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# Effect of itraconazole on mouse mesencephalic neurons

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ABSTRACT

Neuronal cells have complex geometrical shapes, long processes such as axons and dendrites, and as a response to specific stimuli, they go through polarized neuronal migration that influences connectivity and information processing. Recently, it has been discovered that itraconazole, a widely used systemic antifungal drug, has an effect on cell morphology, acting as an inhibitor of the morphogen Sonic Hedgehog (Shh) and of the mammalian target of rapamycin mTOR pathways. In this paper we evaluated the effect of itraconazole on mouse mesencephalic dopaminergic neurons following their neurite outgrowth and functional activity by [$^3$H] DA uptake. Furthermore the expression of several neural markers, the activation of the mTOR and of the morphogenic Shh pathways in the neuronal population was examined. Our results show for the first time a strong alteration of neurons morphology and an inhibitory effect of differentiation by itraconazole, probably due to cholesterol trafficking reduction, mTOR and Shh pathways inhibition. The inhibition of mTOR and Shh pathways by this drug has also been found in other cellular systems such as endothelial cells and lung cancer cells, suggesting a conserved mechanism of intercellular communication. As itraconazole is currently involved in multiple human clinical trials as a prospective anticancer agent, the effect on neuronal differentiation should be taken into account.


## 1. Introduction

Neural connections are the result of axonal outgrowth and branching required for proper nervous system function which can be affected by stress, radiation and other environmental conditions (Goldberg, 2004; Li and Jin, 2010; Kheirbek et al., 2012). Differentiating neurons undergo multiple steps of morphogenesis to form the complex geometrical shapes at the basis of neural circuits (Kim et al., 2011). The study of factors affecting the complex variability of neural morphogenesis could lead to important information on structure-function relationships (Chklovskii, 2004). Recently, itraconazole, normally used in humans as an antifungal drug, was shown to inhibit cell cycle progression of human primary umbilical vein endothelial cells in vitro and fibroblast growth factor-dependent angiogenesis in vivo (Chong et al., 2007). Moreover it has been described to reduce microtubule formation in human dermal microvascular endothelial cells (Del Carratore et al., 2012), induce morphological modification in murine macrophages (Ochi et al., 1998) inhibit basal cell carcinoma growth in mice (Kim et al., 2010) and block lung tumor progression in humans (Aftab et al., 2011). For this reason itraconazole is increasingly employed as an anticancer drug in clinical trials (Shim and Liu, 2014). Overall, itraconazole seems to affect cellular states related to proliferation, movement and morphology changes in a broad range of different cell types, implying a common mechanism of action not yet highlighted. The main target of itraconazole is the lanosterol 14α-demethylase (CYP51), involved in the cholesterol biosynthesis pathway (Fink et al., 2006). The role of cholesterol depletion in cellular shape, related to cell movement and connections, is not yet completely understood. Itraconazole effects on neuronal growth and morphology have never been studied and this approach could contribute to our understanding of the mechanisms regulating neuronal development. In this study we investigated its effect on morphology of mouse mesencephalic dopaminergic neurons (DA neurons) in vitro, by following neurite growth and their functional activity by [$^3$H] dopamine uptake (DA). Mesencephalic cell cultures dissected from mice embryos represent a useful tool for studying mechanisms involved in the morphogenesis and function of neurons during development. Many of the features that

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characterize these neuron progenitors in vivo are retained when they are grown in culture (Vaglini et al., 2008) where it is possible to study the cells during the first days of differentiation.

The expression of several neural markers such as nestin, neural cell adhesion molecule (NCAM), a glial fibrillary acidic protein (GFAP), and neurofilament light biomarkers (NFL) was studied on the whole neuronal population. Furthermore since mTOR and Sonic Hedgehog (Shh) pathways have been shown to be targets of itraconazole (Kim et al., 2010; Xu et al., 2010) and since they play a role in neural differentiation and maturation, we investigated the drug effects on both pathways and a possible correlation between the two pathways and cholesterol depletion. Our results show a strong inhibitory effect of itraconazole on neuronal outgrowth, connected to cholesterol as well as to differentiation markers inhibition. Furthermore analogously to what observed in endothelial cells and lung cancer cells (Chong et al., 2007; Kim et al., 2010) we found in neurons the inhibition of mTOR and Shh pathways.

## 2. Materials and methods

### 2.1. Chemicals

Eagle's minimum essential medium (MEM), Ham's nutrient mixture F-12, L-glutamine, phosphate buffer saline (PBS), itraconazole (I6657), lipoprotein low density from human plasma (LDL) (L8292) were purchased from Sigma–Aldrich (St. Louis, MO, USA). NU-serum V was purchased from Collaborative Research (BD Biosciences, Steroglass, Italy). Rabbit tyrosine hydroxylase (TH) antibody was purchased from Chemicon (Temecula, CA, USA), the Vectastain Elite ABC kit was purchased from Vector Laboratories (Burlingham, CA, USA), CytoScint (ICN Research Products, Costa Mesa, CA, USA). All tissue culture supplies were provided by Falcon.

### 2.2. Mouse mesencephalic cell cultures

Timed pregnant CD1 mice were obtained from our laboratory. The animals were handled in accordance with the Guidelines for Animal Care and Use of the National Institutes of Health, and all efforts were made to minimize animal suffering and to keep the number of animals used to a minimum. Brains were obtained from 13-day-old mouse embryos and placed in cold, sterile, phosphate-buffered saline (PBS). As previously described (Vaglini et al., 2008), the midbrain was dissected out under microscopic control and placed in a nutrient medium composed of F12 and Eagle's minimal essential medium (ratio1:1), supplemented with 2 mM glutamine, 10% of Nu-serum and 33.3 mM glucose. The tissue was mechanically dispersed, centrifuged at $800 \times g$ for 3 min, and suspended in the culture medium. Cells were plated at a density of $5 \times 10^5$ in multiwell plates (16-mm-diameter wells; Costar). Multiwell plate surfaces were pre-coated with 15 μg/ml of poly-D-lysine (high Mw, >300,000). The cultures were maintained in a humidified atmosphere of 5% CO2/95% air at 37 °C. Experiments were performed in the absence of glial cells. To obtain virtually pure neuronal cultures, 10 μM cytosine arabinoside (Ara C) (Upjohn), which suppresses glia proliferation, was added 3 days after plating (Lilliu et al., 2002).

### 2.3. Assay of cell viability

Mesencephalic cell viability was determined by MTT assay kit (Sigma, USA) according to the manufacturer's instructions. Briefly, mesencephalic cells were plated at a density of $45 \times 10^3$ cells/well in a 96-well plate, pre-coated with poly-D-lysine as previously described (Van Meerloo et al., 2011). Cells were treated with the MTT solution (final concentration, 0.5 mg/ml) for 4 h at 37 °C. The medium was replaced with 100 μl of DMSO for each well. The formazan dye crystals were solubilized for 30 min, and absorbance at 570 nm was measured with a microplate reader (Multiscan Spectrum, Thermo Labsystems). Results were expressed as the percentage of MTT reduction, assuming that the absorbance of untreated cells (control) was 100%.

### 2.4. Assay of dopamine uptake

To perform [$^3$H] dopamine uptake, mesencephalic cultures were rinsed with PBS, and incubated for 15 min with 50 nM [$^3$H] DA (23.9 Ci/mmol). Uptake was stopped by removing the reaction mixture containing the radio-ligand, and rinsing the wells three times with ice-cold PBS. The cells were scraped into 0.5 ml of 0.2 N NaOH containing 0.2% Triton X-100; then 0.5 ml of HCl 0.2 N was added to neutralize the pH. Blank values were obtained by incubating cells at 0 °C, a condition that blocked specific uptake in vitro. The radioactivity was counted with 10 ml of Cytoscint (ICN) in a liquid scintillation-counter (Wallac). Results are expressed as per cent of disintegration per minute (dpm), based on three experiments performed in triplicate wells.

### 2.5. Tyrosine-hydroxylase immunocytochemistry

Cultures were washed twice in PBS and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature. After two washes in PBS, they were incubated overnight at 4 °C with a rabbit tyrosine hydroxylase (TH) antibody diluted 1:1000 in PBS (containing 0.2% of Triton X-100). Cultures were then washed with PBS, and incubated with a biotinylated antirabbit IgG, followed by incubation with an avidin-biotin conjugated to peroxidase. The peroxidase was visualized using diaminobenzidine and hydrogen peroxide. Total TH+ cell numbers were counted in ten randomly selected fields (1.13 mm$^2$/field) at 20× magnifications with a Nikon inverted microscope and cell morphology was assessed by microscopic observation of the immunostained mesencephalic monolayer. Micrographs were taken with a Nikon D40x digital camera mounted on the phototube.

### 2.6. Experimental scheme

Mouse mesencephalic cells were grown in 2 ml of culture medium. On day 4 after plating, cells were treated with different doses (0.25, 0.5, 1 and 2 μM) of itraconazole. After 24 or 48 h, cells were functionally tested for [$^3$H] dopamine uptake (control was 54,196 ± 747 dpm/mg of protein after 24 h and 69,401 ± 503 dpm/mg of protein after 48 h). In addition, a separate set of cells were fixed in paraformaldehyde (4% in PBS) and TH immunocytochemistry was performed. In another set of experiments, on day 4 the cells were treated with different concentrations of itraconazole in the presence or absence of LDL (50 μg/ml dissolved in $H_20$) added an hour after the antifungal drug. After 24 h the cells were washed and were grown for a further 72 h in free medium. Then [$^3$H] dopamine uptake was performed (control was 73,219 ± 631 dpm/mg of protein after a further 72 h); a separate set of cells were fixed in paraformaldehyde (4% in PBS), and TH immunocytochemistry was performed. To evaluate the intracellular uptake of itraconazole (0.5 and 1 μM) in cells grown in the presence or absence of LDL, an HPLC set of experiments was performed. 24 h after treatments, the cells were washed three times in NaCl 09% and scraped (each thesis was performed in triplicate). The cell pellets were homogenized in 150 μL of NaCl 09%. The homogenates were centrifuged for 15 min at 8000 rpm; the supernatants (cytosol) and the corresponding growth media were collected and injected in the HPLC.