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Antifungal effect of all-trans retinoic acid against *Aspergillus fumigatus* in vitro and in a pulmonary aspergillosis in vivo model

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ABSTRACT

AIM: *Aspergillus fumigatus* is the most common opportunistic fungal pathogen and causes invasive pulmonary aspergillosis (IPA), with high mortality among immunosuppressed patients. Fungistatic activity of *all-trans* retinoic acid (ATRA) has been recently described *in vitro*.

We evaluated the efficacy of ATRA *in vivo* and its potential synergistic interaction with other antifungal drugs.

MATERIALS AND METHODS: A rat model of IPA and *in vitro* experiments were performed to assess the efficacy of ATRA against *Aspergillus* in association with classical antifungal drugs and *in silico* studies used to clarify its mechanism of action.

RESULTS: ATRA (0.5 and 1 mM) displayed a strong fungistatic activity in *Aspergillus* cultures, while at lower concentrations, synergistically potentiated fungistatic efficacy of sub-inhibitory concentration of Amphotericin B (AmB) and Posaconazole (POS). ATRA also enhanced macrophagic phagocytosis of conidia. In a rat model of IPA, ATRA reduced mortality similarly to Posaconazole.

CONCLUSION: Fungistatic efficacy of ATRA alone and synergistically with other antifungal drugs was documented *in vitro,* likely by inhibiting fungal *Hsp90* expression and Hsp90-related genes. ATRA reduced mortality in a model of IPA *in vivo*. Those findings suggest ATRA as suitable fungistatic agent, also to reduce dosage and adverse reaction of classical antifungal drugs, and new therapeutic strategies against IPA and systemic fungal infections.

Keywords: Trans-retinoic acid, *Aspergillus*, invasive pulmonary aspergillosis

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55 **Introduction**

56 Invasive Fungal infection (IFI) is a critical complication in hematologic and neoplastic patients
57 leading to severe morbidity and mortality (1). IFIs are estimated to be responsible for approximately
58 1.5 to 2.0 million deaths every year (2). Over the last decades, the incidence of IFIs has increased
59 due to the concurrent growth of immunocompromised patients, the broad use of antibiotics and
60 chemotherapies, bone marrow, organ transplants and corticosteroids (2). *Aspergillus fumigatus* is
61 among the most invasive opportunistic pathogens in immunocompromised patients and the
62 incidence of *Aspergillus*-related IFIs is progressively growing (3). The genus *Aspergillus* has been
63 includes four subgenera (*Aspergillus, Circumdati, Fumigati,* and *Nidulantes*) and 20 sections
64 including 339 recognized species(4). Nevertheless, only a few well-known species (including
65 *Aspergillus fumigatus* and *Aspergillus flavus*) are considered important opportunistic pathogens in
66 both humans and animals(5). In humans, *Aspergillus fumigatus* is the most common and life-
67 threatening airborne opportunistic fungal pathogen, with particular significance among
68 immunosuppressed hosts (5). *Aspergillus fumigatus* causes invasive pulmonary aspergillosis (IPA),
69 which is associated with severe morbidity and mortality (5). Despite the high IPA-related mortality,
70 the most used three classes of antifungal drugs (polyenes, azoles, and echinocandins) display a
71 limited clinical efficacy and are often unsuccessful, despite the noteworthy side effects associated
72 with their long-term use, the critical pharmacokinetic profile and lastly the drug-drug interactions
73 (6). An important challenge for the future is to discover novel drugs that can also interact with
74 specific fungal targets or modify the existing ones by optimizing their antifungal action by reducing
75 dosages and consequently side effects.

76 In the last decades, the role of Vitamin A in the regulation of the immune response has been
77 confirmed by several studies and its deficiency is associated with increased susceptibility to severe
78 infectious diseases (7). Vitamin A is a nutrient obtained through the diet either as provitamin-A
79 (carotenoids) or as preformed vitamin A (retinol and retinyl esters), and liver dehydrogenases

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80 converts retinol into retinoic acid, its biologically active metabolite (7). Retinoids are key
81 modulators of cell growth, differentiation, and apoptosis through their receptor-regulated signaling
82 pathways (8). In particular, retinoic acid controls the normal immune system development as a
83 modulator of both innate and adaptive immune responses (9). We recently documented that the
84 topical use of the retinoid derivative tazarotene favors rapid healing of fungal nails infections (10);
85 moreover, *all-trans* retinoic acid (ATRA) demonstrated fungistatic activity *in vitro* (11). Based on
86 those evidences, we sought to evaluate ATRA synergistic interaction with conventional antifungal
87 drugs such as Amphotericin B and Posaconazole in *in vitro* studies and it's therapeutic efficacy in a
88 preclinical model of IPA.

89

90 **METHODS**

91 **Microorganism, fungal cultures and treatments**

92 *Aspergillus fumigatus* strain obtained from a clinical isolate, according to routine hospital laboratory
93 procedures, was grown on Sabouraud dextrose agar (SDA) (Difco, Detroit, MI) supplemented with
94 chloramphenicol. Resting conidia were obtained from the SDA grown fungal culture, as previously
95 described (11). For *in vitro* studies, ATRA (Sigma Aldrich R2625, Milan, Italy) and Amphotericin B
96 (Fungizone) (Sigma Aldrich, Milan, Italy) were tested in the range of 1-0.06 mM (300-37.5 μg/ml)
97 and in the range of 50 to 0.09 μg/ml, respectively. To investigate the potential synergistic interaction
98 between ATRA and AMB on the conidial germination, resting conidia were plated at the density of
99 $1x10^5$ in 96 well-microplate in RPMI 1640 with 10% fetal calf serum (FCS; Euroclone, Milan,
100 Italy), supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) and treated with
101 different concentrations of ATRA or AmB, alone or in combination. In the combined treatment,
102 ATRA was given 30 min. before Amphotericin B. Fungal cultures were maintained at 37° C and
103 monitored during the first 30 hours after the treatments.

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105 **Evaluation of fungistatic activity**

106 *Aspergillus fumigatus* development was followed using an optical microscope (Carl Zeiss, United
107 Kingdom) with a 40x magnification objectives lens. *Aspergillus* conidia vitality was evaluated
108 microscopically by the trypan blue dye exclusion method.

109 **In vitro antifungal susceptibility testing**

110 The synergistic antifungal activity of Atra in combination with the antifungals AmB or POS (Sigma
111 Aldrich) against *Aspergillus fumigatus* was also assessed using the broth microdilution method as
112 described in M38-A2, a document produced by the Clinical and Laboratory Standards Institute
113 (CLSI, 2008) (12). RPMI 1640 2% glucose with L-glutamine (Sigma-Aldrich, St. Louis, MO, USA)
114 buffered to pH 7.0 was used as fungal culture medium. *A. fumigatus* strain was grown on Sabouraud
115 dextrose agar (Difco Laboratories, Detroit, MI, USA) supplemented with chloramphenicol. Resting
116 conidia were harvested by washing the slant cultures with sterile saline as previously described (11).
117 *Aspergillus* inoculum was set to $0.4x10^4$-$0.5x10^5$ CFU/ml, standardized spectrophotometrically. 100
118 µl of this suspension were added to each 96 well flat bottom plates. Twofold serial dilutions of
119 ATRA and antifungals were prepared in RPMI 1640 medium, in order to obtain final concentrations
120 ranging from 150 to 37.5 µg/ml, from 50 to 0.09 µg/ml and from 4 to 0,00005 µg/ml, for ATRA,
121 AmB and POS, respectively. *Aspergillus* conidia were treated with 100 µl of each antifungal drug
122 either alone or in combination with ATRA. Positive (100µl of RPMI medium with 100µl of conidia)
123 and negative controls (200µl of RPMI containing ATRA alone or in association with the antifungals
124 without conidia) were included in all experiments. The plates were incubated with agitation at 30°C
125 for 24 h. The Minimun Inhibitory Concentration (MIC) was determined spectrophotometrically at
126 510 nm with an ELISA reader. For each compound the MIC was defined as the lowest drug
127 concentration that produced a significant inhibition (MIC≥50) of fungal growth when compared

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128 with the drug-free control. The MICs were tested in three independent experiments carried out in
129 triplicate.

130 **Animals, fungal infection and treatment**

131 Sprague-Dawley rats (Envigo; *Huntingdon, UK*) were immunosuppressed by cortisone 21-acetate
132 (C3130, Sigma-Aldrich, St. Louis, USA) treatment subcutaneously administered at 150 mg/kg body
133 weight 7 days before the day of infection; immunosuppression was maintained with 80 mg/kg of
134 cortisone 21-acetate for three days a week until the end of the experiment. As the
135 immunosuppression treatment started and throughout the study, rats received a low protein (8%)
136 diet as well as drinking water containing tetracycline hydrocloride (T3383, Sigma-Aldrich)
137 antibiotic (260 mg/L) supplied ad libitum. Rats were then infected with $5x10^5$ Aspergillus conidia in
138 0.1 mL of saline solution by oropharyngeal aspiration. For treatment, ATRA (Sigma-Aldrich) was
139 resuspended in 100% DMSO at 50 mg/mL and stored at -20 °C in the dark. Before using, ATRA
140 was diluted in Peanut oil (P2144, Sigma-Aldrich) in order to obtain a 2 mg/kg/2 mL suspension,
141 ready for in vivo administration. The Posaconazole oral suspension was obtained diluting in water
142 at concentration of 4 mg/10ml the clinical drug Noxafil (Merck Sharp & Dohme, *Kenilworth, USA*).
143 Posaconazole treatment started concurrently with the infection (day 0) and it was administered for 5
144 days a week until the end of the study. As negative control, vehicle was prepared by adding 2%
145 DMSO in Peanut oil and 5% glucose to water. In the prophylaxis schedule optimization experiment,
146 three groups of rats (n=10) were treated with ATRA by intraperitoneal injection (i.p.) for 7 days
147 before infection (day 0), and then for 5 days a week up to the end of the study.

148 The mortality rate was recorded daily until the end of the studies. All animal procedures were
149 carried out in accordance with the standards established by the Animal Ethical Committee of Takis.

150
151 **Phagocytosis assay**

152 For phagocytosis assay (13), human macrophages U-937 ($1x10^6$ cells/ml) were incubated overnight

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153 in 6-ml polypropylene tubes in Dulbecco's modified Eagle's medium (DMEM; Gibco, Milan, Italy)
154 supplemented with 2% (v/v) FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml of
155 streptomycin, with vehicle (DMSO) or different ATRA concentrations (1, 5 and 10 µM).. After
156 overnight incubation, the cells were incubated for an additional 1 h at 37°C with unopsonized
157 resting *Aspergillus* conidia, at an effector to fungal cells ratio of 1:10, before being evaluated for
158 internalization, visualized by light microscopy. Phagocytic cells were separated from non
159 phagocytosed *A. fumigatus* by centrifugation on a fetal serum gradient. A 0.1-ml sample of the
160 harvested phagocytic cells was used for cytospin preparation. After Diff-Quik staining, fungal cell
161 internalization was expressed according to the following formula: percentage of internalization =
162 number of cells containing one or more fungal cells/100 cells counted. Parallely, macrophage
163 phagocytosis was also performed in 24-well plates for a real time analysis of phagocytosis by
164 microscopy. U-937 cultured at $1x10^6$ cells/ml were treated overnight with ATRA and incubated for
165 an additional 1 h at 37°C with *Aspergillus* conidia, as described above.
166

167 **Western Blot analysis**

168 For western blot analysis, after extraction 20 micrograms of total proteins were blotted onto
169 nitrocellulose membranes and incubate with rabbit anti-Hsp90 (1:1000; Cell Signaling
170 Technologies Danvers, USA) and rabbit anti-Gapdh (1:20000; GenTex, Zeeland USA), followed by
171 incubation with HRP-conjugated anti-rabbit IgG (1:2500; Santa Cruz Biotechnology Dallas USA).
172 Revelation and densitometric blots were performed in three different experiments.
173

174 **RNA extraction and Real-time reverse transcription-PCR (qRT-PCR)**

175 A sample of frozen biomass was placed into a 2 mL extraction tube containing beads and disrupted
176 using TissuLyser. RNA was isolated using TRI® reagent (Sigma Aldrich), according to the
177 manufacturer's instructions. To verify the general purity of the RNA Nanodrop instrument was

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178 used. One microgram of total RNA, was reverse-transcribed by a Quantitec reverse transcription kit
179 (Qiagen, Hilden Germany), following the manufacturer's instructions. qRT-PCR analyses were
180 performed using 20 ng cDNA per well in triplicate with the SYBR green master mix (Applied
181 Biosystems Foster City USA) (14)(15) according to the manufacturer's instructions. Reactions were
182 run on 7900HT system (Applied Biosystems).
183

184 **Heat shock protein 90 simulation methods**

185 Protein-ligand molecular docking has been used to evaluate binding mode and energy between *A.*
186 *fumigatus* Hsp90, ATRA (2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohexen-1-yl) nona-
187 2,4,6,8-tetraenoic acid) and ATP, its natural cofactor. The docking simulations have been executed
188 using the AutoDock Vina 1.1.2 program, through the AutoDock/Vina PyMOL plugin
189 (http://wwwuser.gwdg.de/~dseelig/adplugin.html) (The PyMOL Molecular Graphics System
190 Version 1.5.0.4. Schrödinger, LLC; (16)). The ATRA and ATP structural files (SDF file) have been
191 obtained from the PubChem compound database (https://pubchem.ncbi.nlm.nih.gov), and have been
192 converted into mol2 files and filled with hydrogens using the Open Babel.Org program. The crystal
193 structure of Hsp90 from *S. cerevisiae*, has been used as a template to generate the *A. fumigatus*
194 Hsp90 model structure. The model has been generated using the SWISS-MODEL protein modelling
195 tool (swissmodel.expasy.org) (17). After a structural check, the model has been used as a receptor
196 for the molecular docking simulations of ATP and ATRA in the ATP-binding site. Thirteen side
197 chains (Leu34, Asn37, Lys44, Asp79, Ile82, Met84, Asp88, Lys102, Gln103, Phe124, Val136,
198 Thr171, Ile173), belonging to the Hsp90 ATP-binding site, have been considered rotatable to
199 improve the conformational search during the docking simulations.

200 **Statistical analysis**

201 For in *in vitro* experiments statistical analysis was performed using unpaired two-tailed *t* test
202 between control and experimental groups. For in *vivo* studies, survival curves were determined by

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203 the Kaplan-Meier method and the log-rank test was used to conduct comparison between the
204 groups. Test of statistical significance was two-sided; a value of $P < 0.05$ was considered to be
205 statistically significant.
206

207 **RESULTS**

208 **ATRA displays fungistatic activity on *Aspergillus fumigatus* germination i*n vitro***

209 At 0.5-1 mM concentration, ATRA exerted a strong inhibitory effect on swelling and germination of
210 *Aspergillus* conidia *in vitro* (Figures 1A-B), as previously reported (11). After 18h of treatment with
211 lower ATRA concentration (0.25 and 0.125 mM), *Aspergillus* conidia appeared swollen (Figures
212 1C-D) and similar to control, but without the development of short germ tubes typical of control
213 cultures (Figure 1E). The inhibitory effect on conidial germination was maintained until 30 h
214 (Figures 1F-G). At that time, ATRA at concentration lower than 0.5 mM did not interfere with
215 *Aspergillus* conidia germination, although the development of germ tubes appeared delayed
216 (Figures 1 H-I). Only control *Aspergillus* cultures (Figure 1L) displayed elongated hyphal
217 structures. The fungistatic effect of ATRA was reversible, since conidia were able to germinate and
218 to grow in the hyphal form 7 days after ATRA removal (data not shown).
219

220 **ATRA exerts a synergistic fungistatic activity with Amphotericin B and Posaconazole *in vitro***

221 In order to investigate the synergistic fungistatic activity of ATRA with standard antifungal drugs,
222 we determined a sub-inhibitory concentration of AmB. As shown in Fig 2A, 3.15 μg/ml was the
223 minimal AmB concentration capable to inhibit *Aspergillus* conidia germination, while at 1.57 μg/ml
224 concentration AmB did not interfere with germination (Fig. 2B), and only a delayed hyphal growth
225 was observed. Interestingly, the association of low and non-effective doses of ATRA (0.25 or 0.125
226 mM, Fig. 2C and D) with that sub-inhibitory dose of AmB (1.57 μg/ml) displayed a synergistic

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227 efficacy against *Aspergillus* conidial swelling and germination after 24h (Fig.2E and F), To confirm
228 the synergism of ATRA in combination with AmB or POS, currently used as primary therapy
229 against aspergillosis, antifungal susceptibility tests were also performed *in vitro*. As shown in Table
230 1, the lowest concentration of ATRA that caused prominent growth inhibition (MIC≥50) of the
231 fungus was 75 µg/ml (250 mM), while MIC values of AmB and POS were 3.15 µg/ml and 0,0018
232 µg/ml, respectively. Of note, both the antifungal drugs showed a synergistic inhibitory effect toward
233 *A. fumigatus* in combination with a low ineffective dose of 35 µg/ml (125mM) of ATRA. Notably,
234 this synergistic interaction was more remarkable with POS. Indeed, the MIC values of AmB and
235 POS combined with ATRA were one dilution and three dilutions lower, respectively, in
236 comparison to the MICs of the drugs alone (MIC AmB plus ATRA 1.5 µg/ml *vs* MIC AmB alone
237 3.15 µg/ml; MIC POS plus ATRA 0.00022 µg/ml *vs* MIC POS alone 0.0018 µg/ml). These results
238 suggest a promising interaction *in vivo* and the therapeutic possibility to reduce classical antifungal
239 drug dosage.

240

241 **ATRA reduced mortality in a rat model of invasive pulmonary aspergillosis**

242 We investigated the efficacy of ATRA in a rat model of IPA, also in comparison with antifungal
243 drug Posaconazole, commonly used in the clinical practice against IPA. Thirty rats were divided in
244 three experimental groups (n=10): control, ATRA-treated and Posaconazole-treated group. ATRA
245 dosage and administration protocol (2 mg/kg, intraperitoneally), starting 8 days before the infection,
246 was established in a preliminary study (data not shown). Posaconazole treatment (4 mg/kg, per os)
247 started concurrently with the infection (18). As shown in Figure 3, both drugs impacted IPA
248 progression compared to the control group. In particular, ATRA-treated rats were 100% alive 6 day
249 after the infection. During the second week, ATRA and Posaconazole displayed a very similar effect
250 on survival, with 60% of rats alive at the end of the study ($p<0.05$ *vs* control). Those *in vivo*
251 findings further supported ATRA efficacy as promising for IPA therapy.

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252 **ATRA enhances macrophage phagocytosis of *Aspergillus* conidia *in vitro***

253 Macrophages play a major role in controlling *Aspergillus* infection (19). In the present study we
254 also evaluated the capability of ATRA to modulate macrophage phagocytic activity *in vitro*. As
255 reported in Fig. 4A, ATRA significantly increased conidia phagocytosis by U-937 human
256 macrophages compared to control (at 5 or 10 µM, around 20 and 40%, respectively). Image analysis
257 of macrophage phagocytosis (Figure 4B), confirmed the increased phagocytosis and adherent
258 macrophage-conidia complex after ATRA treatment (5 and 10 µM). Those data suggested ATRA
259 improves macrophage phagocytosis that likely contribute to antifungal activity against IPA *in vivo*.

260

261 **ATRA inhibits *Aspergillus* Hsp90 expression and Hsp90-related genes *in vitro***

262 To confirm whether ATRA is an inhibitor of *Hsp90*, *Aspergillus* conidia were cultured in ATRA-
263 supplemented medium for 24h. Immunoblot analysis showed that after 24 and 48h of ATRA (1mM)
264 treatment, Hsp90 protein expression was almost suppressed (Figure 5A-B). In the presence of the
265 reduced density and growth rate of *Aspergillus* conidia, qRT-PCR documented in ATRA-treated the
266 strong decrease (about 70%; $P<0.05$) of *Hsp90* transcripts compared to untreated *Aspergillus*
267 conidia (Figure 5C). It was previously reported that the expression of the conidiation-specific genes
268 *AbaA* and *WetA* is reduced in the presence of *Hsp90* inhibitor (20). To confirm that fungistatic
269 activity of ATRA is Hsp90-mediated, we also quantified the expression of *AbaA*, and *WetA*; qRT-
270 PCR analysis showed that ATRA-induced-reduced conidia formation associates to the inhibition of
271 *Hsp90* expression and decreased *AbaA* (about 60%) and *WetA* (about 60%) transcripts in *Aspergillus*
272 cultures (Fig. 5D). Similar results were also observed at 62µM ATRA concentration (data not
273 shown). Hsp90 is also reported to interact with the catalytic subunit of calcineurin (21), necessary
274 for correct hyphal growth(22). We investigated ATRA-induced inhibition of the calcineurin pathway
275 and quantified the expression of *CrzA*, a major signaling component of the zinc finger transcription
276 factor. *CrzA* expression was strongly reduced (about 40%) in presence of ATRA (Figure 5D). Those

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277 data demonstrated that ATRA is a potent inhibitor of fungal *Hsp90* expression and Hsp90-related
278 genes and that its inhibition likely contributes to the fungistatic activity of ATRA against
279 *Aspergillus in vitro.*

280

281 **Heat shock protein 90 ATP-binding site is a target of ATRA**

282 Hsp90 is a chaperone protein required for the activation and stabilization of a wide variety of client
283 proteins, many of them involved in crucial cellular pathways (23). In the absence of *A. fumigatus*
284 Hsp90 X-ray structure, the crystal structure of Hsp90 from *S. cerevisiae*, with PDB ID 2CG9 (24),
285 whose sequence shares around 78% of identity and 95% of query cover with *A. fumigatus*, has been
286 used as a template to generate the *A. fumigatus* Hsp90 model structure (Fig. 6A). A structural
287 bioinformatic analysis was performed to investigate whether Hsp90 may be an ATRA-specific
288 pharmacological target against *Aspergillus* growth. The ATRA structure, as observed with the ATP
289 molecule, completely filled the Hsp90 ATP-binding site in the N terminal domain of the *Aspergillus*
290 *fumigatus* model structure (Fig. 6 B and C). In particular, the trimethylcyclohexen moiety was
291 located at the bottom of the ATP-binding site, fully stabilized by hydrophobic contacts with the
292 residues (Fig. 6 B and C;(25)), while the carboxyl terminal group was involved in hydrogen bonds
293 with residue Arg98 located at the external border of the ATP-binding site. The interaction energy,
294 evaluated by molecular docking simulation, was around -8.9 kcal/mol, a value only slightly below
295 that obtained using the same procedure with the ATP molecule (-9.7 kcal/mol). These results
296 strongly supported that ATRA molecule may be considered a competitive inhibitor of the Hsp90
297 ATP binding site, in particular at high concentrations, and further confirm Hsp90 as a target of
298 ATRA-induced fungistatic activity.

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300 **DISCUSSION**

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301 Over the last decades, the incidence of IFIs constantly increased. Moreover, the use of fungicidal
302 drugs in the treatment of fungal diseases led to the development of resistance burden(26). Here we
303 documented that ATRA displayed a strong fungistatic activity in *Aspergillus* cultures and reduced
304 mortality in a model of IPA in cortisone acetate immunosuppressed rats. Our preliminary data also
305 showed that therapeutic benefit of ATRA is dose-dependent and most importantly, the outcome
306 comparable to that obtained with Posaconazole, used as current standard care for the treatment of
307 IPA. Our *in vitro* and *in vivo* findings help to explain some observations previously reported in the
308 clinical practice. In leukemic patients treated with ATRA and other drug, a lower occurrence of
309 overall fungaemic events has been observed (27,28). Thus, the low clinical incidence of fungal
310 infections is likely linked to ability of ATRA to rapidly differentiate leukemic promyelocytes (29)
311 but, more importantly, to its direct antifungal and immunomodulatory properties. In this light, we
312 also documented a synergistic therapeutic activity of suboptimal doses of ATRA combined with
313 sub-inhibitory concentration of the antifungal drug Amphotericin B against *Aspergillus* conidia
314 germination. This promising interaction could provide an important potential advantage with the
315 use of ATRA in the clinical practice allowing to reduce the dosage of classic antifungal drugs with a
316 greater control of their side effects as well as a potential better control of the development of fungal
317 drug-resistance. However, further experimental *in vivo* studies are required to establish an optimal
318 therapeutic protocol for the use of ATRA in association with antifungal drugs against IPA and
319 systemic *Aspergillus* infections.

320 It is well known that conidial germination represents a crucial step in the pathogenesis and
321 progression of IPA. Alveolar macrophages are the first important line of the host's immune defense
322 against *Aspergillus* conidia. In healthy individuals, lung resident macrophages are able to rapidly
323 engulf and kill inhaled conidia and are not capable of internalizing hyphal cells(30). In
324 immunocompromised patients, non-phagocytosed conidia produce germ tubes that elongate into
325 hyphae in the lung tissue, causing a systemic infection for their capacity to invade of blood vessels.
326 Here, we demonstrated that ATRA increases in a dose-dependent manner, the number of adherent

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327 and internalized resting *Aspergillus* conidia by macrophages, suggesting its modulatory action on
328 the innate immunity. Our results were in line with previous studies showing that ATRA is able to
329 enhance macrophage phagocytosis against *Candida* yeasts and to favour healing of pneumonia by
330 *Pneumocystis carinii* in an affected patient(29, 31). ATRA, by suppressing the conidial germination
331 and hyphal growth could exert a crucial role in facilitating the conidia uptake and their elimination
332 by phagocytic cells, preventing the spread of infection as well as host tissue damage caused by
333 hyphal invasion. Considering the crucial role of macrophages in *Aspergillus* conidia clearance and
334 that the phagocytic activity is impaired in severely immunocompromised patients, the impact of
335 ATRA on phagocytic activity of macrophages is quite relevant. Recent studies support also the
336 hypothesis that efficacy of vitamin A and ATRA alone or in combination with other drugs are
337 related to the capacity to stimulate the monocyte-mediate immune response (32).

338 Recent works indicate that fungal Hsps are implicated in several processes, including pathogenicity,
339 phase transition, and antifungal drug resistance(33). The function of Hsp90 in *Aspergillus* has been
340 also investigated and genetic and pharmacologic studies indicate an important role of Hsps in
341 conidiation and in cell wall stress-compensatory mechanisms(33). Reduced Hsp90 expression in
342 ATRA-treated *Aspergillus* conidia cultures we reported an important target for inhibiting replication
343 and controlling fungal drug resistance. It was demonstrated that Hsp90 itself is subjected to
344 posttranslational modification in a cell cycle-dependent manner. In *S. cerevisiae*, during S phase of
345 the cell cycle the tyrosine kinase Swe1 phosphorylates a residue in the N-terminus of Hsp90 that
346 facilitates chaperone interaction with numerous client proteins (34). A role for Hsp90 in *C. albicans*
347 cell cycle progression has been also established and a molecular link between Hsp90, cell cycle
348 regulation and morphogenesis defined, identifying Hsp90 as an important player in cell cycle
349 progression (35). Besides Hsp90, our results showed that ATRA down-regulates transcription of
350 other genes, in particular AbaA and WetA; Their inhibition is reported in association with decreased
351 conidia formation (36). We documented the down-regulation of CrzA expression in ATRA-treated
352 *Aspergillus* cultures. CrzA is involved in the calcineurin pathway and hyphal growth (37).

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353 In order to better elucidate how ATRA targets the expression of Hsp90, molecular docking
354 demonstrated that ATRA can fit the Hsp90 ATP-binding site in the N terminal domain of the
355 *Aspergillus fumigatus* model structure. Hsp90 is an ATP-dependent molecular chaperone which is
356 essential in eukaryotes. Hsp90 is required for the activation and stabilization of a wide variety of
357 client proteins, and many of them are involved in important cellular pathways(38). Structurally,
358 Hsp90 is a flexible dimeric protein composed of three different domains which adopt structurally
359 distinct conformations. ATP-binding triggers directionality in these conformational changes and
360 leads to a more compact state. Post-translational modifications of Hsp90, such as phosphorylation
361 and acetylation may provide another level of its regulation. Phosphorylation and acetylation
362 influence the conformational cycle, co-chaperone interaction, and inter-domain
363 communications(39). Targeting Hsp90 to reduce stress response compensatory pathways with
364 fungal-specific inhibitors of ATRA may represent an attractive novel antifungal strategy (40)

365 In conclusion, we documented the efficacy of ATRA in a rat model of IPA and its synergistic
366 capacity to improve efficacy of antifungal agents *in vitro*. ATRA, for its direct fungistatic action
367 targeting the expression of fungal Hsp90, combined with its immunoadjuvant effect against
368 opportunistic fungi, may represent a promising novel candidate in the armamentarium of systemic
369 therapeutic strategies for the treatment and/or prophylaxis of IPA and IFIs either alone or in
370 association with conventional antifungal drugs.

371

372 **Acknowledgements**

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374

375 **DISCLOSURE**

376 The Authors declare no conflicts of interest.

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500

501 **FIGURE LEGENDS**

502 **FIG. 1. ATRA inhibits germination of *Aspergillus fumigatus* conidia.** Representative images of
503 the effect of different ATRA concentrations on *Aspergillus fumigatus* conidia germination. The
504 conidia germination was followed using an optical microscope with a 40x magnification objectives
505 lens. Microscopic images were recorded 18 and 30 hrs after the treatments. One of three
506 representative experiments is shown**.** Bars indicate 50 µm.

507 **FIG. 2. Synergic effect of ATRA and Amphotericin B on *Aspergillus fumigatus* conidia**
508 **germination**. (A) Representative images of the effect of AmB and ATRA alone and in combination
509 on *Aspergillus fumigatus* conidia germination, followed using an optical microscope with a 40x
510 magnification objectives lens. Microscopic images were recorded 30 hrs after the treatments. One
511 of three representative experiments is shown**.** Bars indicate 50 µm.

512

513 **FIG. 3. ATRA and Posaconazole show a similar efficacy in a rat model of invasive pulmonary**
514 **aspergillosis**. Kaplan-Meier analysis of overall survival of 10 rats/group treated with ATRA (2

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515 mg/kg, ip), Posaconazole (4 mg/kg, per os) or vehicle (control group). Statistical significance of
516 ATRA and Posaconazole treatments versus control group was evaluated by Log-Rank test analysis:
517 *P=0.05.
518

519 **FIG. 4. Effect of ATRA on macrophage phagocytosis of *Aspergillus fumigatus* conidia *in vitro*.**
520 (A) Representative image of bar graph showing an increased phagocytosis of *Aspergillus fumigatus*
521 conidia by human macrophage cell line U-937 incubated overnight with DMSO (control) or
522 different concentrations of ATRA, and exposed to unopsonized *Aspergillus* conidia., Data collected
523 from three independent experiments and expressed as percentage of control. Student's t test was
524 used to determine the significance of values in experimental groups and defined as $P < 0.05$. *$P <$
525 0.05; ** $P < 0.01$. (B) Representative image of macrophage phagocytosis also performed in 24-well
526 plates. All images of the cell culture plates were recorded at the end of the incubation period using
527 an optical microscope with a 40x magnification objectives lens. One of three representative
528 experiments was shown. Bars indicate 50 µm.
529

530 **FIG 5. ATRA inhibits the heat shock Hsp90 protein and mRNA expression of *Aspergillus*.** (A)
531 Representative blots and (B) bar graphs of densitometric evaluation of Hsp90 protein expression on
532 *Aspergillus* conidia treated with ATRA. *Aspergillus* conidia were cultured in L15 with 2% FCS at
533 37°C for 24h, in the presence of 1 mM of ATRA. The average and SEM of triplicate experiments
534 are shown. Significant changes reported $^{***}P < 0.001$ at Student's t-test. (C) Hsp90 mRNA
535 expression level assessed by qRT-PCR and normalized to untreated *Aspergillus* conidia. (D). *CrzA,*
536 *WetA* and *AbaA* mRNA levels assessed by qRT-PCR and normalized to untreated *Aspergillus*
537 conidia. The expression ratios were normalized to elongation factor 1α expression, and were
538 calculated according to the DDCt method. All experiments were performed in triplicate, and data
539 were presented as the mean ± standard error (SEM). ** $P < 0.01$; *** $P < 0.001$, in a Student's t
540 test.

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542 **FIG. 6. Heat shock protein 90 is a pharmacological target of ATRA.** (A) Structural model of
543 *Aspergillus fumigatus* Hsp90 dimeric structure. On the right, a magnification of *Aspergillus*
544 *fumigatus* ATP binding site including the ATP molecule from the template, colored by atom type.
545 (B) Schematic view of the best molecular docking complexes between Hsp90 *Aspergillus*
546 *fumigatus.* ATP (left panel) and ATRA molecule (right panel). The hydrogen bonds have been
547 indicated with green dashed lines between the interaction partners. The residues composing the
548 active sites that are in proximity of the molecules are shown. Images have been produced using the
549 LigPlot+ software. (C) Best molecular docking complexes between *Aspergillus fumigatus* Hsp90
550 with ATP (left panel), or ATRA molecules (right panel). The α-helices and the loops are shown as
551 light blue spirals and wires, respectively. The molecular surface is shown and the ligand molecules,
552 hosted in the Hsp90 ATP binding site, are depicted by sphere representations. Panels A and C have
553 been produced using the Chimera program.

554

Activty of ATRA alone and combined with Amphotericin B or Posaconazole on *A. fumigatus* growth

MIC (µg/ml) 24 h			
Substances alone		Substances combined	
	µg/ml		µg/ml
ATRA	75		-
AmB	3.15	ATRA + AmB	1.57
POS	0.0018	ATRA + POS	0.00022

555 **Table 1**. MIC: (minimal inhibitory concentration) is the lowest concentration of ATRA, AmB or
556 POS alone or in combination, at which a prominent decrease in turbidity is observed when
557 compared with the drug-free control (≥50% growth inhibition) after 24 h of incubation. In the
558 combination antifungal treatments, ATRA was used at a suboptimal dose of 37.5 µg/ml.

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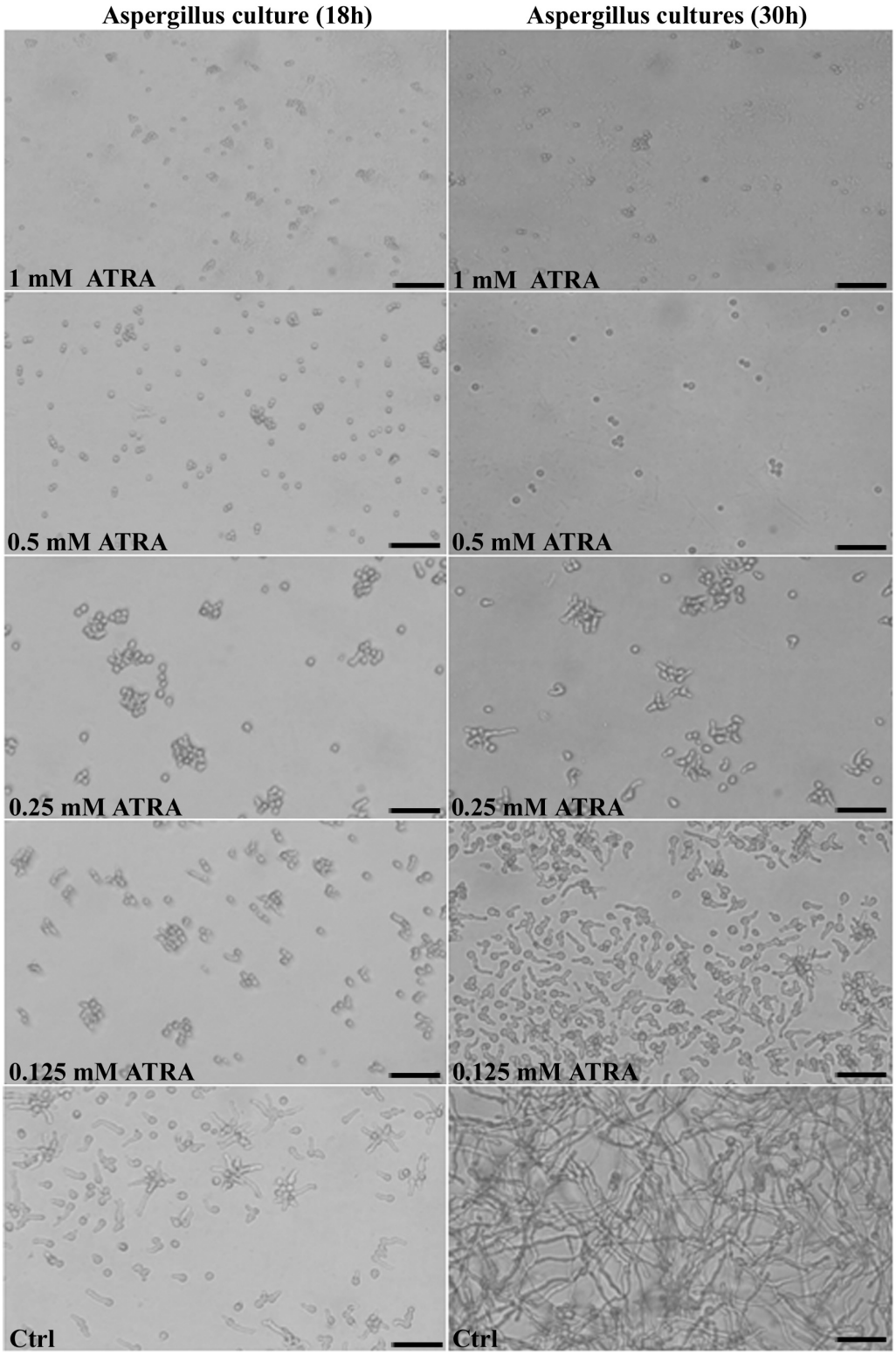

Fig. 1

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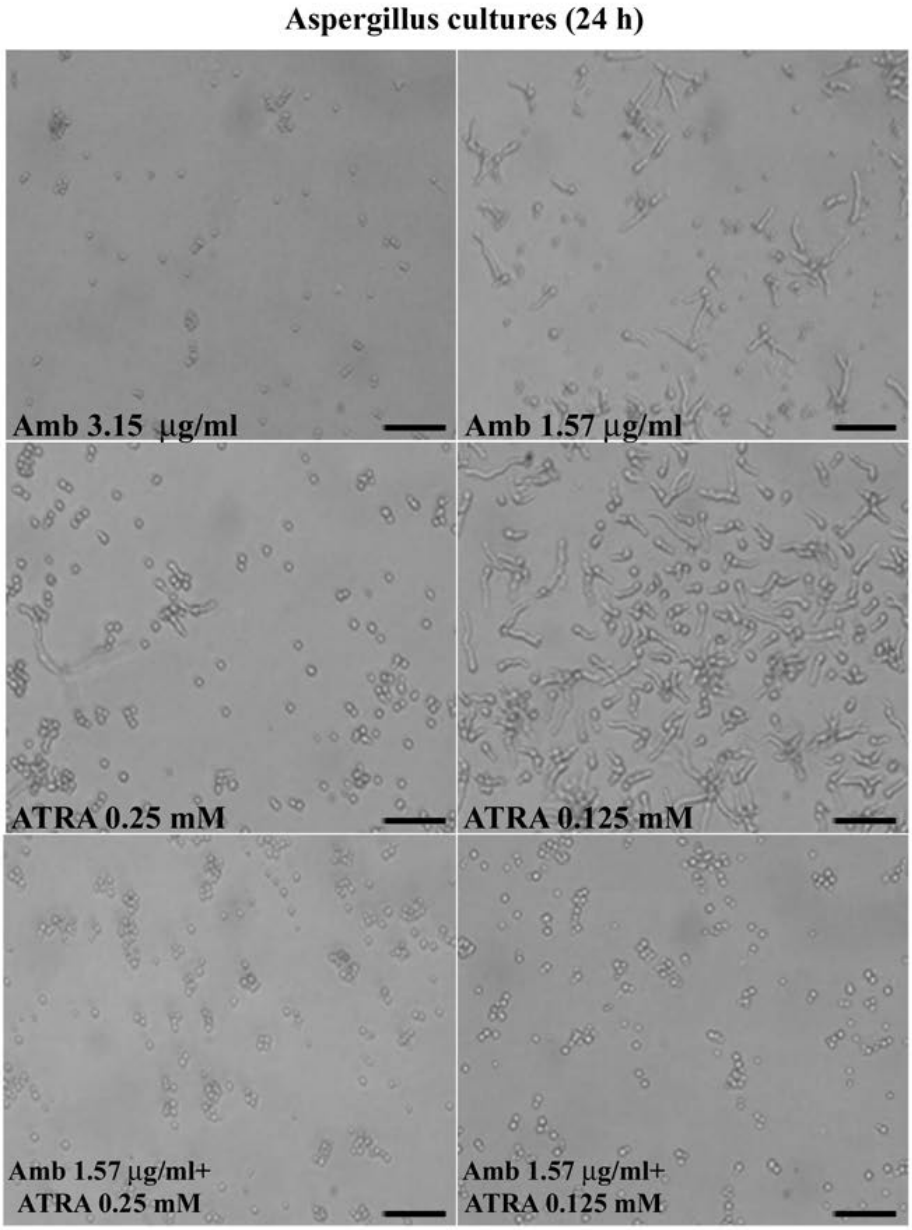

Fig. 2

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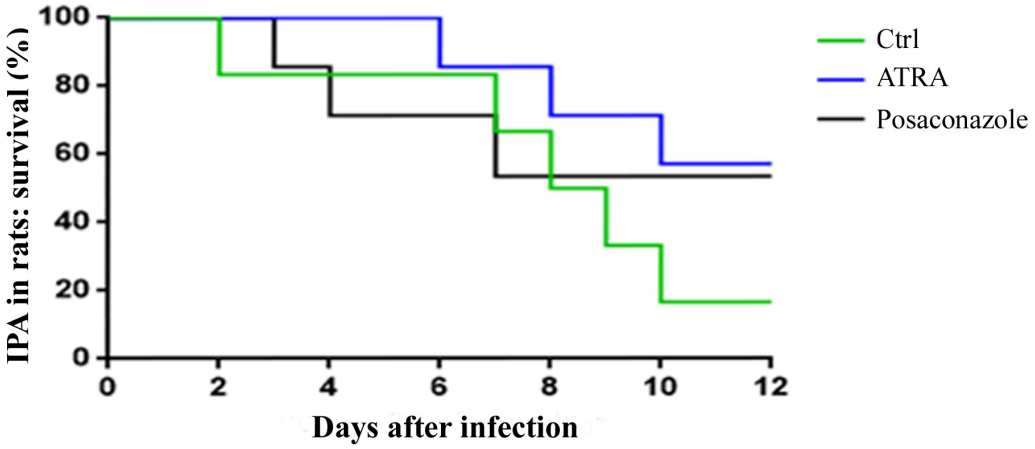

Fig. 3

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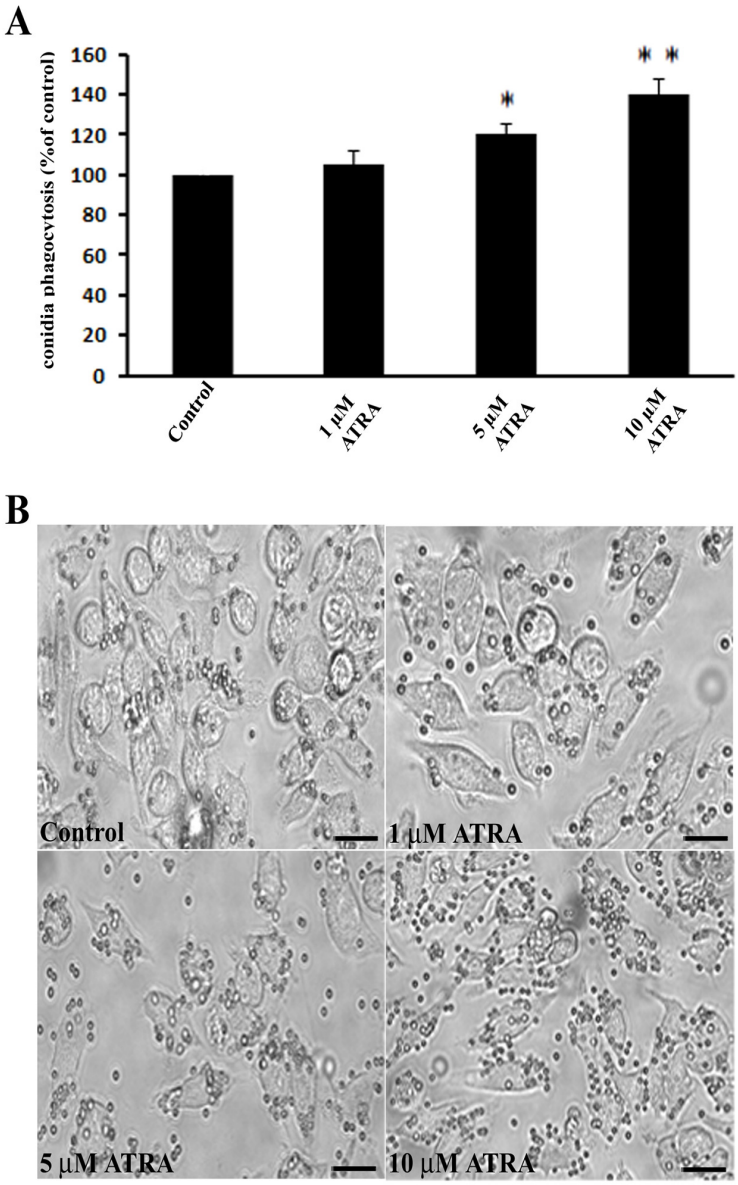

Fig. 4

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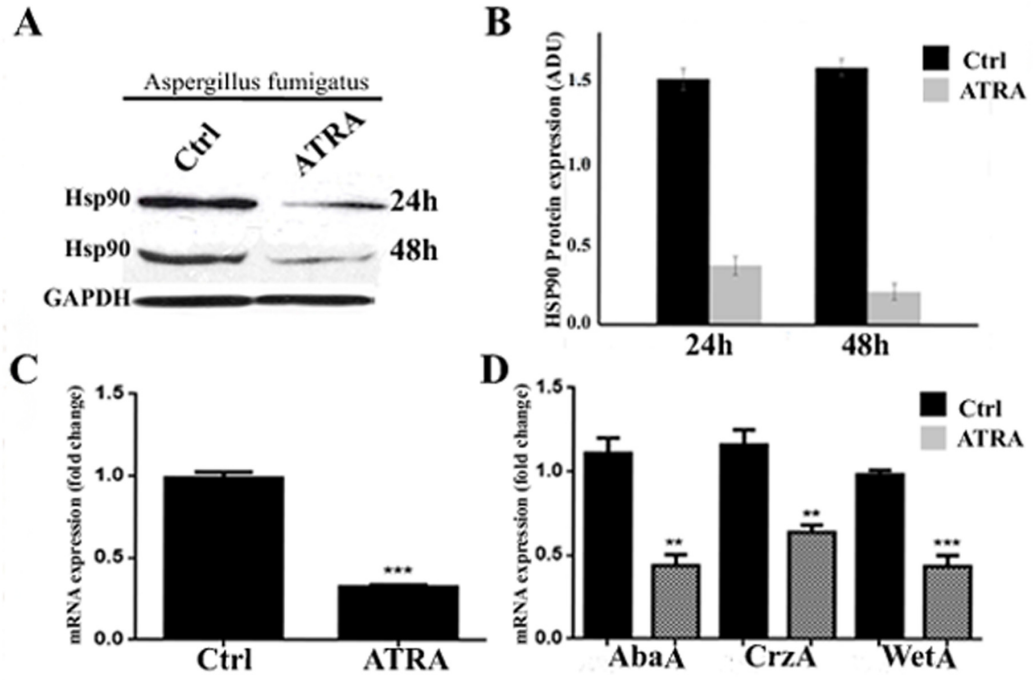

Fig.5

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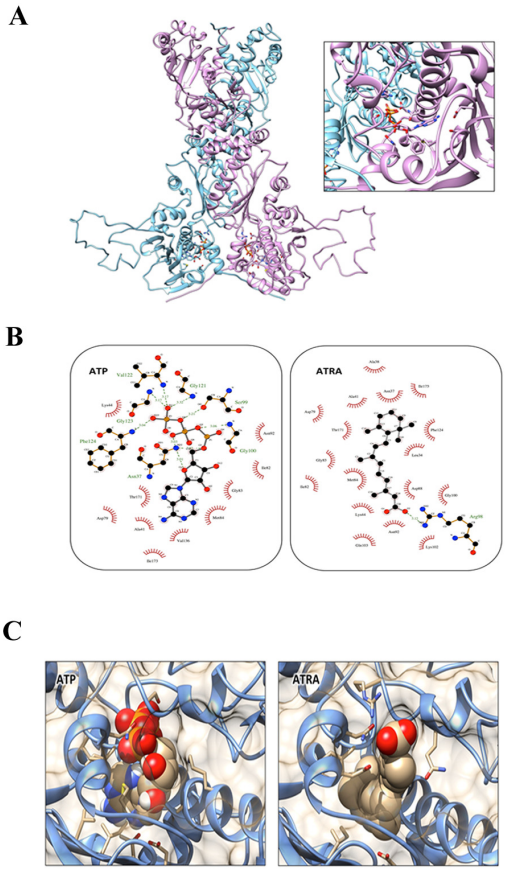

Fig. 6

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