JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986

JPET#155986

A novel indole-3-propanamide exerts its immunosuppressive activity by inhibiting JAK3 in T cells

Delphine Carbonnelle, Muriel Duflos, Pascal Marchand, Catherine Chauvet, Jean-Yves Petit and François Lang

Immuno-pharmacology Department, UPRES EA-1155-IICiMed, Nantes-Atlantique University, UFR of Pharmaceutical Sciences, 1 rue Gaston Veil, 44035 Nantes, France (DC, CC, JYP, FL)

Pharmacochemistry Department, UPRES EA 1155 IICiMed, Nantes-Atlantique University, UFR of Pharmaceutical Sciences, 1 rue Gaston Veil, 44035 Nantes, France (MD, PM)

UMR INSERM U892, 8 quai Moncousu, BP 70721 44007 Nantes cedex, France (FL)

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

A novel indole-3-propanamide inhibits JAK3 in T cells

Address correspondence to: Pr. François Lang, Inserm U892, 8 quai Moncousu, BP70721 44007 Nantes cedex France . Phone number: (33) 2 28 08 02 06. Fax number: (33) 2 40 35 66 97.

E-mail: flang@nantes.inserm.fr

Number of pages: 27

Number of tables : 0

Number of figures: 7

Number of references: 38

Abstract: 212 words

Introduction: 535 words

Discussion: 860 words

Abbreviations: **CsA**: cyclosporin A; **MFI**: mean fluorescence intensity; γ_c : common γ-chain

Section: Inflammation, Immunopharmacology, and Asthma

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

We previously identified an indole-3-propanamide derivative, AD412, as a potential immunosuppressive agent. Here, we document that AD412 inhibited the proliferative response of CD3/CD28-stimulated human T cells without inhibiting their IL-2 production and also inhibited the proliferation of CTL-L2 cells in response to IL-2. These results prompted us to analyse the effect of our compound on the three main signaling pathways coupled to the IL-2 receptor. We provide evidence that AD412 inhibited the JAK1/3 dependent phosphorylations of Akt, STAT5a/b, and ERK1/2 in IL-2-stimulated CTL-L2 cells. In contrast, AD412 had little effect on the JAK1/2 dependent INF-γ-induced phosphorylation of STAT1 in U266 cells. This suggested a preferential inhibition of JAK3 over JAK1 or JAK 2 activities by AD412, that was confirmed by *in vitro* kinase assays with purified JAK2 and JAK3 kinases. In addition, we provide evidence that the inhibition of IL2 response by AD412 was not due to inhibition of IL2Rα upregulation since neither AD412 nor previously described JAK3 inhibitors (WHI-P154 and AG-490) significantly inhibited IL2-induced IL2Rα over-expression. Finally, we further document the immunosuppressive activity of AD412 *in vivo* by showing that its administration *per os* significantly prolonged heart allograft graft survival. This molecule may thus represent an interesting lead compound to develop new immunosuppressive agents in the field of transplantation and autoimmune diseases.

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

The efficacy of immunosuppression after allograft transplantation is limited by a variety of side effects that endanger long-term graft survival (Hariharan, 2003; Boots et al., 2004; Sulanc et al., 2005). The main reason for drug-induced toxicities is the widespread distribution of their biochemical target. Therefore, inhibiting targets exclusively expressed in lymphocytes should diminish collateral toxicities. In this purpose, intense screening efforts have been performed to characterize novel lymphoid-specific immunosuppressive drugs.

A first pharmacological approach consists in interrupting the signal 1 (TCR-dependent) activation cascade in lymphocytes that triggers the transition from the G0 to the G1 phase of the cell cycle. The development of antagonists of lymphocyte cell kinase (lck), whose expression is restricted to T and NK cells, yielded encouraging results with the characterisation of A-420983 (Waegell et al., 2002) and more recently second generation lck antagonists, A-770041 and derivatives (Burchat et al., 2006; Abbott et al., 2007).

The second strategy consists in blocking the costimulatory signal 2 that allows full T lymphocyte activation. In this respect, clinical trials of co-stimulation blockade in renal transplantation with betalacept (LEA29Y), the second generation fusion protein CTLA4-Ig, gave encouraging results (Larsen et al., 2005).

The first two activation signals 1 and 2 will induce signal 3 i.e. the action of lymphokines on their specific receptors. Thus, immunosuppression can also be achieved by inhibiting signal 3 as exemplified by sirolimus (Rapamycin) or its analogs that block the mTOR complex 1 (TORC1) activated by IL-2R activation (Wullschleger et al., 2006). Activation of mTOR is critically dependent on the activation of Janus protein tyrosine kinases (JAKs) which are associated with the intracellular portions of many cytokines and growth factor receptors. In

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

the case of IL-2, binding of this cytokine to its multimeric high-affinity receptor recruits and activates the IL-2Rβ-associated JAK1 and the common γ-chain (γ_c)-associated JAK3 resulting in activation of STAT5 but also in the initiation of PI3K/Akt and ERK1/2 signaling pathways (Gaffen, 2001). Janus Kinase 3 is a particularly attractive target because, unlike other JAK family members that are widespread, JAK3 expression is restricted to haematopoietic cells (Borie et al., 2004; Papageorgiou and Wikman, 2004). In addition, human or mice genetically deficient in JAK3 display a SCID phenotype (Russell et al., 1995; Baird et al., 1998). A number of inhibitors of JAK3 have already been described such as prodigiosin PNU156804 (Mortellaro et al., 1999), dimethoxyquinazolines WHI-P131 and WHI-P154 (Sudbeck and Uckun, 1999), the tyrphostin AG-490 (Kirken et al., 1999; Wang et al., 1999) or more recently, CP- 690,550 (Changelian et al., 2003), the Mannich base NC1153 (Stepkowski et al., 2005), a natural flavonoid compound, Kaempferol (Cortes et al., 2007) and other synthesis compounds (Clark et al., 2007).

Our laboratory has previously reported novel immunosuppressive derivatives acting on signal 1 or 2 (Sabourin et al., 2004; Carbonnelle et al., 2005). More recently, we described the synthesis and immunosuppressive potential of synthetic *N*-pyridinyl(methyl)indol-3-ylpropanamide compounds (Carbonnelle et al., 2007) (Fig. 1) that inhibited the proliferative response to IL-2. In the present study, we provide evidence that the representative compound of this series, AD412, exerts its immunosuppressive activity by inhibiting IL-2-induced JAK3 activation. Moreover, we demonstrate the *in vivo* immunosuppressive potential of our compound by showing that it significantly prolonged rat heart allograft survival.

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

METHODS

Drugs, mAbs and reagents. AD412: 3-[1-(4-chlorobenzyl)indol-3-yl]- *N*-(pyridin-4-yl)propanamide, JAK inhibitor I: 2-(1,1-Dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one, WHI-P154: 4-[(3-Bromo-4-hydroxyphenyl)amino]-6,7-dimethoxyquinazoline (Calbiochem, VWR, Pessac, France) and AG-490: α-Cyano-(3,4-dihydroxy)-N-benzylcinnamide were solubilized in DMSO. Ciclosporin (CsA) (Tocris, Illkirch, France) was dissolved in absolute ethanol containing 2% Tween 80. IL-2 was obtained from Chiron (Amsterdam, The Netherlands) and IFN-γ from Tebu (Paris, France). Antibodies against total and phosphorylated Akt (Ser 473), GSK3, STAT5a/b, ERK1/2 and STAT1 were purchased from Cell Signaling Technology (Ozyme, St Quentin-Yvelines, France). FITC anti-mouse CD25 mAb was purchased from BioLegend (Ozyme).

Cell isolation and culture. PBMC were isolated from healthy donors by Ficoll-Hypaque gradient centrifugation and resuspended in RPMI medium (Sigma, St Quentin Fallavier, France) with 1% L-glutamine (Sigma) and 10% heat inactivated FCS (Sigma) referred to as complete medium. CTL-L2 murine cells were maintained in complete medium supplemented with IL-2 (50 IU/ml) and 2-ME (50 µM). The U266 myeloma cell line was maintained in complete medium.

Proliferation assays. Freshly isolated PBMC (60 x 10^3/well) were stimulated with biotinylated anti-CD3 and/or anti-CD28 mAbs precoated on streptavidin microbeads M280 (20 x 10^3/well) (Dynal Biotech SA, Compiegne, France) with or without AD412 (30 µM or 90 µM). Proliferation was assessed in sextuplicate after 72 h of culture. CTL-L2 (5 x 10^3/ well) were washed and incubated with a range of IL-2 concentrations (0-125 IU/ml) with or

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

without AD412 (3 to 30 µM) or rapamycin (50 nM). Proliferation was assessed in triplicate after 18 h of culture by thymidine uptake.

Detection of IL-2. Levels of IL-2 in supernatants of PBMC cultures stimulated for 48 h with anti-CD3 and anti-CD28 beads were quantified using ELISA kits (eBioscience, Cliniscience, Montrouge, France) according to the manufacturer's instructions.

Expression of IL-2R. CD25 surface expression on CTL-L2 cells was induced by IL-2 stimulation (25 IU/ml). Cells were cultured for 18 h with or without inhibitors, stained with FITC-conjugated anti-mouse CD25 mAb and analyzed with a FACSCalibur (BD Biosciences). Dead cells were excluded on the basis of Topro-3-iodide staining.

Western blot Analysis. CTL-L2 cells (10×10^6) were incubated with AD412 (90, 30, or 10 µM) and IL-2 (100 IU/ml), washed and starved of IL-2 for 8 h, and stimulated for 0.5 h or 1 h by IL-2 with or without AD412. U266 myeloma cells (10×10^6) were incubated with AD412 (90, 30, or 10 µM) and INF-γ (500 IU/ml) for 0.5 h. After culture, cell pellets were treated with lysis buffer (10 mM Tris (pH7,5), 150 mM NaCl, 5 mM EDTA (pH8), 0,5% Nonidet P-40, 5 mM NaF, 2 mM, $NaVO_4$, 25 mM b̃glycerophosphate and protease inhibitor mixture) and clarified by centrifugation. Proteins were separated on 12% SDS-PAGE gels and transferred to PVDF membranes (Sigma). Immunostaining was performed with indicated mAbs at 1:1000 overnight at 4°C followed by an anti-rabbit IgG horseradish peroxydase-conjugated secondary Ab (1:2000). Proteins were visualized by ECL.

Kinase activity assay. Recombinant JAK2 and JAK3 activities (Invitrogen) was measured with The Omnia® kinase Assay (Invitrogen) that uses an enhanced fluorophore (Sox) incorporated into a substrate Tyr peptide. Phosphorylation of the peptide substrate by JAK results in an increase in fluorescence measured at 485 nm. Kinase activities were assayed

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

using 10 µM of Tyr peptide 7 in the presence or not of AD412 (3 to 90 µM) or WHI-P154 (30 µM). Fluorescence intensities were measured every 30 sec. with a fluorimeter (Fluoroskan, Thermo Electro Corp., France).

Heart allograft transplantation. Eight to twelve weeks old male LEW.1W (RT1.^{u}) rats were used as heart donors, and LEW.1A (RT1.^{a}) rats were used as recipients. Heterotopic cardiac transplantations were performed as described by Ono and Lindsey (Ono and Lindsey, 1969). These congenic strains were purchased from the Centre d'élevage Janvier (Laval, France) and maintained according to European and Institutional guidelines. Recipients Lewis 1A were treated daily *per os* for 21 days with 60 mg/kg of AD412 starting the day before the graft. Grafts were evaluated daily for function by palpation through the abdominal wall and rejection was defined as the day of cessation of heartbeat.

Statistical analysis. Results are expressed as mean ± SD. All results were compared using either ANOVA analysis followed by Dunnett post-tests when samples passed the normality test or the non parametric Kruskal-Wallis test followed by Dunn post-tests when they did not. A Chi square analysis was performed to compare graft survival curves.

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Effect of AD412 on T cell activation

Fresh PBMC from healthy donors were stimulated with microbeads precoated with anti-CD3 and anti-CD28 mAb. The effect of AD412 at two concentrations (30 µM or 90 µM) on the proliferative response of T lymphocytes was evaluated at 72 h by tritiated thymidine incorporation using CsA (0.5 µM) as a positive control. Cell viability after incubation with AD412 or CsA for 72 h was above 95% in all experiments. Results are presented in Fig. 2A and showed that our product significantly inhibited lymphocyte proliferation (40229 ± 3783 cpm for untreated stimulated PBMC *vs* 16876 ± 1237 cpm, $p<0.01$ and *vs* 24715 ± 1845 cpm, $p<0.01$ for stimulated PBMC treated with 90 µM and 30 µM of AD412, respectively).

Next, supernatants of each culture well of CD3/CD28 stimulated PBMC were collected at 48 h and IL-2 concentrations were measured by ELISA. As shown in Fig. 2B, CD3/CD28 stimulation of PBMC induced a significant increase in IL-2 production (507.9 ± 67.7 pg/ml *vs* 20.3 ± 11.4 pg/ml, $p<0.001$ for stimulated *vs* unstimulated PBMC, respectively) while treatment with CsA significantly inhibited IL-2 secretion after stimulation (507.9 ± 67.7 pg/ml *vs* 30.2 ± 14.4 pg/ml, $p<0.001$, for untreated *vs* CsA treated, respectively). In contrast, treatment with AD412 did not significantly alter IL-2 secretion (530.5 ± 120.8 pg/ml at 90 µM and 514.7 ± 88.6 pg/ml at 30 µM).

Since IL-2 production was not inhibited by AD412, we tested whether our compound inhibited the response to IL-2. For this purpose, we assessed the proliferative response to IL-2 of the murine IL-2-dependent CTL-L2 line in the presence of AD412. As shown in Fig. 2C, AD412 significantly inhibited proliferation of CTL-L2 in a dose dependent manner. This inhibition was total at 30 µM (98% of inhibition), partial but still significant at 10 µM (25%)

and disappeared at 3 µM. In this experiment, rapamycin at 50 nM, used as a positive control, completely inhibited IL-2-dependent proliferation of CTL-L2 (Fig. 2C). Since IL-2 response was significantly inhibited by our compound, we investigated which activation pathways downstream of the IL-2-receptor (IL-2R) were inhibited by AD412.

AD412 inhibited IL-2 receptor-dependent pathways

To analyze the effect of our compound on the pathways activated by IL-2 (i.e PI3K/Akt, JAK/STAT and ERK1/2) (Gaffen, 2001), we studied its effect on the phosphorylation patterns induced by IL-2R activation in the CTL-L2 line. We first examined the effect of AD412 on the phosphorylation status of Akt on Ser473 which is critical for its activation. As shown in a representative experiment (Fig. 3A, left panel), phosphorylated Akt was very low in cells starved of IL-2 for 8 h (first lane) while exposure to IL-2 for 0.5 h or 1 h induced significant phosphorylation of Akt (second and fourth lanes). Treatment of cells with 90 µM AD412 during IL-2 stimulation significantly inhibited phosphorylation of Akt without affecting the total amount of Akt. In order to confirm the inhibition of Akt activation, we tested the effect of our compound on the phosphorylation status of GSK3, a known Akt substrate. We observed a strong inhibition of GSK3 phosphorylation by AD412 at 90 µM (Fig. 3A) after 0.5 and 1 h of IL-2 stimulation. These observations were confirmed in three distinct experiments as shown on the right panel of Fig. 3.

We next tested the effect of our compound on STAT5a/b phosphorylation. Starved cells showed a total loss of phosphorylation of STAT5a/b (Fig. 3B, first lane) while stimulation of cells with IL-2 for 0.5 h or 1 h strongly induced STAT5a/b phosphorylation (Fig. 3B). Treatment of cells with AD412 at 90 µM during IL-2 stimulation strongly inhibited STAT5a/b phosphorylation at 30 min but much less after 1 hour in three distinct experiments

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

(Fig. 3B, right panel). As shown in Fig. 3A, this inhibition of STAT5 phosphorylation by AD412 was dose-dependent.

Finally, we tested the effect of our compound on the phosphorylation status of ERK1/2 (Fig. 3C). Similarly to Akt, IL-2-starved cells showed little activation of ERK1/2 as shown by the absence of ERK1/2 phosphorylation (first lane). However, activation of ERK phosphorylation by IL-2 was delayed when compared to Akt or STAT5a/b phosphorylation. Indeed, ERK phosphorylation was detectable at 0.5 h but reached its maximum only after 1 h of stimulation. Treatment of CTL-L2 cells with AD412 during IL-2 stimulation had two opposite effects on ERK phosphorylation: it strongly enhanced ERK1/2 phosphorylation at 0.5 h but inhibited this phosphorylation after 1 h in three experiments (Fig. 3C).

AD412 had little effect on INF-γ-induced STAT1 phosphorylation

Since our compound AD412 inhibited the three pathways linked to the IL-2R, we reasoned that it probably inhibited kinases located upstream of these pathways. We thus focused on the Janus Kinases (JAK), particularly JAK1 and JAK3 which are associated to the IL-2R. To delineate which of the two IL-2R-associated JAKs was inhibited by our compound, we analyzed the effect of AD412 on a cytokine stimulation model known to be independent of JAK3. In this respect, the recruitment and phosphorylation of STAT1 induced by INF-γ in lymphoid cells has been shown to depend on JAK1 and JAK2 activation (Ramana et al., 2002). Because stimulation of CTL-L2 cells with murine INF-γ induced no detectable phosphorylation of STAT1, we used the U266 myeloma cell-line (Wong et al., 2002) to explore the JAK1/2 dependent phosphorylation of STAT1. As shown on Fig. 4B, a 30 min stimulation of U266 cells with INF-γ was enough to induce a strong phosphorylation of STAT1 (second lane). In these conditions, addition of AD412 at 90 μM resulted in a very

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

weak inhibition of STAT1 phosphorylation while lower doses of 30 µM and 10 µM had no effect (Fig. 4B). Longer incubations of U266 (2 and 24 hours) with INF-γ in the presence of AD412 showed no inhibition of STAT1 phosphorylation either (not shown). Thus, AD412 did not significantly inhibit JAK1 or JAK2 activation. This strongly suggested that JAK3 is the major target of AD412 following activation of the IL-2 receptor.

AD412 inhibited JAK3 activity *in vitro*

To validate the hypothesis that JAK3 is a target of AD412, we assayed our derivative on purified JAK3 kinase. We performed *in vitro* kinase experiments using a fluorescent peptide substrate in the presence of increasing concentrations of AD412 (3 to 90 µM) or WHI-P154 used as positive control. As shown on Fig. 5A, AD412 inhibited JAK3 kinase activity in a dose dependent manner. At 90 µM, AD412 induced a major inhibition of JAK3 activity (81 ± 4.2 %, calculated at the kinetic plateau from three independent experiments) that decreased to 36 ± 4.1 % at 30 µM and was still detectable at 10 µM. At 30 µM, WHI-P154 showed a 23 ± 4% inhibition of JAK3.

Next, we examined the effect of AD412 on JAK2, the closest homologue of JAK3. Data are presented in Fig. 5B. At 90 µM, AD412 induced a 29 ± 2,6 % inhibition that disappeared at 30 µM while WHI-P154 at 30 µM still inhibited JAK2 (16.5 ± 3.2 %). Taken together, these data thus demonstrated that AD412 inhibited JAK3 much better than JAK2 in agreement with our biochemical analyses of the JAK-dependent pathways.

AD412 did not strongly inhibit IL-2-induced IL-2Rα expression

One of the effect of IL-2 binding to its high affinity receptor is the up-regulation of IL-2Rα (CD25) expression (Waldmann, 1989). Therefore, we tested the effect of AD412 on the IL-2-dependent up-regulation of CD25 in the CTL-L2 cell-line. As shown in Fig. 6, IL-2

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

stimulation of starved CTL-L2 for 18 h induced significant CD25 up-regulation as assessed by the increase in the geometric mean fluorescence intensity (MFI of 26.1 ± 6.0 for IL-2-starved CTL-L2 *vs* MFI of 115.6 ± 8.2 for IL-2 stimulated cells, $p<0.01$) (Fig. 6). Treatment with AD412 during IL-2 stimulation induced no significant decrease in CD25 expression (MFI of 99.0 ± 20.3 at 90 µM and 105.6 ± 25.0 at 30 µM of AD412 vs MFI of 115.6 ± 8.2 for IL-2 stimulated untreated cells, $p > 0.05$). Similarly, the JAK3 inhibitor WHI-P154 at 15 µM induced a moderate but not significant decrease in IL-2R expression (MFI of 75.3 ± 7.6 vs 115.6 ± 8.2, $p > 0.05$) that disappeared at 5 µM (MFI of 105.7 ± 14.3). The other JAK3 inhibitor, AG-490 at both doses induced a slight increase in IL-2R expression that was not significant (MFI of 132.0 ± 24.1 at 90 µM and 139.3 ±18.2 at 30 µM vs 115.6 ± 8.2, $p > 0.05$). In marked contrast, treatment of CTL-L2 cells with JAK I, known to inhibit JAK1, 2 and 3 (Thompson et al., 2002) significantly inhibited IL-2-induced CD25 up-regulation (MFI of 27.6 ± 7.7 at 0.6 µM and MFI of 25.4 ± 7.8 at 0.2 µM vs 115.6 ± 8.2 for IL-2 stimulated untreated cells , $p< 0.05$ and $p< 0.01$, respectively) (Fig. 6).

Immunosuppressive effects of AD412 *in vivo*

To evaluate the immunosuppressive potential of our compound *in vivo*, we tested the effect of AD412 therapy on allograft survival. We used the previously described model of LEW.1W heart allograft into a LEW.1A recipient (Chiffoleau et al., 2002) in which we chose to treat all animals for 21 days with 60 mg/kg *per os* of AD412, starting the day before the graft. In figure 7 is shown the graft survival curves in 5 untreated rats and in 8 AD412-treated rats. The median survival of untreated rats was 7 days in accordance with previous reports whereas AD412-treated rats showed a median graft survival of 23.5 days, $p<0.001$. These data further confirmed the immunosuppressive effect of AD412 *in vivo*.

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

There is a pressing need for the development of novel immunosuppressants that selectively inhibit signal 3 pathways of immune response, in order to block clonal expansion of T lymphocytes without affecting other cells. In the present study, we describe such a compound, AD412, that inhibits the proliferative response to IL-2 without affecting IL-2 production. We provide evidence that all three pathways activated by the binding of IL-2 to its receptor i.e. the JAK/STAT, ERK1/2 and PI3K/Akt pathways were inhibited by AD412 at 90 μM, a dose that induced total inhibition of CTL-L2 proliferation. However, we could evidence differences in the intensities and/or kinetics of these inhibitions by AD412. For Akt, a partial inhibition of phosphorylation was observed after 30 min that was maintained after 1 hour. This partial inhibition of phosphorylation was yet sufficient to significantly decrease kinase activity as evidenced by the strong inhibition of GSK3 phosphorylation observed at both time points. Since GSK3 is mainly involved in the negative regulation of cell cycle entry and since its phosphorylation by Akt is inhibitory (Jope and Johnson, 2004), we hypothesize that inhibition of GSK3 phosphorylation by AD412 contributes in a major part to its anti-proliferative effect. Concerning STAT5a/b, our data showed that AD412 induced a significant yet short-lasting inhibition of phosphorylation since inhibition was more pronounced at 30 min than at 1 hour. This may also contribute to the inhibition of CTL-L2 proliferation by AD412 since STAT5a/b are involved in T cell proliferation, either directly (Moriggl et al., 1999) or through IL-2Rα up-regulation as discussed underneath.

In the case of ERK1/2, AD412 induced an up-regulation of its phosphorylation at 30 min followed by a strong inhibition at 1 hour. These opposite effects could be explained by the existing cross-talk between the PI3K/Akt and the Raf/MEK/ERK pathways. In fact, Akt

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

phosphorylates Raf at a highly conserved serine residue in its regulatory domain resulting in an inhibition of this protein and thus an inhibition of the Raf/MEK/ERK pathway (Zimmermann and Moelling, 1999). We reason that in a first phase, AD412 indirectly stimulated ERK1/2 phosphorylation by inhibiting Akt-dependent Raf phosphorylation but later on, exerted a direct inhibition of Raf activation through inhibition of IL-2R tyrosine phosphorylation. The resulting inhibition of ERK1/2 activation should also contribute to the anti-proliferative effect of AD412 since these kinases are critically involved in cell proliferation (Seger and Krebs, 1995).

Overall, the above experiments revealed that our compound down-regulated all three pathways triggered by IL-2R and so provided indirect evidence that it inhibited kinase(s) located upstream of these cascades. In our case, two Janus kinases could be considered, the IL-2Rβ-associated JAK1 and the common γ-chain (γ_c)-associated JAK3, which are both recruited after binding of IL-2 to its multimeric high-affinity receptor (Gaffen, 2001). To delineate which JAK was targeted by AD412, we tested the effect of our compound on the JAK1/JAK2 dependent phosphorylation of STAT1 induced by INF-γ (Ramana et al., 2002). The very weak effect of AD412 on INF-γ -induced STAT1 phosphorylation suggested that our compound preferentially inhibited JAK3. Our *in vitro* kinase experiments confirmed that AD412 could significantly inhibit JAK3 activity while showing only minor inhibition of JAK2.

Considering that the JAK/STAT pathway has been shown to be critical for IL-2-induced up-regulation of IL-2Rα (CD25) (Lin and Leonard, 2000), we reckoned that AD412 may inhibit this up-regulation, an effect which could contribute to its immunosuppressive activity. However, neither AD412 nor the JAK3 inhibitor WHI-P154 induced any significant

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

inhibition of CD25 up-regulation while AG-490, a JAK2 and JAK3 inhibitor (Wang et al., 1999) showed a tendency to increase IL2R expression that did not reach statistical significance. Thus, the inhibition of IL2 responses by AD412 was not due to IL-2R down regulation.

In marked contrast, the pan JAK inhibitor JAK I totally abrogated IL-2R up-regulation. Altogether, our data strongly suggest that JAK1 but not JAK3 activation was crucial for IL-2 induced IL-2R up-regulation in CTL-L2 cells, in agreement with the previous report of Farrar's group in the IL2- dependent D10 cell-line (Wang et al., 1999).

JAK3 inhibitors have been shown to be efficient immunosuppressive drugs in animal models (Sudbeck and Uckun, 1999; Changelian et al., 2003; Stepkowski et al., 2005) and are considered for clinical use (Yabu and Vincenti, 2007). However, the strict JAK3 specificity of a number of inhibitors among which WHI-P154, has been recently questioned (Changelian et al., 2008) and, thus AD412 will have to be tested on a panel of kinases to ascertain its JAK3 selectivity.

Nonetheless, our *in vivo* data on the effect of AD412 on heart allograft rejection clearly demonstrated a significant prolongation of graft survival during treatment. However, rejection occurred shortly after treatment interruption, thus suggesting that AD412 did not favor tolerance induction. These data were very similar to those obtained with CsA in the same model where rejection was prevented during CsA administration but occurred shortly after the end of treatment (Pr.Soulillou, personal communication).

In conclusion the present study provides evidence that AD412 decreased IL-2-induced proliferation by inhibiting JAK3 in T lymphocytes and thus AD412 represents a promising lead compound to develop new immunosuppressive drugs that could be used alone or in

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

combination with calcineurin inhibitors to prevent allograft rejection and autoimmune diseases.

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES

Abbott L, Betschmann P, Burchat A, Calderwood DJ, Davis H, Hrnciar P, Hirst GC, Li B, Morytko M, Mullen K and Yang B (2007) Discovery of thienopyridines as Src-family selective Lck inhibitors. *Bioorg Med Chem Lett* **17**:1167-1171.

Baird AM, Thomis DC and Berg LJ (1998) T cell development and activation in Jak3-deficient mice. *J Leukoc Biol* **63**:669-677.

Boots JM, Christiaans MH and van Hooff JP (2004) Effect of immunosuppressive agents on long-term survival of renal transplant recipients: focus on the cardiovascular risk. *Drugs* **64**:2047-2073.

Borie DC, O'Shea JJ and Changelian PS (2004) JAK3 inhibition, a viable new modality of immunosuppression for solid organ transplants. *Trends Mol Med* **10**:532-541.

Burchat A, Borhani DW, Calderwood DJ, Hirst GC, Li B and Stachlewitz RF (2006) Discovery of A-770041, a src-family selective orally active lck inhibitor that prevents organ allograft rejection. *Bioorg Med Chem Lett* **16**:118-122.

Carbonnelle D, Ebstein F, Rabu C, Petit JY, Gregoire M and Lang F (2005) A new carboxamide compound exerts immuno-suppressive activity by inhibiting dendritic cell maturation. *Eur J Immunol* **35**:546-556.

Carbonnelle D, Lardic M, Dassonville A, Verron E, Petit JY, Duflos M and Lang F (2007) Synthetic N-pyridinyl(methyl)-indol-3-ylpropanamides as new potential immunosuppressive agents. *Eur J Med Chem* **42**:686-693.

Changelian PS, Flanagan ME, Ball DJ, Kent CR, Magnuson KS, Martin WH, Rizzuti BJ, Sawyer PS, Perry BD, Brissette WH, McCurdy SP, Kudlacz EM, Conklyn MJ, Elliott EA, Koslov ER, Fisher MB, Strelevitz TJ, Yoon K, Whipple DA, Sun J, Munchhof

MJ, Doty JL, Casavant JM, Blumenkopf TA, Hines M, Brown MF, Lillie BM, Subramanyam C, Shang-Poa C, Milici AJ, Beckius GE, Moyer JD, Su C, Woodworth TG, Gaweco AS, Beals CR, Littman BH, Fisher DA, Smith JF, Zagouras P, Magna HA, Saltarelli MJ, Johnson KS, Nelms LF, Des Etages SG, Hayes LS, Kawabata TT, Finco-Kent D, Baker DL, Larson M, Si MS, Paniagua R, Higgins J, Holm B, Reitz B, Zhou YJ, Morris RE, O'Shea JJ and Borie DC (2003) Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. *Science* **302**:875-878.

Changelian PS, Moshinsky D, Kuhn CF, Flanagan ME, Munchhof MJ, Harris TM, Doty JL, Sun J, Kent CR, Magnuson KS, Perregaux DG, Sawyer PS and Kudlacz EM (2008) The specificity of JAK3 kinase inhibitors. *Blood* **111**:2155-2157.

Chiffoleau E, Beriou G, Dutartre P, Usal C, Soulillou JP and Cuturi MC (2002) Induction of donor-specific allograft tolerance by short-term treatment with LF15-0195 after transplantation. Evidence for a direct effect on T-cell differentiation. *Am J Transplant* **2**:745-757.

Clark MP, George KM, Bookland RG, Chen J, Laughlin SK, Thakur KD, Lee W, Davis JR, Cabrera EJ, Brugel TA, VanRens JC, Laufersweiler MJ, Maier JA, Sabat MP, Golebiowski A, Easwaran V, Webster ME, De B and Zhang G (2007) Development of new pyrrolopyrimidine-based inhibitors of Janus kinase 3 (JAK3). *Bioorg Med Chem Lett* **17**:1250-1253.

Cortes JR, Perez GM, Rivas MD and Zamorano J (2007) Kaempferol inhibits IL-4-induced STAT6 activation by specifically targeting JAK3. *J Immunol* **179**:3881-3887.

Gaffen SL (2001) Signaling domains of the interleukin 2 receptor. *Cytokine* **14**:63-77.

Hariharan S (2003) Case 4: cardiovascular risk in renal transplantation. *Transplantation* **75**:1610-1614.

Jope RS and Johnson GV (2004) The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* **29**:95-102.

Kirken RA, Erwin RA, Taub D, Murphy WJ, Behbod F, Wang L, Pericle F and Farrar WL (1999) Tyrphostin AG-490 inhibits cytokine-mediated JAK3/STAT5a/b signal transduction and cellular proliferation of antigen-activated human T cells. *J Leukoc Biol* **65**:891-899.

Larsen CP, Pearson TC, Adams AB, Tso P, Shirasugi N, Strobertm E, Anderson D, Cowan S, Price K, Naemura J, Emswiler J, Greene J, Turk LA, Bajorath J, Townsend R, Hagerty D, Linsley PS and Peach RJ (2005) Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant* **5**:443-453.

Lin JX and Leonard WJ (2000) The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. *Oncogene* **19**:2566-2576.

Moriggl R, Sexl V, Piekorz R, Topham D and Ihle JN (1999) Stat5 activation is uniquely associated with cytokine signaling in peripheral T cells. *Immunity* **11**:225-230.

Mortellaro A, Songia S, Gnocchi P, Ferrari M, Fornasiero C, D'Alessio R, Isetta A, Colotta F and Golay J (1999) New immunosuppressive drug PNU156804 blocks IL-2-dependent proliferation and NF-kappa B and AP-1 activation. *J Immunol* **162**:7102-7109.

Ono K and Lindsey ES (1969) Improved technique of heart transplantation in rats. *J Thorac Cardiovasc Surg* **57**:225-229.

Papageorgiou AC and Wikman LE (2004) Is JAK3 a new drug target for immunomodulation-based therapies? *Trends Pharmacol Sci* **25**:558-562.

Ramana CV, Gil MP, Schreiber RD and Stark GR (2002) Stat1-dependent and -independent pathways in IFN-gamma-dependent signaling. *Trends Immunol* **23**:96-101.

Russell SM, Tayebi N, Nakajima H, Riedy MC, Roberts JL, Aman MJ, Migone TS, Noguchi M, Markert ML, Buckley RH, O'Shea JJ and Leonard WJ (1995) Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. *Science* **270**:797-800.

Sabourin C, Robert JM, Robert-Piessard S, Carbonnelle D and Lang F (2004) Synthesis and evaluation of disubstituted N1- and N3-imidazolidin-2-ones acting as potential immunosuppressive agents. *J Enzyme Inhib Med Chem* **19**:459-465.

Seger R and Krebs EG (1995) The MAPK signaling cascade. *Faseb J* **9**:726-735.

Stepkowski SM, Kao J, Wang ME, Tejpal N, Podder H, Furian L, Dimmock J, Jha A, Das U, Kahan BD and Kirken RA (2005) The Mannich base NC1153 promotes long-term allograft survival and spares the recipient from multiple toxicities. *J Immunol* **175**:4236-4246.

Sudbeck EA and Uckun FM (1999) Recent advances in JAK3 kinase inhibitors. *IDrugs* **2**:1026-1030.

Sulanc E, Lane JT, Puumala SE, Groggel GC, Wrenshall LE and Stevens RB (2005) New-onset diabetes after kidney transplantation: an application of 2003 International Guidelines. *Transplantation* **80**:945-952.

Thompson JE, Cubbon RM, Cummings RT, Wicker LS, Frankshun R, Cunningham BR, Cameron PM, Meinke PT, Liverton N, Weng Y and DeMartino JA (2002)

Photochemical preparation of a pyridone containing tetracycle: a Jak protein kinase inhibitor. *Bioorg Med Chem Lett* **12**:1219-1223.

Waegell W, Babineau M, Hart M, Dixon K, McRae B, Wallace C, Leach M, Ratnofsky S, Belanger A, Hirst G, Rossini A, Appel M, Mordes J, Greiner D and Banerjee S (2002) A420983, a novel, small molecule inhibitor of LCK prevents allograft rejection. *Transplant Proc* **34**:1411-1417.

Waldmann TA (1989) The multi-subunit interleukin-2 receptor. *Annu Rev Biochem* **58**:875-911.

Wang LH, Kirken RA, Erwin RA, Yu CR, Farrar WL, Kirken RA, Erwin RA, Taub D, Murphy WJ, Behbod F, Wang L, Pericle F and Farrar WL (1999) JAK3, STAT, and MAPK signaling pathways as novel molecular targets for the tyrphostin AG-490 regulation of IL-2-mediated T cell response

Tyrphostin AG-490 inhibits cytokine-mediated JAK3/STAT5a/b signal transduction and cellular proliferation of antigen-activated human T cells. *J Immunol* **162**:3897-3904.

Wong LH, Sim H, Chatterjee-Kishore M, Hatzinisiriou I, Devenish RJ, Stark G and Ralph SJ (2002) Isolation and characterization of a human STAT1 gene regulatory element. Inducibility by interferon (IFN) types I and II and role of IFN regulatory factor-1. *J Biol Chem* **277**:19408-19417.

Wullschleger S, Loewith R and Hall MN (2006) TOR signaling in growth and metabolism. *Cell* **124**:471-484.

Yabu JM and Vincenti F (2007) Novel immunosuppression: small molecules and biologics. *Semin Nephrol* **27**:479-486.

Zimmermann S and Moelling K (1999) Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science* **286**:1741-1744.

FOOTNOTES

This study was supported by a grant [EFG#FL2003] from "Agence de Biomédecine", Saint-Denis, France.

Reprint requests: Pr. François Lang, Inserm U892, 8 quai Moncousu, BP 70721 44007 Nantes cedex France. Phone number: (33) 2 28 08 02 06. Fax number: (33) 2 40 35 66 97. E-mail: flang@nantes.inserm.fr

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

LEGENDS FOR FIGURES

Fig. 1. Chemical structure of AD412.

Fig. 2. Effect of AD412 on PBMC or CTL-L2 activation.

(A) Human PBMC were incubated with or without (unstim. PBMC) microbeads coated with anti-CD3/CD28 mAb (stimulated PBMC) in the presence or not of CsA or AD412. Proliferation was evaluated at 72 h by [^{3}H]-thymidine uptake. Mean ± SD of three independent experiments. (B) IL-2 production of the same PBMC was measured by ELISA in supernatants after 48 h of stimulation. Mean ± SD of three independent experiments. (C) Murine CTL-L2 were stimulated for 18 h with a range of IL-2 concentrations either alone (open circles) or with 3.3 µM (closed losanges), 10 µM (closed squares) or 30 µM (closed triangles) of AD412 or with 50 nM rapamycin (crosses). Mean ± SD of three independent experiments. ***$p<0.001$, **$p<0.01$ by ANOVA and Dunnett post-test comparing to untreated stimulated cells.

Fig. 3. Effect of AD412 on IL-2-induced phosphorylations.

CTL-L2 cells (10 x 10^6) were washed and starved of IL-2 for 8 h, and then stimulated for 0.5 h or 1 h by IL-2 (100 IU/ml) in the presence or not of AD412. (A) Western blot analyses of cell lysates were performed with antibodies to phospho-Akt, phospho-GSK3 or total Akt as first antibodies followed by anti-rabbit IgG horseradish peroxydase-conjugated secondary Ab and revealed by chemiluminescence. Left panel shows a representative experiment and right panel shows the densitometric blot analyses of phosphorylated Akt, phosphorylated GSK3 and total Akt from three experiments, expressed in arbitrary units as mean ± SD. (B) Same as

in A, with primary antibodies specific for phospho-STAT5a/b or total STAT5a/b. (C) Same as in A, with primary antibodies specific for phospho-ERK1/2 or total ERK1/2

Fig. 4. Dose-dependent effect of AD412 on IL2-induced STAT5 phosphorylation and on INF-γ-induced STAT1 phosphorylation. (A) IL-2 starved CTL-L2 cells were stimulated for 30 min with IL-2 (100UI/ml) with or without ranging concentrations of AD412 and Western blot analyses were performed as in figure 4B with antibodies against total STAT5 or phospho-STAT5. (B) U266 myeloma cells were incubated with INF-γ (500 IU/ml) in the presence or not of ranging concentrations of AD412 for 30 min. Western blot analyses of cell lysates were performed with antibodies to phospho-STAT1 or total STAT1. One representative experiment out of two performed is presented on each panel.

Fig. 5. Effect of AD412 on JAK3 and JAK2 kinase activity.

Kinetic analyses of JAK3 (A) and JAK2 (B) kinase activity were performed in the absence (full circles) or in the presence of increasing concentrations of AD412 (3 µM: open losanges, 10 µM: open triangles, 30 µM: open circles and 90 µM: open squares), or WHI-P154 at 30µM: full squares. Results are presented as relative fluorescence units *vs* time. Data are from one representative experiment out of four performed.

Fig. 6. Effect of AD412 and several JAK inhibitors on IL-2Rα expression.

CD25 surface expression on CTL-L2 cells was induced by IL-2 stimulation in the presence or not of various inhibitors and analyzed by flow cytometry. Bars represent geometric mean fluorescence intensities ± SD. Number of experiments are indicated. ** $p<0.01$ and * $p<0.05$ comparing each group to IL-2 stimulated untreated CTL-L2 cells (black bar) by Kruskal-Wallis test with Dunn post-test.

JPET Fast Forward. Published on August 26, 2009 as DOI: 10.1124/jpet.109.155986
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 7. Effect of AD412 on heart allograft survival.

Heterotopic allografts were performed as described in Material and methods and evaluated daily for function by palpation through the abdominal wall. Rats were treated orally with AD412 at 60 mg/kg for 21 days starting 24h before the graft. Rejection was defined as cessation of heartbeat and expressed in days. p=0.0002, Chi square.

Figure 1

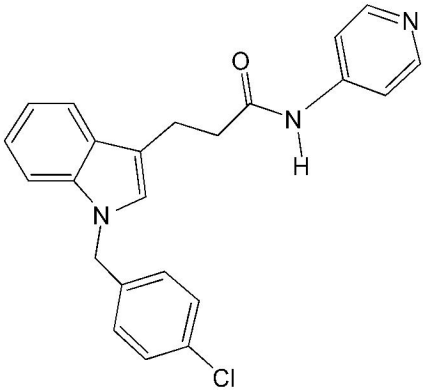


Figure 2

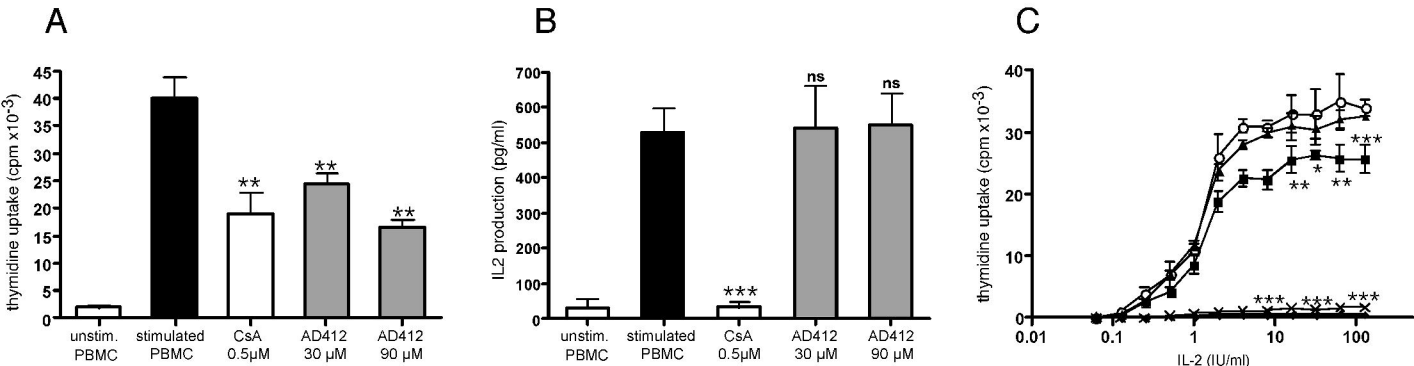

A
thymidine uptake (cpm x10-3)
45
40
35
30
25
20
15
10
5
0
**
**
**
unstim. PBMC
stimulated PBMC
CsA 0.5µM
AD412 30 µM
AD412 90 µM
B
IL-2 production (pg/ml)
700
600
500
400
300
200
100
0
ns
ns
***
unstim. PBMC
stimulated PBMC
CsA 0.5µM
AD412 30 µM
AD412 90 µM
C
thymidine uptake (cpm x10-3)
40
30
20
10
0
***
*
**
**
***
***
***
0.01
0.1
1
10
100
IL-2 (IU/ml)

Figure 3

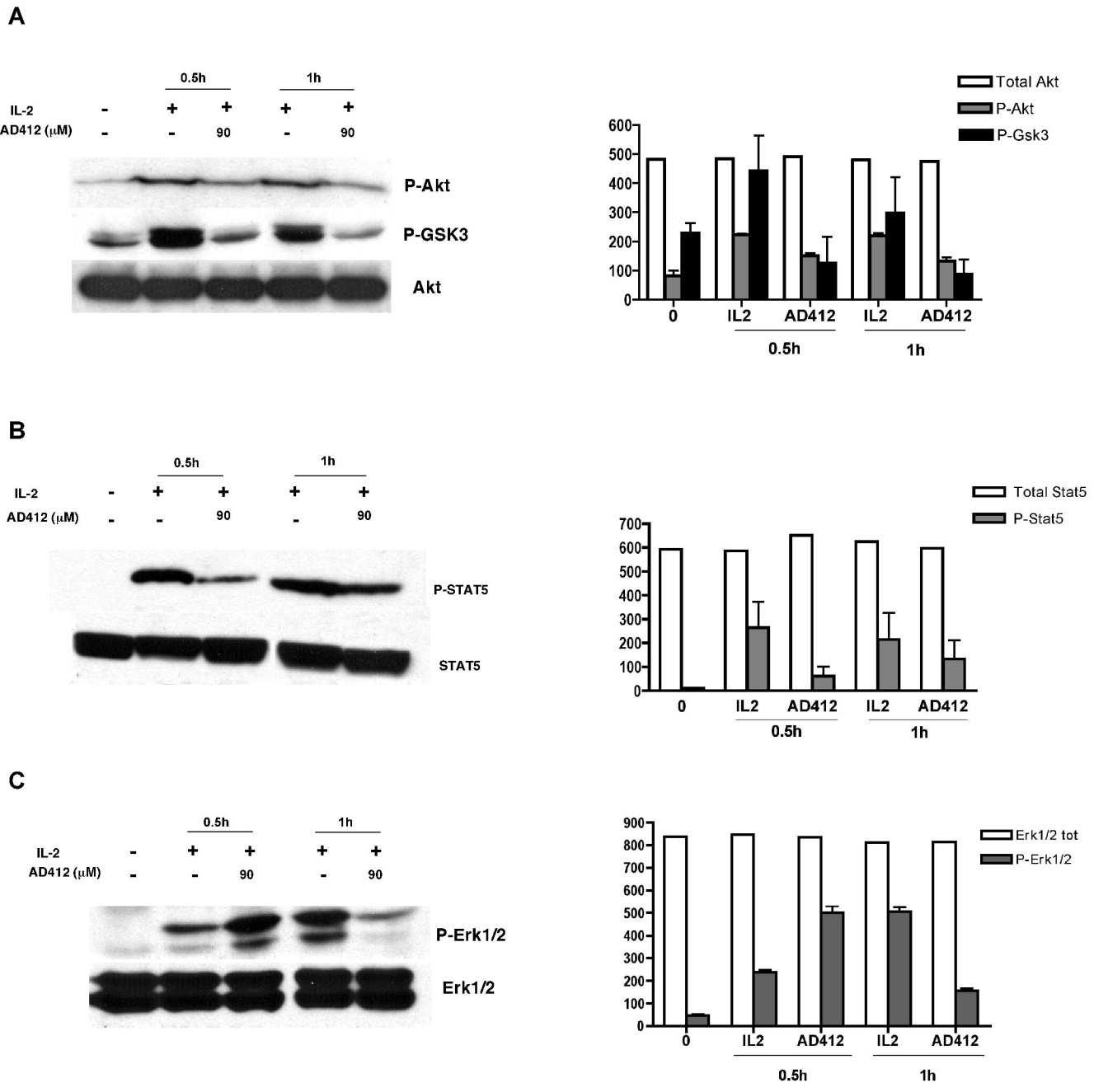

A
0.5h
1h
IL-2 - + + + +
AD412 (μM) - - 90 - 90
P-Akt
P-GSK3
Akt
Total Akt
P-Akt
P-Gsk3
600
500
400
300
200
100
0
0
IL2
AD412
IL2
AD412
0.5h
1h
B
0.5h
1h
IL-2 - + + + +
AD412 (μM) - - 90 - 90
P-STAT5
STAT5
Total Stat5
P-Stat5
700
600
500
400
300
200
100
0
0
IL2
AD412
IL2
AD412
0.5h
1h
C
0.5h
1h
IL-2 - + + + +
AD412 (μM) - - 90 - 90
P-Erk1/2
Erk1/2
Erk1/2 tot
P-Erk1/2
900
800
700
600
500
400
300
200
100
0
0
IL2
AD412
IL2
AD412
0.5h
1h

Figure 4

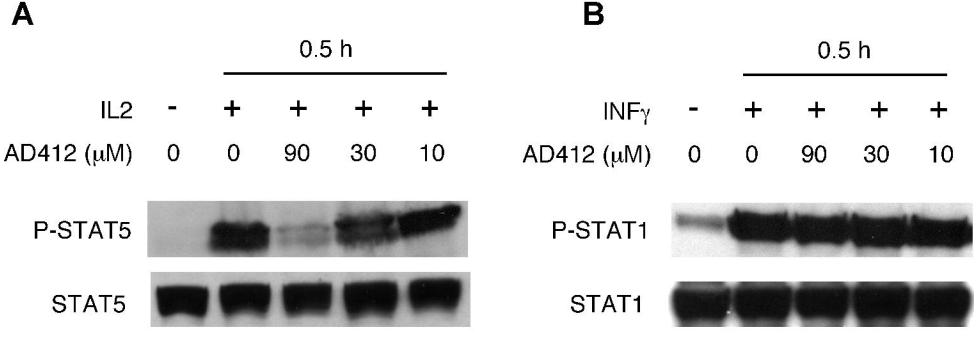
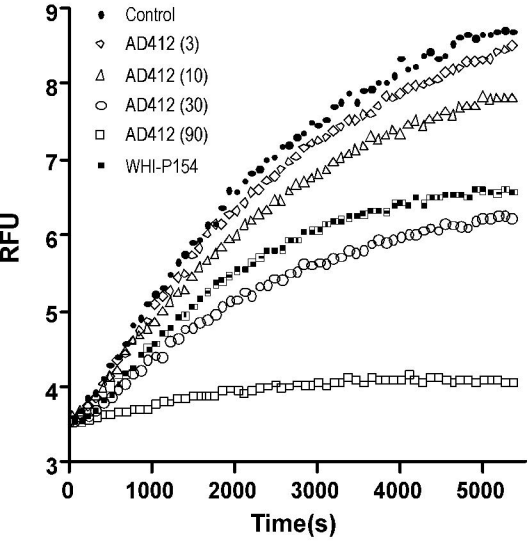

A
0.5 h
IL2 - + + + +
AD412 (μM) 0 0 90 30 10
P-STAT5
STAT5
B
0.5 h
INFγ - + + + +
AD412 (μM) 0 0 90 30 10
P-STAT1
STAT1

Figure 5


Control
AD412 (3)
AD412 (10)
AD412 (30)
AD412 (90)
WHI-P154
RFU
Time(s)
9
8
7
6
5
4
3
0
1000
2000
3000
4000
5000

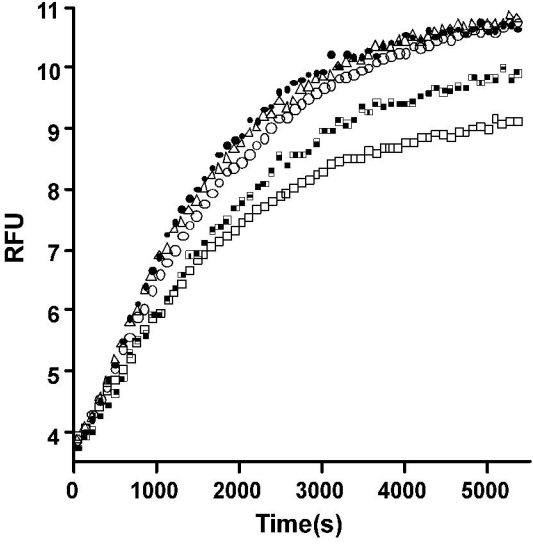

RFU
Time(s)
11
10
9
8
7
6
5
4
0
1000
2000
3000
4000
5000

Figure 6

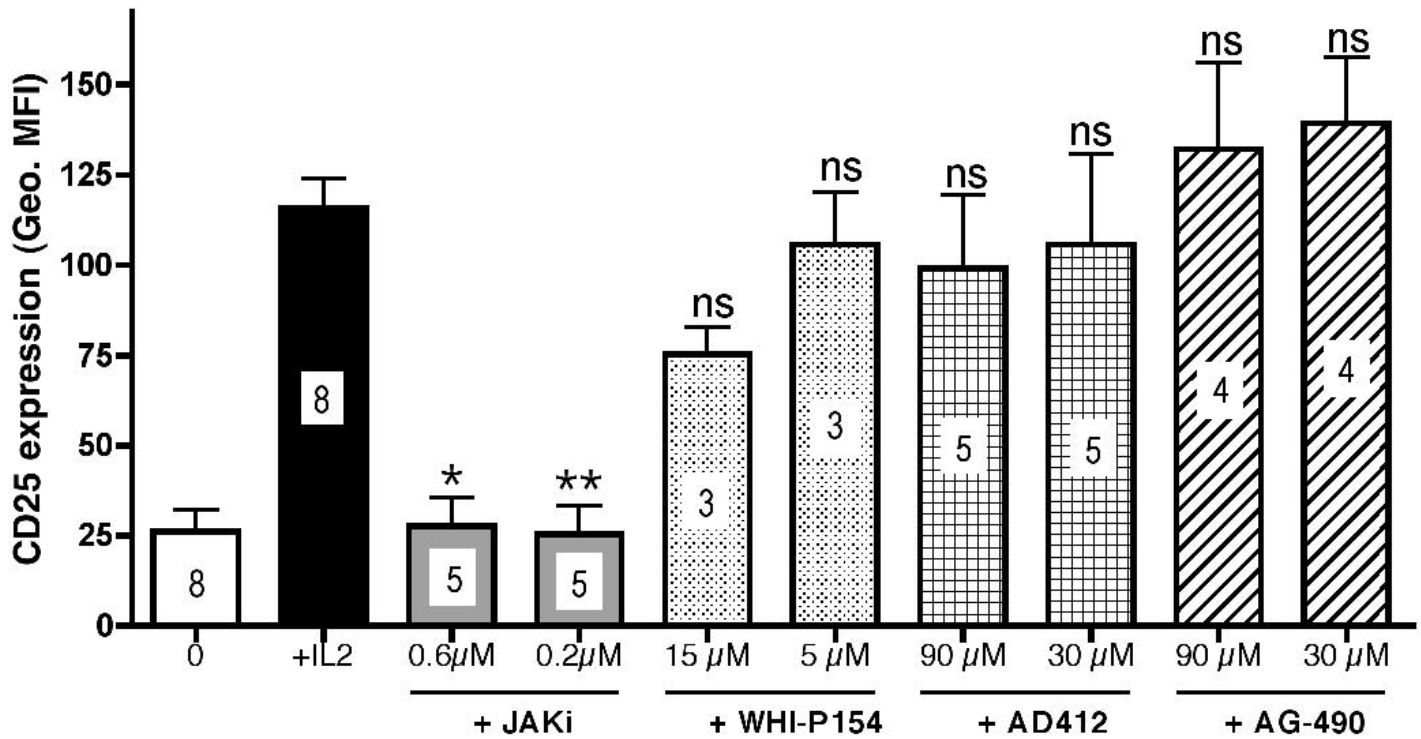
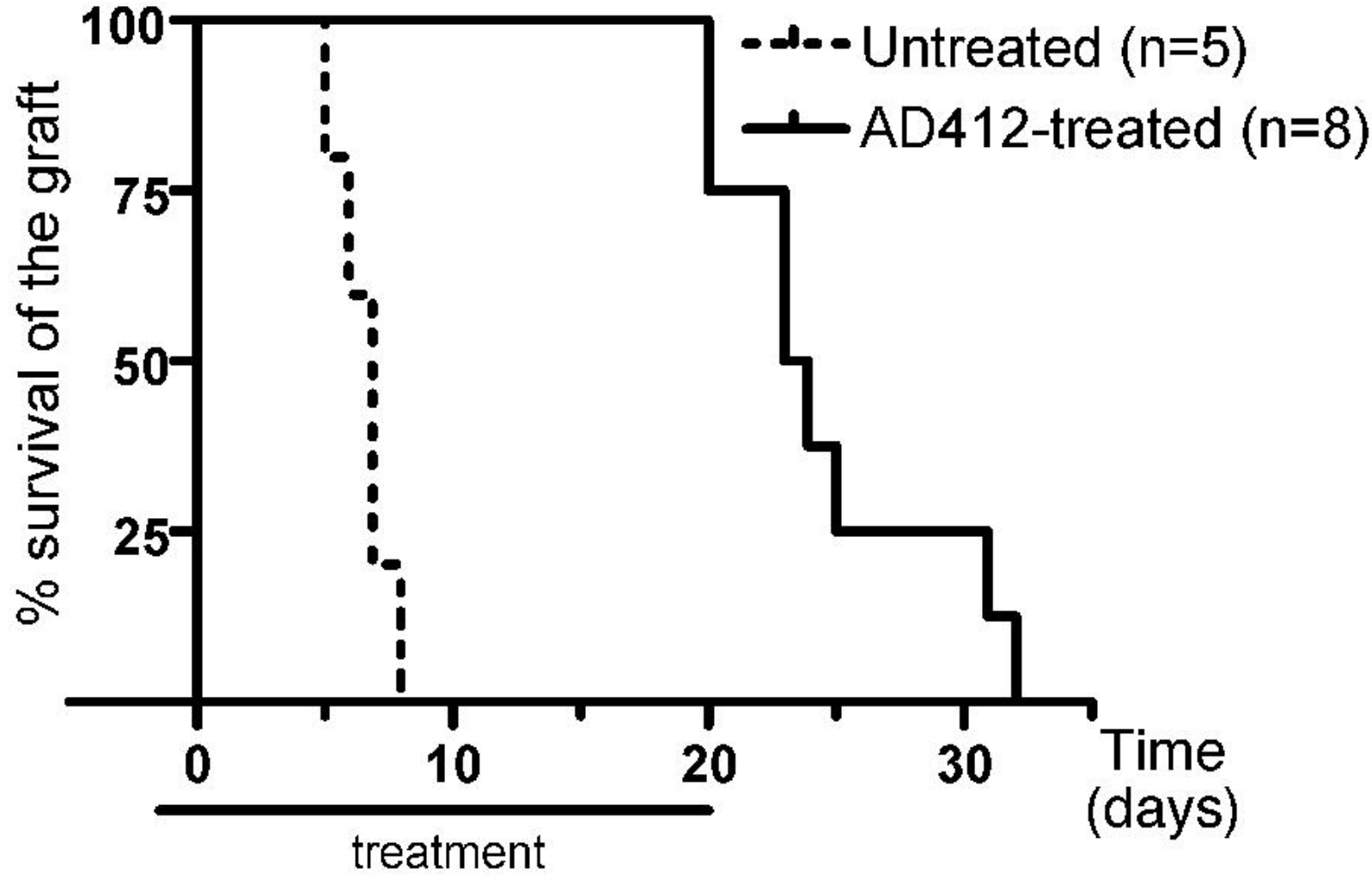

CD25 expression (Geo. MFI)
150
125
100
75
50
25
0
8
8
5
5
3
3
5
5
4
4
*
**
ns
ns
ns
ns
ns
ns
0
+IL2
0.6μM
0.2μM
15 μM
5 μM
90 μM
30 μM
90 μM
30 μM
+ JAKi
+ WHI-P154
+ AD412
+ AG-490

Figure 7


100
75
50
25
% survival of the graft
Untreated (n=5)
AD412-treated (n=8)
0
10
20
30
Time
(days)
treatment