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Synthesis of *N*-aryl-3-(indol-3-yl)propanamides and their immunosuppressive activities

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ABSTRACT

N-Aryl-3-(indol-3-yl)propanamides were synthesized and their immunosuppressive activities were evaluated. This study highlighted the promising potency of 3-[1-(4-chlorobenzyl)-1*H*-indol-3-yl]-*N*-(4-nitrophenyl)propanamide **15** which exhibited a significant inhibitory activity on murine splenocytes proliferation assay in vitro and on mice delayed-type hypersensitivity (DTH) assay in vivo.

Cyclosporin A (CsA), tacrolimus (FK506), and sirolimus (rapamycin) are known to be potent immunosuppressive agents. They act by inhibiting T lymphocyte proliferation during immune response.[1] All three compounds are current chemotherapeutic agents, limiting chronic rejection after organ transplantation and improving lifetime of grafts receivers, and successfully used for treatment of autoimmune diseases in clinic.[2] However, these molecules suffer from several side effects such as nephrotoxicity, neurotoxicity, infection, cancer, hyperlipidemia, and hypertension.[3] Thus, the search for new immunosuppressants with a comparable efficacy but with lower toxicity is an important task for medicinal chemistry.

We previously described a series of *N*-pyridinyl(methyl)-(indol-3-yl)propanamides with a promising immunosuppressive activity.[4,5] Our initial structure–activity investigations showed the importance of the benzyl moiety and the nature of the pyridine ring for immunosuppressive potential and this study disclosed compound **1** as a promising lead (Fig. 1). This compound exerted a potent inhibitory activity on murine splenocytes proliferation (87% inhibition at 90 μM, 19% inhibition at 30 μM, compared to 90% inhibition obtained with CsA at 0.5 μM). Previous experiments[5] highlighted its antiproliferative activity in vitro on T lymphocytes (IC_{50} = 17 μM) and a significant immunosuppressive effect in vivo in a model of delayed-type hypersensitivity (DTH) in mouse. We also showed its non-toxicity and its oral bioavailability.

Previously, compound **1** was found to decrease IL2-induced T lymphocyte proliferation by inhibiting preferentially JAK3 kinase over JAK2 (IC_{50} = 52.0 μM and 133.6 μM, respectively).[6] Moreover, compound **1** significantly prolonged rat heart allograft survival demonstrating its in vivo immunosuppressive potential.

The Janus kinases (JAKs), consisting of JAK1, JAK2, JAK3, and TYK2, are an important family of cytoplasmic tyrosine kinases as a consequence of their essential role in cytokine signal transduction.[7] Janus Kinase 3 is a particularly attractive target for therapeutic intervention in the treatment of autoimmune disorders, inflammatory diseases, and organ transplant rejection because, unlike other JAK family members that are widespread, JAK3 expression is restricted to haematopoietic cells.[8] A number of inhibitors of JAK3 have already been described.[9]

Herein, we will report our continuous work consisting of synthesis and immunosuppressive activity evaluation of 3-[1-(4-chlo-

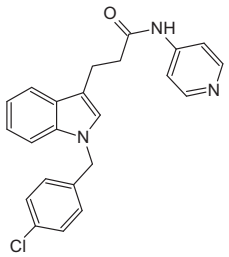

Figure 1. Chemical structure of lead compound **1**.

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robenzyl)-1*H*-indol-3-yl]propanamide derivatives in order to explore the effect of *N*-aryl substitution with compound **1** as the initial lead.

Synthesis of *N*-aryl-3-(indol-3-yl)propanamides **6–15** was performed, as previously described,[4,5] in four or in five steps from commercially available 3-(1*H*-indol-3-yl)propanoic acid **2** (Scheme 1).

To obtain the target amides, the first step was the esterification of 3-(1*H*-indol-3-yl)propanoic acid **2** in ethanol–hydrogen chloride medium at reflux. Ester **3** was further reacted with 4-chlorobenzyl chloride in the presence of Cs_2CO_3 in anhydrous acetonitrile to afford the corresponding *N*-(4-chlorobenzyl)indole derivative **4**. Subsequent hydrolysis under basic conditions followed by the amidation of the resulting propanoic acid **5** with Mukaiyama reagent in refluxing CH_2Cl_2 gave the *N*-aryl-3-(indol-3-yl)propanamides **6–15** in low to moderate yields (17–58%) depending on the nature of the aromatic amines.

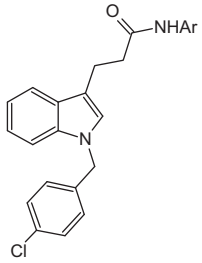
All starting amines were commercially available except 4-aminoquinoline that was prepared by treatment of the 4-quinolone with $POCl_3$ at reflux, in a first step, to afford 4-chloroquinoline in quantitative yield.[10] Nucleophilic displacement of the chlorine atom was performed in the presence of ammonia in phenol by following a literature procedure.[11]

Moreover, catalytic reduction of the nitro functional group of compound **15**, using palladium charcoal in THF at room temperature, yielded amino derivative **16**.

The new *N*-aryl-3-(indol-3-yl)propanamides **6–16** were tested in vitro for their inhibitory activity on concanavalin A (ConA)-induced T cell proliferation with compound **1** as the standard. Freshly isolated murine spleen cells were stimulated with 1 μg/mL ConA for 72 h in the presence of two different doses of the tested compound.[12] The pharmacological results are summarized in Table 1.

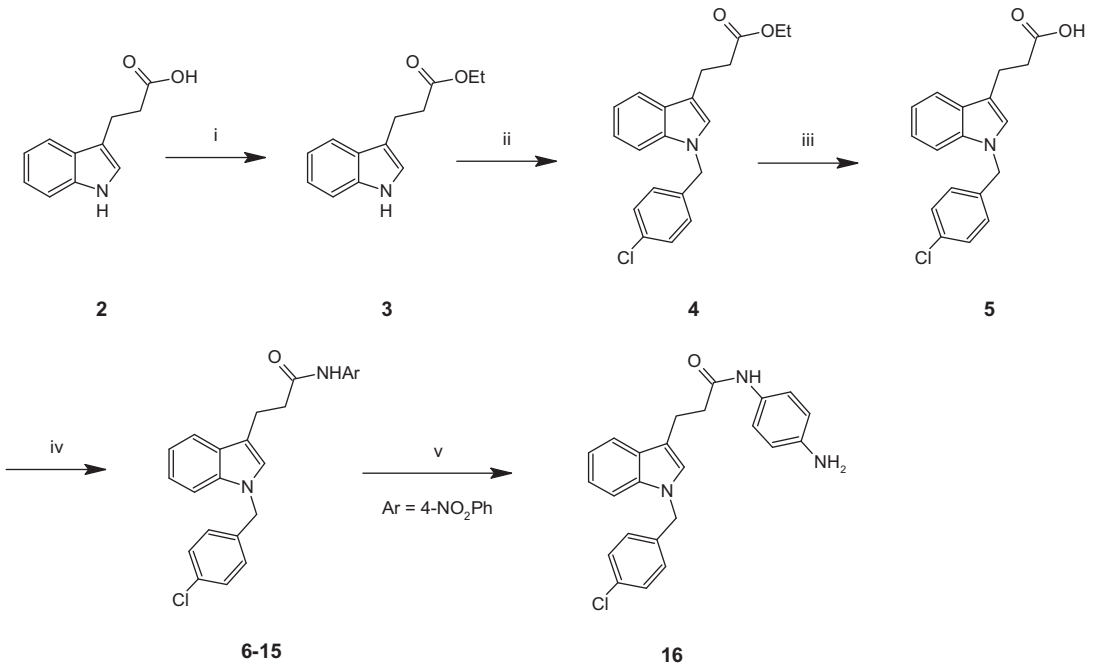
To understand the incidence of the *N*-aryl substitution in the pharmacological activity, diverse alternatives to the 4-pyridyl group (compound **1**) were studied. Replacement of the heteroaromatic ring by the phenyl ring (compound **12**) or by 4-substituted phenyls revealed that the presence of a polar and electro-withdrawing group (compounds **13** and **15**) was a more suitable modification for getting an active entity except for **14** bearing a trifluoromethyl moiety. Indeed, the amino analog **16** displayed no activity but stimulated the proliferation response of cells (data not shown).

Table 1
Splenocytes proliferation assay data for the *N*-aryl-3-(indol-3-yl)propanamide compounds **1**, **6–16**



Compounds	Ar	Splenocytes proliferation	
		Inhibition% ± SEM at 90 μM[a]	Inhibition% ± SEM at 30 μM[a]
1	Pyridin-4-yl	87 ± 1.7	19 ± 3.2
6	Pyridin-3-yl	40 ± 1.6	28 ± 2.8
7	Pyridin-2-yl	26 ± 1.4	na
8	Quinolin-4-yl	37 ± 1.6	20 ± 2.0
9	Quinolin-5-yl	na	na
10	Quinolin-6-yl	86 ± 1.2	na
11	Isoquinolin-5-yl	17 ± 2.1	na
12	Phenyl	na	na
13	4-Cyanophenyl	78 ± 1.0	30 ± 1.2
14	4-Trifluoromethyl-phenyl	18 ± 1.7	nd
15	4-Nitrophenyl	92 ± 1.5	87 ± 1.7
16	4-Aminophenyl	na	na

na = not active.
nd = not determined.
[a] Cell assay results of one representative experiment out of three performed.



Scheme 1. Reagents and conditions: (i) EtOH, HCl, reflux, 95%; (ii) Cs_2CO_3, 4-ClPhCH$_2$Cl, CH_3CN, reflux, 81%; (iii) NaOH 1 N, EtOH, reflux, 94%; (iv) Et_3N, CNMPI, Ar-NH_2, CH_2Cl_2, reflux, 17–58%; (v) H_2 (10 bars), Pd/C 10%, THF, rt, 45%.

Introduction of fused-pyridine heterocyclic scaffolds such as quinoline (compounds **8** and **9**) or isoquinoline (compound **11**) had deleterious impact on inhibition of splenocytes proliferation, even for **8**, the direct analog of **1**. Incorporation of bulky heteroaromatic amines led to a large decrease of potency. We suspected that the activity of derivative **10** (86% inhibition at 90 μM) was the consequence of cellular toxicity since it was inactive at 30 μM.

Interestingly, shifting the nitrogen atom of the pyridine ring from the *para*- to the *meta*- or *ortho*-position (compounds **6** and **7**, respectively) resulted in a significant loss of activity. In the case of ATP-competitive JAK3 inhibitor **1**, the orientation of the pyridine ring would be favorable for a hydrogen-bond between the pyridine nitrogen atom and a suitable amino acid in the hinge region of the ATP-binding site of the kinase.

In addition, the lack of activity of phenyl derivative **12** would confirm a possible interaction between the heteroatom or the substituent at the 4-position and an amino acid residue in the hinge region of the target enzyme. Very low activity of quinoline or isoquinoline derivatives would be rationalized by a steric hindrance at the level of the binding site.

One possible explanation for keeping activity with compounds **13** and **15** would be that cyano or nitro groups may display hydrogen-bond acceptor properties and may mimic the pyridine nitrogen interaction with the protein kinase target. Although there is ample crystallographic, physicochemical, and theoretical evidence that the nitrile group is a reasonably strong hydrogen-bond acceptor, there are few examples where it is known to act as the conserved acceptor in a kinase ligand.[13] In the literature, it was also hypothesized that farnesyltransferase inhibitors might benefit from the presence of a polar moiety with hydrogen-bond acceptor properties like nitro group.[14]

The results showed that, among the tested compounds, *N*-(4-nitrophenyl)propanamide **15** exhibited the highest bioactivity. The antiproliferative effect of **15** was not due to direct toxicity since 95% of the cells were still viable after 48 h incubation with 90 μM of **15** (data not shown).

Based upon previous data concerning the mechanism of action of compound **1** involving JAK3 inhibition, compound **15** was submitted to JAK 3 and JAK 2 kinases inhibition assay. Interestingly, 4-nitrophenyl derivative exhibited better activity than compound **1** against JAK3 enzyme with an IC_{50} of 34.2 μM (IC_{50} = 52.0 μM for **1**) and showed a threefold selectivity in favor of JAK3 relative to JAK2 (IC_{50} = 106.7 μM). These results were consistent with previous conclusion reporting the correlation between JAK3 inhibition and immunosuppressive activity.

On the basis of screening data, compound **15** was subjected to more in-depth characterization. We first investigated its effect on proliferation of murine splenocytes using a large range of doses in order to confirm its antiproliferative activity on T cells. As shown in Figure 2, we found that it inhibited mitogen-dependent T cell proliferation in a dose-dependent manner on murine splenocytes with an IC_{50} of 19.8 ± 1.9 μM, similar to compound **1** (IC_{50} = 17.0 μM).[5] These data confirmed its immunosuppressive potential.

Finally, compound **15** has been selected for in vivo evaluation on delayed-type hypersensitivity reaction (DTH) in mice. Experiments were performed as previously described with slight modifications.[5] Animals were sensitized with 5×10^6 sheep red blood cells (SRBC) by iv and challenged four days later by injection of SRBC into the hind footpad. Compounds were administrated orally from the day of priming until the day of challenge. Figure 3 shows the inhibition of DTH footpad swelling in mice treated with compound **15** at 50 mg/kg, 80 mg/kg, and 120 mg/kg.[15]

Derivative **15** significantly inhibited the DTH response in a dose-dependent manner. At the highest dose (120 mg/kg), inhibition of DTH (75.5%) was comparable to compound **1** (69%, Fig. 3) and CsA (73.9%, data not shown). Moreover during the 5-days treatment, no acute toxicity was observable in treated mice compared to untreated control mice. These results thus demon-

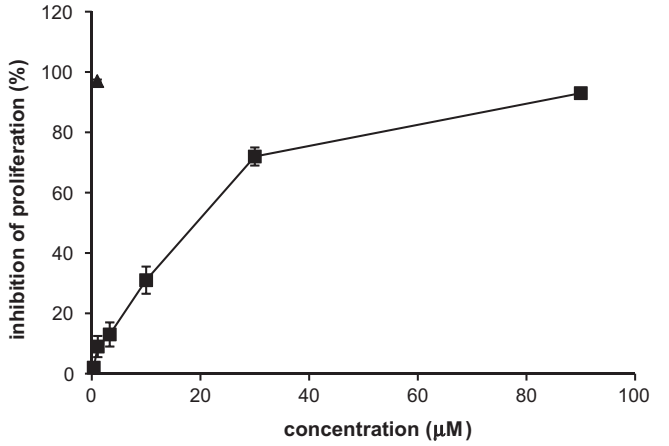


Figure 2. Effect of compound **15** on T cell proliferation. Murine splenocytes were stimulated with ConA in the presence of increasing concentrations of compound **15** (filled squares) or CsA at 1 μM (filled triangle). Mean ± SEM of three independent experiments is presented.

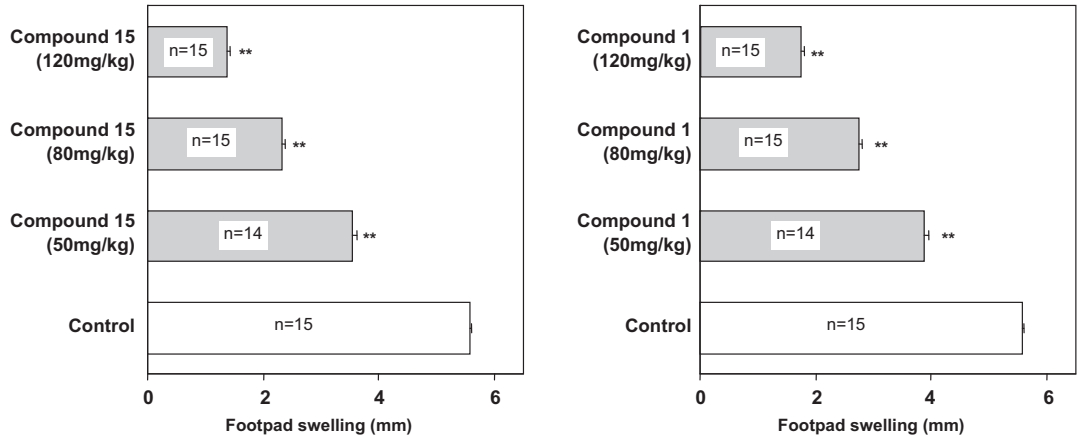


Figure 3. Effect of compounds **15** and **1** on DTH response. Each bar represents the mean difference ± SEM in swelling between the two hind footpads, one injected with saline, the other with SRBC, measured 24 h after challenge. Compounds were administered daily per os from the time of priming until SRBC challenge. The number of mice (n) in each group is indicated. ** p <0.01 compared to the control group.

Table 2
Kinase selectivity of compounds **1** and **15**—IC_{50} values (μM)

	JAK3	JAK2	Erk	HIPK1	Aurora-A	Pim1	c-Abl	KDR	TrkA	Yes
1	52.0	133.6	>100	>100	>100	>100	>100.0	26.0	>100.0	30.0
15	34.2	106.7	>100	>100	>100	>100	>100.0	33.2	>100.0	38.1

strated that compound **15** exerted significant immunosuppressive effects in vivo.

Compound **1**, the reference analog, and the most active compound **15** were tested for selectivity against several kinases, and these results are summarized in Table 2.[16]

Kinase profiling demonstrated that, in addition to their JAK3 micromolar activity, compounds **1** and **15** were also inhibitors of tyrosine kinases KDR and Yes in the micromolar range. Compounds **1** and **15** were inactive for other kinases, including Erk, HIPK1, Aurora-A, Pim1, c-Abl, and TrkA.

In conclusion, among the tested compounds, 3-[1-(4-chlorobenzyl)-1*H*-indol-3-yl]-*N*-(4-nitrophenyl)propanamide **15** showed promising in vitro and in vivo immunosuppressive activity. Nevertheless, JAK kinase docking study needs to be performed to confirm our SAR hypothesis. Further modification of compounds **1** and **15**, namely by introducing various substituents on the benzene ring of the indole core, in order to improve their potency is currently in progress. In addition, promising activity towards KDR and Yes kinases will be also studied more in-depth.

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12. *Splenocytes proliferation assay*: All compounds were solubilized in DMSO and further diluted in RPMI medium (Sigma, St Quentin Fallavier, France) complemented with 1% L-glutamine (Gibco BRL, Paisley, Scotland) and 10% heat inactivated FCS (Sigma) referred as complete medium. Splenocytes were isolated from two spleens of 8-week-old female C57BL/6 (Janvier, Laval, France) mice. Spleens were aseptically harvested and homogenized in a Petri dish containing HBSS medium (Sigma) and splenocytes suspension was hemolysed by buffer containing 20 mM Tris–HCl and 140 mM NH_4Cl. Cells were washed twice with RPMI, subsequently suspended in complete RPMI medium and seeded at densities of 1.5×10^5/well in U-bottom 96-well culture plates (Falcon). Cells were incubated with 1 μg/mL concanavalin A (Sigma) in the presence of several concentrations of studied compounds and cultured at 37 °C in 5% CO_2 in a final volume of 150 μL of complete RPMI medium supplemented with 50 μM mercaptoethanol. Cell proliferation was assessed in sextuplicate after 72 h of culture, by colorimetric detection. Briefly, cells were incubated with 12.5 μg/well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h at 37 °C. Formazan products were solubilized by 100 μL of lysis buffer (dimethylformamide (1 V), SDS 20% (2 V), pH 4.7) and overnight incubation at 37 °C. Cell growth was assessed using a MRX microplate reader (Dynex Technologies, Chantilly, USA) with the test wavelength at 570 nm and expressed as optical density (OD) values. The inhibition of splenocytes proliferation was expressed as inhibitory rate [(OD value of control untreated cells − OD value of treated cells)/OD value of control untreated cells group] × 100.
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15. *Delayed-type hypersensitivity assay*: Compound **15** was solubilized in olive oil and CsA was dissolved in olive oil solution containing 3% absolute ethanol. Groups of five female BALB/C mice (Janvier, Laval, France), 8–9 weeks of age, were injected with 5×10^6 sheep red blood cells (SRBC) (BioMérieux, Marcy-l'Etoile, France) in 200 μL PBS into the tail vein. Four days after immunisation, mice were tested for DTH with a subinflammatory challenging dose ($3–4 \times 10^8$ SRBC suspended in 40 μL) injected into the right hind footpad. The left footpad was used as a control and injected with PBS alone. The size of both hind footpads was measured with an 'Oditest' (Kroeplin, Germany) 24 h after challenge. DTH reaction was determined by the difference in size between the SRBC- and PBS-injected footpads. Mice were daily treated orally for 5 days with different doses of compound **15**, from the time of priming until the SRBC challenge. The DTH inhibition index was calculated as the ratio of DTH reaction in the treated animal versus the DTH reaction in control mice. Results are expressed as mean ± SEM. All results were compared statistically using Dunnett unpaired multiple.
16. *Kinase inhibition assay*: Recombinant kinases were purchased from Millipore or ProQinase. AlphaScreen Protein-A-Detection Kit from Perkin–Elmer was used to quantify the kinase activity. For the assessment of IC_{50} values, compounds were tested at 10 final concentrations between 3.16 nM and 100 μM. Kinase, 10 μM ATP, kinase substrate and the test compound were incubated for 1 h on a 384-well Optiplate in a final volume of 15 μL. The kinase reaction was stopped by adding 10 μL ALPHA-Beadmix. The read out was done on the next morning using an Envision reader (Perkin–Elmer). IC_{50} values were calculated using Graph Pad Prism software.