts of ROS and proinflammatory cytokines ( $\text{TNF-}\alpha$  and IL-6) from glial cells to sustain the neuroinflammatory state. Enhanced ROS generation by reactive glial cells trigger mitochondria dysfunction in neuron, which induces neuronal apoptosis, the prerequisite for a diverse number of neurodegenerative conditions.

## SysBio Inflammation

## Tibolone

## 1.2. Material and Methods

### Tissue Specific Model Construction

The tissue specific model construction process started with the identification of all enzyme-coding genes expressed over the mean in at least 50 % of samples for healthy human astrocytes indexed in the GEO database [16] as GSE73721 [17]. Gene identifiers conversion from GeneCards[18] to ENTREZ [19] was performed through ‘UniProt.ws’ R Package [20]. Reactions associated with the identified genes were mapped from the Human Genome Scale Metabolic Reconstruction RECON 2.04 downloaded from the VMH Lab (<https://vmh.uni.lu>) [21]. The R package ‘g2f’ [22] was used to identify and fill the gaps using all no gene associated reactions included in RECON 2.04, as well as to identify and remove all blocked reactions from the reconstruction. All reactions involved in the conversion of extracellular glutamate, glycine, cysteine and glucose to extracellular glutamine, glycine, serine-D, reduced glutathione, lactate and ATP respectively were added. Exchange reactions were limited to components of the Dulbecco’s Modified Eagle Medium (DMEM) as input and gliotransmitters (glutamine, D-serine, ATP, glutamate), reduced glutathione, lactate, glucose, nitric oxide, prostaglandins and leukotrienes as output. Finally, syntax, mass-charge validation and creation of SBML files were carried out through the ‘minval’ R Package [23]. Reaction limits (upper and lower bounds) were constrained proportional to the mean gene expression reported for genes included in Gene-Protein-Reaction (GPR) [24] associated to each reaction in samples of 47 to 63 years old using ‘exp2flux’ R package [25]. All analysis were done by the ‘sybil’ [26] R Package running under R 3.3.1 [27].

### Flux Balance Analysis

Flux Balance Analysis (FBA) is a linear optimization method for simulating metabolism that allows to identify the set of reactions involved in the production of a biological response within a metabolic model [28]. The metabolic reactions are represented internally as a stoichiometric matrix ( $S$ ), of size  $m * n$ , where  $m$  represents the compounds and  $n$  the reactions; the entries in the matrix are the stoichiometric coefficients of the metabolites participating in a reaction [29]. The flux through all of the reactions in a network is represented by the vector  $v$ , which has a length of  $n$ . The concentrations of all metabolites are represented by the vector  $x$ , with length  $m$ . The systems of mass balance equations at steady state,  $\frac{d_x}{d_t} = 0$  or  $S * v = 0$ . FBA seeks to maximize or minimize an objective function which can be any linear combination fluxes, to obtain a flux for each reaction, indicating how much each reaction contributes to the objective function [28]. FBA for healthy, inflamed and medicated scenarios was

100 resolved using GLPK 4.60, setting the generic human biomass reaction included in RECON  
101 2.04 and each one of reactions described in table **1-1** as objective functions. Models were  
102 analyzed by comparing fluxes between scenarios, metabolites production rate and sensitivity  
103 analysis.

**Table 1-1:** Main metabolic capabilities associated to astrocytes represented as the set of  
objective functions used to evaluate neuroprotective effects of Tibolone under  
inflamed scenarios

ID	FORMULA REACTION	DESCRIPTION
Glu2Gln	1 glu_L[e] $\Rightarrow$ 1 gln_L[e]	Glutamate - Glutamine Cycle
Gly2SerD	1 gly[e] $\Rightarrow$ 1 ser_D[e]	Glycine to D-serine conversion
Glc2Lac	1 glc_D[e] $\Rightarrow$ 2 lac_L[e]	Lactate production from Glucose
Glc2ATP	1 glc_D[e] $\Rightarrow$ 36 atp[e]	ATP production from Glucose
Cys2GTHRD	1 cys_L[e] + 1 glu_L[c] + 1 gly[c] $\Rightarrow$ 1 gthrd[e]	Catch of Cysteine to produce re- duced Glutathione

## 104 Metabolic Scenarios

105 To test neuroprotective effects of tibolone during astrocytic metabolic inflammation we define  
106 three different metabolic scenarios. A ‘healthy’ scenario, where palmitate uptake rate was  
107 freely set by optimizer; an ‘inflamed’ scenario, where uptake rate of palmitate was forced  
108 to be stable in the mean of the half maximal inhibitory concentration (IC50) value for all  
109 objective functions included in table **1-1**. IC50 values were calculated through a robustness  
110 analysis performed using uptake of palmitate (‘EX\_hdca(e)’ in RECON 2.04) as control  
111 reaction and a 1000 points in the range from 0 to 1 mMgDW<sup>-1</sup>h<sup>-1</sup> for each objective function.  
112 Uptake value where each objective function reached IC50 was selected and subsequently  
113 averaged. Finally, a medicated scenario, defined as an inflamed scenario that include  
114 279 reactions associated with tibolone and estradiol-derived compounds metabolism. Ten  
115 specific reactions described in table **1-2** associated to specific Tibolone action mechanism  
116 non included in RECON 2.04 were added to medicated scenario.

## 117 Metabolic Changes

Metabolic changes across metabolic scenarios were measured through two different approxi-  
mations. Flux differences for each reaction between optimized scenarios were measured using

**Table 1-2:** Set of reactions associated to tibolone specific action mechanism in brain reported by Kloosterboer, H. J. (2004) added to medicated scenario model.

ID	FORMULA REACTION	DESCRIPTION
T1	tibolone[e] $\Leftrightarrow$	Tibolone exchange reaction
T2	tibolone[e] $\Leftrightarrow$ a3OHTibolone[e]	3 $\alpha$ hydroxytibolone interconversion
T3	tibolone[e] $\Leftrightarrow$ b3OHTibolone[e]	3 $\beta$ hydroxytibolone interconversion
T4	tibolone[e] $\Rightarrow$ d4tibolone[e]	Tibolone $\Delta^4$ isomer formation
T5	b3OHTibolone[e] $\Rightarrow$ d4tibolone[e]	Tibolone $\Delta^4$ isomer formation from 3 $\beta$ -hydroxytibolone
T6	a3OHTibolone[e] $\Rightarrow$ estradiol[c]	Estradiol receptor agonist action mechanism of 3 $\alpha$ -hydroxytibolone
T7	b3OHTibolone[e] $\Rightarrow$ estradiol[c]	Estradiol receptor agonist action mechanism of 3 $\beta$ -hydroxytibolone
T8	d4tibolone[e] $\Rightarrow$ prgstrn[c] + tststerone[c]	Progesterone and androgen receptor activation by tibolone $\Delta^4$ isomer
T9	a3OHTibolone[e] $\Leftrightarrow$ a3SOTibolone[e]	3 $\alpha$ hydroxytibolone interconversion to sulfated inactive compounds
T10	a3SOTibolone[e] $\Rightarrow$	Tibolone inactive form in blood

the fold change calculated as described in equation 1-1.

$$foldChange = \frac{valueModel2 - valueModel1}{|valueModel1|} \quad (1-1)$$

118 Additionally, to obtain a full perspective about inflammation effects in metabolites pro-  
 119 duction, the production of each metabolite was set as objective function in each metabolic  
 120 scenario and differences were evaluated as well as flux differences.

## 121 Proinflammatory, Antiinflammatory and Tibolone Action Mechanism 122 Associated Enzymes

123 Identification of enzymes involved in proinflammatory and antiinflammatory responses as  
 124 well as in the tibolone action mechanism were identified through several sensitivity analysis  
 125 as follows: Proinflammatory enzymes, are those that catalyze reactions that being knocked  
 126 out allows an increase of objective function value. Antiinflammatory enzymes, are those  
 127 associated to reactions that being knocked out reduce even more the objective function  
 128 value. Tibolone action mechanism associated enzymes are those that catalyze reactions that  
 129 being knocked out inhibit entirely the metabolic effect of tibolone.

<sup>130</sup> **1.3. Results**

<sup>131</sup> **1.4. Conclusion**

# Bibliography

- 133 [1] Kazuhiro Takuma, Akemichi Baba, and Toshio Matsuda. Astrocyte apoptosis: Impli-  
134 cations for neuroprotection. *Progress in Neurobiology*, 72:111–127, 2004.
- 135 [2] Sofie C. Lange, Lasse K. Bak, Helle S. Waagepetersen, Arne Schousboe, and Michael D.  
136 Norenberg. Primary cultures of astrocytes: Their value in understanding astrocytes in  
137 health and disease. *Neurochemical Research*, 37:2569–2588, 2012.
- 138 [3] Harold K Kimelberg. Functions of Mature Mammalian Astrocytes: A Current View.  
139 *The Neuroscientist*, 16(1):79–106, feb 2010.
- 140 [4] Nicola J Allen and Ben Barres. Neuroscience: Glia - more than just brain glue. *Nature*,  
141 457(7230):675–677, 2009.
- 142 [5] Michael M Halassa and Philip G Haydon. Integrated brain circuits: astrocytic networks  
143 modulate neuronal activity and behavior. *Annual review of physiology*, 72(2):335–355,  
144 2010.
- 145 [6] Christian Giaume, Annette Koulakoff, Lisa Roux, David Holcman, and Nathalie Rouach.  
146 Astroglial networks: a step further in neuroglial and gliovascular interactions. *Nature*  
147 *reviews. Neuroscience*, 11(fEbRuARy):87–99, 2010.
- 148 [7] Cora H. Nijboer, Cobi J. Heijnen, Vincent Degos, Hanneke L M Willemsen, Pierre Gres-  
149 sens, and Annemieke Kavelaars. Astrocyte GRK2 as a novel regulator of glutamate  
150 transport and brain damage. *Neurobiology of Disease*, 54:206–215, 2013.
- 151 [8] Ben Barres. The mystery and magic of glia: a perspective on their roles in health and  
152 disease. *Neuron*, 60(3):430–40, nov 2008.
- 153 [9] Jun Shen. Modeling the glutamate-glutamine neurotransmitter cycle. *Frontiers in*  
154 *Neuroenergetics*, 5(JAN):1–13, 2013.
- 155 [10] Francesco Petrelli and Paola Bezzi. Novel insights into gliotransmitters. *Current Opinion*  
156 *in Pharmacology*, 26(Table 1):138–145, 2016.
- 157 [11] Andrea R Durrant and Uriel Heresco-levy. D-Serine in Neuropsychiatric Disorders :  
158 New Advances. 2014, 2014.

- 
- [12] Stephen P. Raps, James C K Lai, Leif Hertz, and Arthur J L Cooper. Glutathione is present in high concentrations in cultured astrocytes but not in cultured neurons. *Brain Research*, 493(2):398–401, 1989.
- [13] Mithilesh Kumar Jha, Dong Ho Park, Hyun Kook, In-Kyu Lee, Won-Ha Lee, and Kyoungcho Suk. Metabolic Control of Glia-Mediated Neuroinflammation. *Current Alzheimer research*, 13(4):387–402, 2016.
- [14] Igor Allaman, Mireille Bélanger, and Pierre J. Magistretti. Astrocyte–neuron metabolic relationships: for better and for worse. *Trends in Neurosciences*, 34(2):76–87, feb 2011.
- [15] Xue Feng Wang and Max S. Cynader. Astrocytes provide cysteine to neurons by releasing glutathione. *Journal of Neurochemistry*, 74(4):1434–1442, 2000.
- [16] Ron Edgar, Michael Domrachev, and Alex E Lash. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res*, 30(1):207–210, 2002.
- [17] Ye Zhang, Steven A. Sloan, Laura E. Clarke, Christine Caneda, Colton A. Plaza, Paul D. Blumenthal, Hannes Vogel, Gary K. Steinberg, Michael S B Edwards, Gordon Li, John A. Duncan, Samuel H. Cheshier, Lawrence M. Shuer, Edward F. Chang, Gerald A. Grant, Melanie G Hayden Gephart, and Ben A. Barres. Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse. *Neuron*, 89(1):37–53, 2016.
- [18] Michael Rebhan, Vered Chalifa-Caspi, Jaime Prilusky, and Doron Lancet. GeneCards: integrating information about genes, proteins and diseases. *Trends in Genetics*, 13(4):163, 1997.
- [19] Donna Maglott, Jim Ostell, Kim D Pruitt, and Tatiana Tatusova. Entrez Gene: gene-centered information at NCBI. *Nucleic acids research*, 33(suppl 1):D54—D58, 2005.
- [20] Marc Carlson. *UniProt.ws: R Interface to UniProt Web Services*, 2016.
- [21] Ines Thiele, Neil Swainston, Ronan M T Fleming, Andreas Hoppe, Swagatika Sahoo, Maike K Aurich, Hulda Haraldsdottir, Monica L Mo, Ottar Rolfsson, Miranda D Stobbe, and Others. A community-driven global reconstruction of human metabolism. *Nature biotechnology*, 31(5):419–425, 2013.
- [22] Kelly Botero, Daniel Osorio, Janneth Gonzalez, and Andres Pinzon. *g2f: Find and Fill Gaps in Metabolic Networks*, 2016.
- [23] Daniel Osorio, Janneth Gonzalez, and Andres Pinzon. minval: MINimal VALidation for Stoichiometric Reactions, 2016.



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- 192 [24] Ines Thiele and Bernhard Ø Palsson. A protocol for generating a high-quality genome-  
193 scale metabolic reconstruction. *Nature Protocols*, 5(1):93–121, jan 2010.
- 194 [25] Daniel Osorio, Kelly Botero, Janneth Gonzalez, and Andres Pinzon. *exp2flux: Convert*  
195 *Gene EXPression Data to FBA FLUXes*, 2016.
- 196 [26] Gabriel Gelius-dietrich, Abdelmoneim Amer Desouki, Claus Jonathan Fritzscheier, and  
197 Martin J Lercher. *sybil – Efficient constraint-based modelling in R*. 2013.
- 198 [27] R Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation  
199 for Statistical Computing, Vienna, Austria, 2016.
- 200 [28] Jeffrey D Orth, Ines Thiele, and B O Palsson. What is flux balance analysis? *Nature*  
201 *Biotechnology*, 28(3):245–248, 2010.
- 202 [29] Karthik Raman and Nagasuma Chandra. Flux balance analysis of biological systems:  
203 Applications and challenges. *Briefings in Bioinformatics*, 10(4):435–449, 2009.