Deciphering and modelling remyelinating mechanisms induced by clinically-used azole antifungals with exploitable repurposing properties

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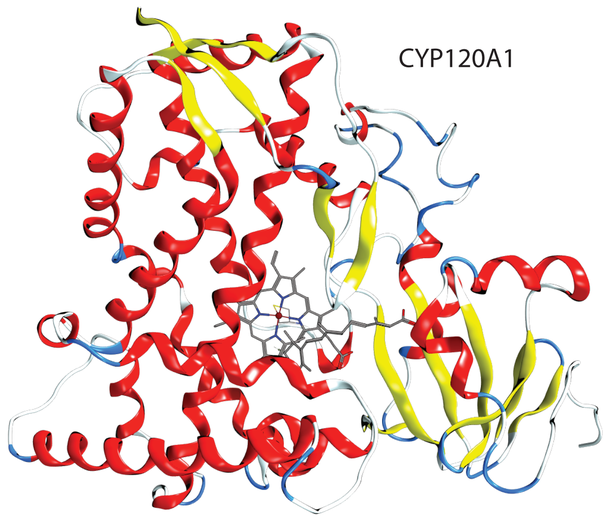
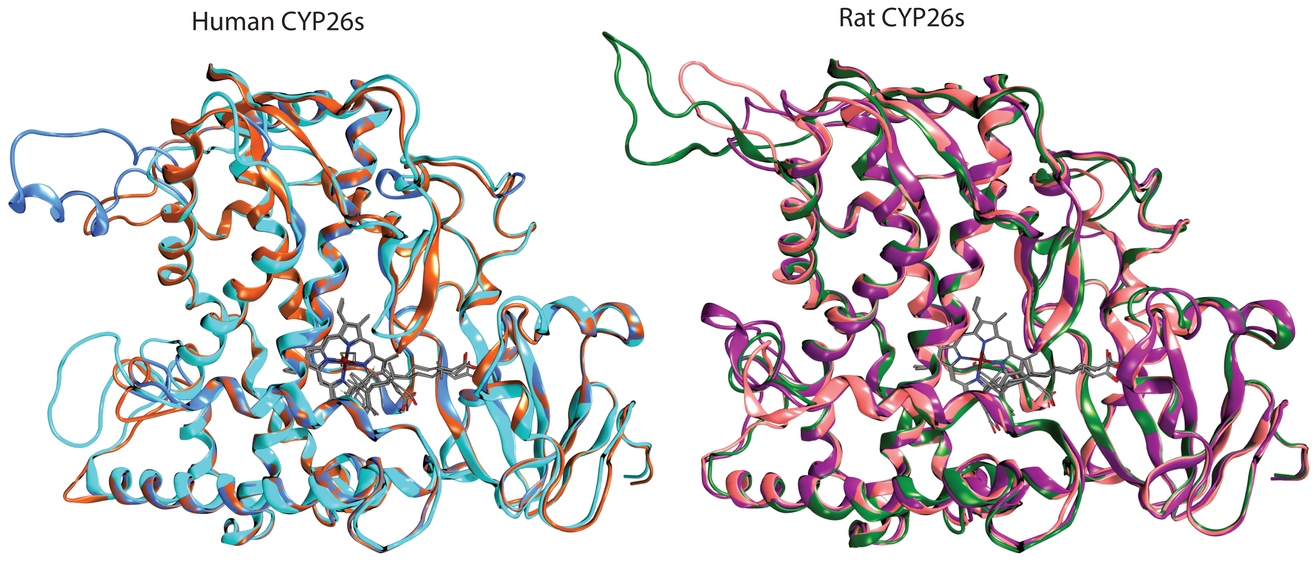
Summary of the research
Recent evidence demonstrated the ability of some antifungals to induce (re)myelination in specific experimental models. These compounds, already marketed for different pathologies, are interesting candidates for a repurposing strategy in neurodegenerative diseases, like multiple sclerosis (MS), characterized by strong demyelination. Our preliminary results show that the inhibition of enzymes involved in retinoic acid (RA) catabolism (CYP26), by miconazole and other azole antifungals, induces an increase of cellular RA concentration and that this increase may be behind the observed oligodendrocyte precursor cell (OPC) maturation and differentiation. On this basis, our proposal is aimed at studying the molecular mechanisms of azole antifungals used in human pharmacology, both *in silico* and *in vitro*, and at identifying the most promising ones according to their ability to increase Myelin Basic Protein (MBP) expression on OPCs and to promote their differentiation and myelination.

In our investigation, the ability of 7 azoles to inhibit CYP26 isozymes was evaluated through molecular docking: *in silico* results were the basis for the selection of the drugs for the *in vitro* experiments. The selected azoles were tested in three different cell models, characterized by increasing complexity: i) OPC cultures, a maturation assay for myelin-producing cells; ii) OPC-dorsal root ganglion (DRG) neuron co-cultures, a myelination assay; iii) cerebral micromass cultures, a cell differentiation assay for CNS.
In parallel, the effects of exogenous RA and of an inhibitor of the synthesis of RA (citral), were evaluated as well. In the next phase of the project, the outcomes from the different *in vitro* models will be statistically correlated with the pharmacological treatments.
Our results will be useful: 1) for identifying the most promising azole antifungals with respect to their pro-myelinating activity, 2) for clarifying the molecular mechanisms underlying their reparative effect and 3) for formalizing mathematical models to identify the most active concentrations for the most potent azoles.

Molecular modeling for CYP26 isoenzymes



Since no crystal structure is yet available for any member of the CYP26 family, models of the 3D structures of the three isoenzymes (CYP26A1, CYP26B1 and CYP26C1) were built for both the human and the rat protein *via* comparative modelling, using the X-ray structure of the RA-bound cyanobacterial CYP120A1 cytochrome as template (sequence identity approx. 34% for all the queries).
Then, *in silico* RA::CYP26s 3D complexes were optimized for further computations and finally relaxed *via* energy minimization (EM).
All the *in silico* procedures were carried out with the MOE Suite 2016.08.



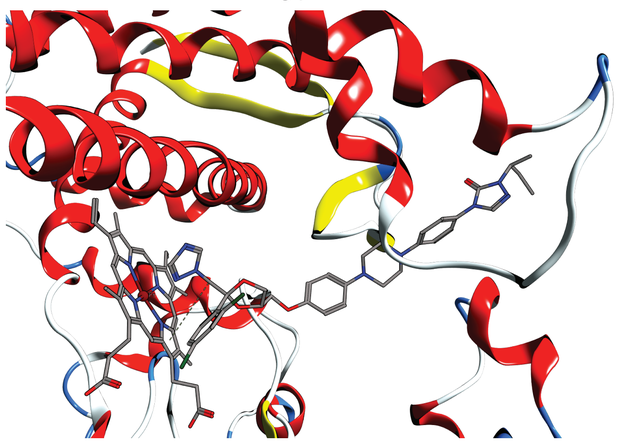
In silico evaluation of azole affinity for CYP26 isoenzymes

Seven different azoles in clinical use for human pathologies were selected and their propensity to inhibit both the human and rat Cyp26 isoenzymes was investigated *in silico* through molecular docking. RA, which is known to be catabolized by CYP26 in humans (Menegola et al. 2006), was included in this procedure as control. All the generated complexes were carefully inspected: as already reported for CYP450 (Li et al. 2012), also in CYP26 the nitrogen atom of the azole ring coordinates the heme iron and the compounds occupy the binding pocket blocking the access of the substrate to the catalytic site. Accurate binding free energy values (affinity) were computed by refining the docking complexes, relaxing the ligand within the binding pocket and applying a constrained forcefield EM. *In silico* dissociation constants (Ki) were estimated on the basis of their binding free energy values to prioritize azoles and to set-up *in vitro* experiments.

Azoles on human CYP26s	Binding free energy (kcal/mol)	Affinity (kcal/mol)	K_i	CYP26 Isoenzyme
Itraconazole	-11.8	-13.4	9.9	CYP26B1
Posaconazole	-11.1	-13.3	9.8	CYP26B1
Ketoconazole	-9.9	-12.3	9.1	CYP26B1
Isavuconazole	-9.3	-9.6	7.1	CYP26A1
Retinoic Acid	-8.3	-8.5	6.3	CYP26C1
Miconazole	-8.2	-8.7	6.4	CYP26A1
Voriconazole	-7.6	-13.4	9.9	CYP26B1
Fluconazole	-7.0	-7.6	5.6	CYP26A1

Azoles on rat CYP26s	Binding free energy (kcal/mol)	Affinity (kcal/mol)	K_i	CYP26 Isoenzyme
Itraconazole	-11.2	-12.1	8.9	CYP26A1
Posaconazole	-9.7	-10.8	7.9	CYP26A1
Ketoconazole	-9.4	-13.7	10.1	CYP26A1
Isavuconazole	-8.5	-9.8	7.2	CYP26A1
Retinoic Acid	-8.3	-8.6	6.3	CYP26A1
Miconazole	-8.0	-8.6	6.3	CYP26A1
Voriconazole	-7.1	-8.4	6.2	CYP26C1
Fluconazole	-7.0	-7.1	5.2	CYP26C1

Itraconazole binding pose for Human CYP26A1

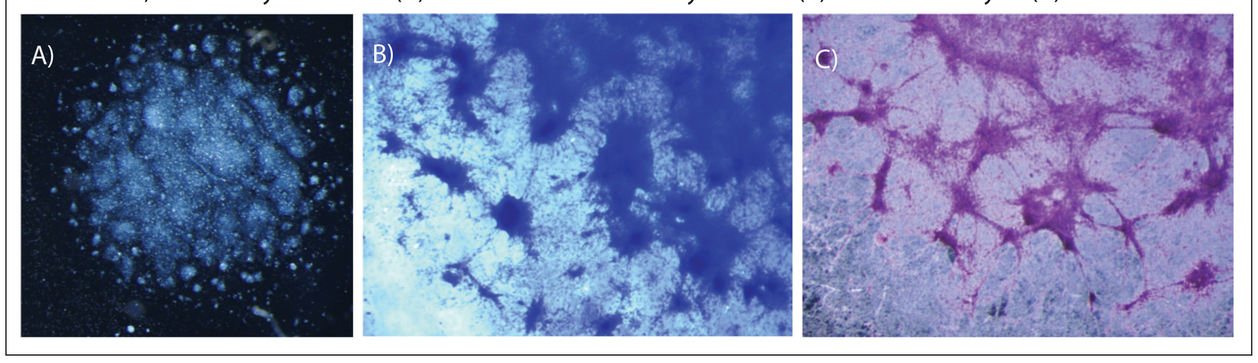


No differences in affinity for the investigated azoles was found between human and rat CYP26s, suggesting that rat OPC cultures are a suitable model for testing clinically used azoles.

According to computed Ki, itraconazole and posaconazole were selected as the most promising compounds to be forwarded to the *in vitro* test. In parallel, the effect of fluconazole, which has the lowest affinity for the CYP26 family, was also evaluated. Predicted Ki values for miconazole are consistent with literature data (Najm et al. 2015).

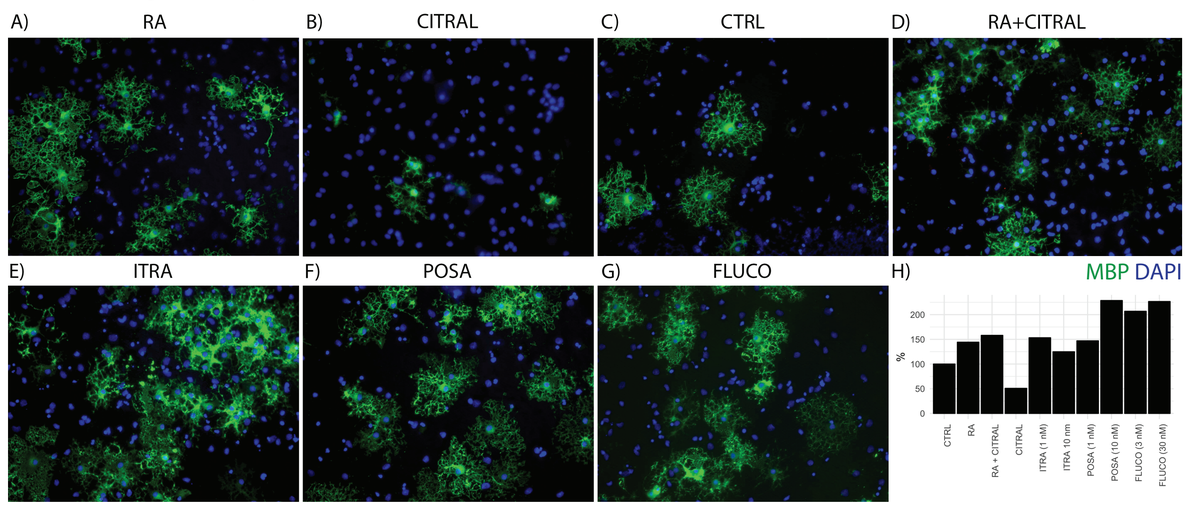
Next steps

Further treatments on cerebral micromass cultures with the selected azoles either alone or in combination with the RA ihibitor citral are currently ongoing. The micrographs show the images of untreated rat cerebral micromass, after 2 day in culture (A) and stained with methylen blue (B) and hematoxylin (C).



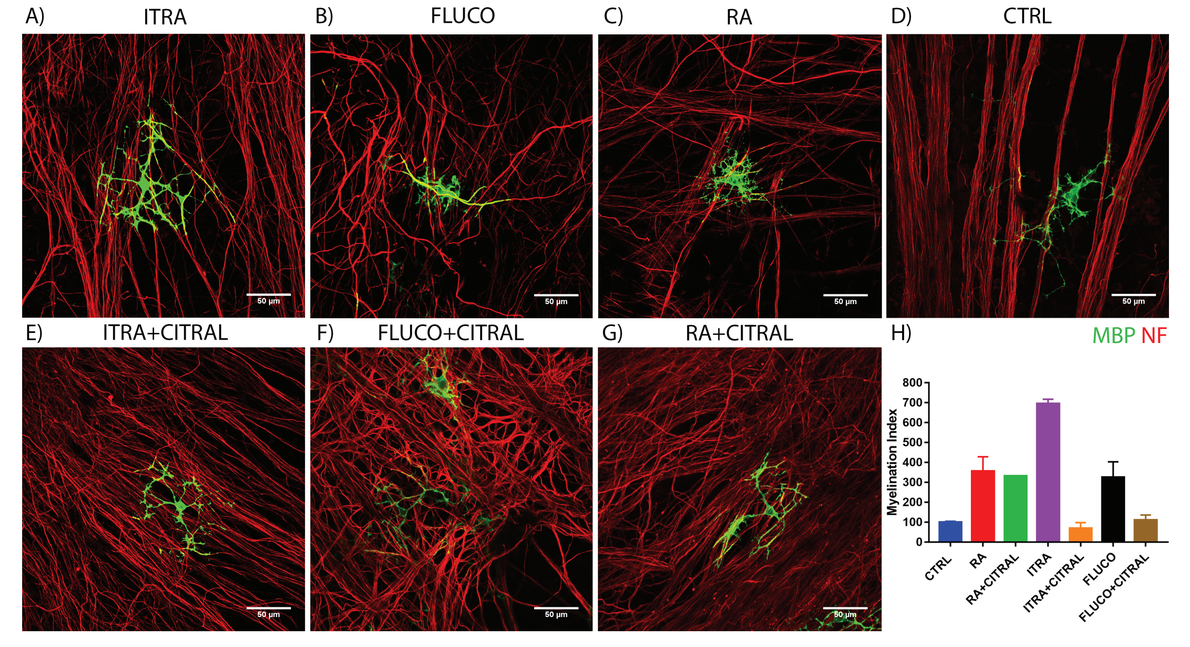
Effect of antifungal azoles on OPC maturation assay

Treatment of *in vitro* OPC cultures with RA (50 nM, A) is able to increase the number of MBP$^+$-expressing cells, whereas the treatment with citral (150 uM, B) produces a decrease in the number of MBP$^+$ cells *versus* vehicle-treated cells (C). When RA and citral are administered in combination to OPCs (D), the effect of RA is reduced. Also the selected azoles (E-G), when administered to OPCs at selected concentrations (1-30 nM), significantly increase the number of MBP$^+$ cells *versus* control.



Effect of antifungal azoles on OPC-DRG myelination assay

OPC cultured on DRG neurons were exposed to itraconazole (10 nM) and fluconazole (30 nM). Both azoles (A, B), similarly to RA (50 nM, C), were able to significantly increase the formation of double positive MBP/NF-myelinated axons *versus* vehicle-treated cultures (D). Also in this case, when azoles or RA were administered to OPC-DRG cultures in combination with the RA inhibitor citral (150 uM, E-G), the effect on myelination was reduced with respect to the same compound alone.



Conclusions

Overall, these results demonstrate that antifungal azoles are able to increase MBP expression and the formation of myelinating fibers on both OPC and OPC-DRG *in vitro* cultures, as already demonstrated for miconazole (Najm et al. 2015). These data suggest that the pro-myelinating effect of antifungal azoles may be connected with the increased local bioavailability of RA.

As soon as the analysis will be completed, all the *in silico* and *in vitro* results produced within this project will be comprehensively integrated via statistical and numerical analysis. The elucidation of the role of azoles in RA-mediated (re)myelination will provide the basis for the identification of new promising candidates for repurposing strategies in MS.

Acknowledgements

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