THE SUSCEPTIBILITY OF BABOONS TO THE NOVEL IMMUNOSUPPRESSANT, FTY720.

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DECLARATION

I, Patricia Lynn Semple, (Fullard) declare that the work on which this thesis is based is original (except where acknowledgements indicate otherwise) and that neither the whole-work nor part of it has been, is being, or is to be submitted for another degree in this or any other University.

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DEDICATION

To my husband Andrew, for your love and support.

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PUBLICATION

The following publication relates to this work:

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1. ABSTRACT

The success of allotransplantation has encouraged many physicians to refer ever-increasing numbers of patients to transplant units to be considered for organ transplantation. Since there is a major scarcity of donor organs world-wide, the experimental search for human organs has focused on two alternatives; mechanical devices and cross-species transplants. The use of mechanical devices as substitute organs is understandably limited due to complications from trying to duplicate the function of complex organs such as the liver. This has resulted in a renewed interest in xenotransplantation.

Organs from non-human primates would arguably be the organs of choice but ethical consideration prevents this. The transplantation of organs from pigs or sheep to humans i.e. xenotransplants, results in hyperacute rejection. The development of immunosuppressive agents such as Cyclosporine A and Tacrolimus have significantly improved the survival of organ transplants. However, although there is a good 1-5 year survival, the recurrent problem of chronic rejection still remains, and unresponsiveness to allografts has never been induced by these immunosuppressive agents. More importantly, the presence of adverse side effects including immunological complications and drug toxicity e.g. nephrotoxicity, remains a serious problem.

Since the drugs currently available for allotransplantation preferably target T-cells, and are therefore unlikely to be sufficient for xenotransplantation where there is a

strong B-cell driven response, there is a need for new immunosuppressive agents. FTY720 (2 amino-2-(2-[4-octylphenyl] ethyl)-1,3-propanediol hydrochloride), a novel, immunosuppressive drug active in rodent and dog transplantation models, has shown no toxic side effects in pre-clinical studies although no long-term patient studies exist. The aim of this study was to evaluate the effect of FTY720 on the various components of the immune system in baboons as they are the species of choice for the pig-to-primate cardiac orthotopic xenotransplant. Three doses of FTY720 were studied in order to establish a dose response. The administration of 0.3mg/kg FTY720 resulted in a marked decrease in circulating lymphocytes within four hours of treatment, reaching a 60-80% decrease within 24 hours. The effect of FTY720 was seen on both T and B-cells, the effect being more pronounced on T-cells but more sustained by B-cells. The reduction in CD4+ T-cells was slightly more rapid and more sustained than CD8+ T-cells. FTY720 was then tested at lower doses of 0.1mg/kg and 0.03mg/kg. Although the response was more rapid and prolonged using a higher dosage, the maximum peripheral lymphodepletion was comparable.

Ex-vivo mitogen-induced whole blood cell proliferation was drastically decreased within hours after FTY720 treatment which suggests that the drug has a significant effect on the function of lymphocytes even at a low dose. The blood drug levels measured after the administration of FTY720 correlated favourably with the dose applied but not with the extent of lymphodepletion, suggestive of a high tissue distribution. FTY720 was well tolerated and no side effects were encountered. The drug was effective in terms of peripheral T and B-cell lymphodepletion in baboons indicating it could be used in xenotransplantation.

The mode of action of FTY720 has not been elucidated although its molecular mechanism of immunosuppression has been reported to be either due to apoptosis of activated lymphocytes, or by homing to peripheral lymphoid tissue (lymph nodes, Peyer's Patches) through lymphocyte homing receptors including L-selectin. LTα mice deficient in lymph nodes and Peyer's Patches, L-selectin knockout mice and control C57Bl/6+129SV mice were administered 1mg/kg FTY720 in order to try to determine the mode of action of the drug. All the mice that received FTY720 showed a drastic reduction in both their white blood cell counts and in lymphocyte counts while those that received the vehicle i.e. distilled water, showed a very slight response. These results suggest that, in mice, the lymphocytes are eliminated rather than recruited to peripheral tissues, however, no apoptotic assays were performed to confirm these findings.

2. INTRODUCTION

2.1 Introduction to xenotransplantation

The major scarcity of donor organs world-wide has resulted in a renewed interest in transplantation of organs across species. Xenotransplantation was pioneered by Reemtsma *et al.* (1964) who transplanted chimpanzee kidneys into human beings. Although the kidneys remained functional for nine months, attempts at transplanting other non-human primate organs were met with hyperacute rejection sometimes within hours of surgery. In addition, the use of non-human primates as organ donors for man is controversial and ethical consideration limits this.

The transplantation of organs from disparate species e.g. pigs or sheep into humans would solve the critical shortage of allogeneic organs. The ease with which they can be bred in large numbers, their relative lack of pathogens, and the similarity of organ size attributes to pigs being a good alternative source for human organs. However, transplantation of organs from disparate species results in hyperacute rejection (HAR). Humans, apes and Old World monkeys have pre-existing natural antibodies that recognise oligosaccharides with a terminal Galα1-3Gal structure (αGal epitopes) which are present on porcine endothelial cells. This leads to complement (C^1)–mediated lysis of the graft endothelium and consequent rejection of the graft. The problem of HAR has, to some extent, been successfully addressed by the neutralisation of these antibodies (Reding *et al.*, 1989), C^1 inhibition or deletion (Leventhal *et al.*, 1993), and donor organ hemoperfusion (Cooper *et al.*, 1988). Although rejection of the xenografts was delayed considerably, graft rejection did

however occur within 3-4 days by a process known as delayed xenograft rejection (DXR).

It is yet to be determined whether DXR can be overcome with conventional immunosuppression. Xu *et al.* (1997) achieved a nineteen-day survival in a discordant cardiac pig-to-baboon xenograft with pre-transplant total lymphoid irradiation combined with cyclosporine A (CsA) and methotrexate. Minanov *et al.* (1997) reported a slight prolongation of a cardiac pig-to-newborn baboon xenograft using cyclosporine-based triple immunosuppression, but documented that the protocol was not able to prevent a humoral response which led to graft rejection. Waterworth *et al.* (1998) reported a three-week survival of a heterotopic heart xenotransplant (pig-to-baboon) using an immunosuppressive regimen of CsA, cyclophosphamide and steroids.

It is becoming more evident that the prevention of graft rejection in xenotransplantation involves not only the inhibition of T-cells, but also other cell types, as the cells that infiltrate the xenografts consist mainly of B cells, natural killer cells (NK) and macrophages. Thus, DXR rejection is mediated by both humoral and cellular mechanisms. An optimum immunosuppressive regimen that could inhibit the production of induced anti-xenograft antibodies should result in long-term survival and is therefore, essential to successful xenotransplantation.

2.2 Immunosuppression in allotransplantation

Immunosuppressive drugs are classified into categories based on their principal mechanisms. CsA and Tacrolimus (FK506) inhibit the production of interleukin-2 (IL-2) and interferon-γ (INF-γ) expression by activated T-cells (Kaufmann *et al.*, 1984; Kino *et al.*, 1987; Bishop and Hall, 1988). Both CsA and FK506 have shown profound immunosuppressive effect on the prevention of acute rejection in clinical and experimental organ transplantation. Although these immunosuppressive drugs act on the same transduction pathway in the T-lymphocyte, namely by inhibiting calcineurin, some of their effects could be different, e.g. FK506 appears to decrease CD8 and local NK cell infiltration which could be mediated by FK506-induced IL-10 suppression (Jiang *et al.*, 1999).

Rapamycin (Rapa) is structurally similar to FK506 and binds to the same intracellular binding protein but acts later than FK506 in blocking lymphokine gene transcription (Watson *et al.*, 1999). Rapa blocks IL-2-dependent proliferation and the stimulation caused by cross-linkage of CD28 (Pai *et al.*, 1994). Azathioprine, and Mizoribin inhibit DNA biosynthesis and Cyclophosphamide preferentially affects B-cell proliferation. However, regardless of the immunosuppressive efficacy of each of these drugs, they all have severe side effects which limit their potential use in organ transplantation. The challenge facing any researcher in transplantation is to find an immunosuppression regimen that mitigates side effects especially nephrotoxicity, neurotoxicity, thrombocytopenia and altered glucose metabolism.

2.3 FTY720: a novel immunosuppressant

Isaria sinclairii, an ascomycete, acts as a parasite on the larva of the cicada *Meimuna opalifera Walker*. Myriocin (ISP-1), a drug with immunosuppressive properties, was purified from the culture filtrates of this fungus. In mouse models, ISP-1 was shown to inhibit allogeneic mixed lymphocyte reactions (Fujita *et al.*, 1995), interleukin-2-dependent proliferation (Miyake *et al.*, 1995), and to reduce alloreactive cytotoxic T lymphocytes (Fujita *et al.*, 1994). However, the administration of ISP-1 at a dose of 1mg/kg induced severe digestive disorders resulting in the death of the experimental animals (Fujita *et al.*, 1995). Due to the immunosuppressive potential of this compound, Professor T. Fujita (Taito Co. Ltd) in collaboration with Yoshitomi Pharmaceuticals Ltd, performed synthetic modifications of ISP-1 to produce a less toxic yet more active compound, FTY720 (2-amino-2-(2-[4-octylphenyl] ethyl)-1,3-propanediol hydrochloride).

The immunosuppressive effect of FTY720 is associated with a decrease in circulating lymphocytes, affecting both T- and B-lymphocytes. The mechanism of action of FTY720 has not been elucidated but the immunosuppressive effect may result from homing of lymphocytes to peripheral organs and/or apoptosis.

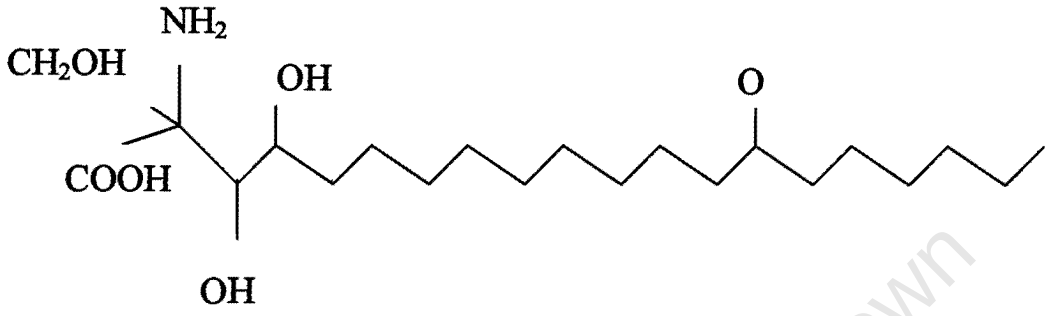


Figure 1A: The chemical structure of ISP-1

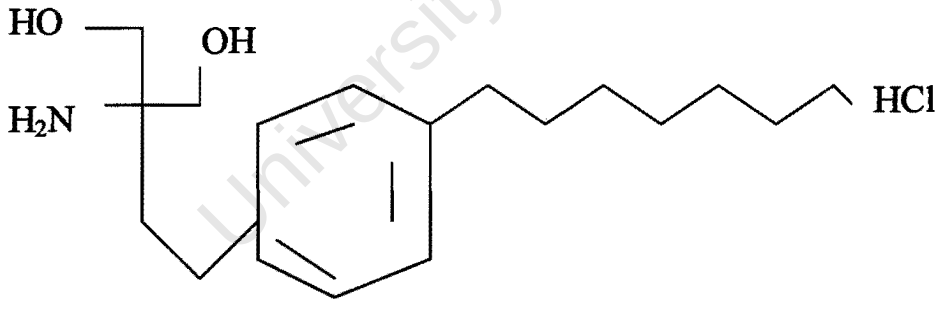


Figure 1B: The chemical structure of FTY720

2.4 Immunosuppressive effect of FTY720: *in vivo*

Suzuki *et al.* (1996) reported that a single dose of 10mg/kg FTY720 administered to ACI rats resulted in a rapid decrease in peripheral lymphocytes which returned to pre-drug values within two weeks. Adachi *et al.* (1995) described the efficacy of FTY720 in prolonging rat skin allografts in a major histocompatibility complex (MHC)-compatible rat strain. They reported that a dose of >0.03mg/kg FTY720 by intraperitoneal administration was effective in increasing graft survival. The prolonged survival of the rat skin allograft in a major histocompatibility complex (MHC)-compatible rat strain was supported by Chiba *et al.* (1996) who evaluated the oral immunosuppressive efficacy of FTY720 in a MHC-compatible and MHC-incompatible rat skin allograft model. They found that FTY720 significantly prolonged graft survival in a dose-dependent manner in both models. Administration of the same dose of FTY720, given either orally or intraperitoneal, resulted in graft survival which indicates a good bio-availability of the drug. FTY720 selectively decreased peripheral mature T-cells but did not affect bone marrow cells, thymocytes or polymorphonuclear cells. Unlike CsA and FK506 the drug had no effect on IL-2 production even at concentrations of 1000nM. In addition, the authors reported that FTY720 did not inhibit colony-forming bone marrow cells in response to erythropoietin, IL-3 or IL6. They concluded that FTY720 is an orally active compound which prevents acute rejection and that the immunosuppressive mechanisms of FTY720 seems to be related to the selective decrease in the circulating T-cells.

Suzuki *et al.* (1996) tested the effect of FTY720 in a rat liver allotransplant model by implanting livers from ACI rats into LEW rats. The recipients were divided into four groups. Group one did not receive any immunosuppressive therapy; group two were given 0.5mg/kg FTY720 for 14 days; group three received 5mg/kg FTY720 one day before and on the day of transplanting as a pre-transplant dose; and group four were administered 5mg/kg FTY720 three and four days after the transplant. The results showed that the rats which did not receive FTY720 had a median survival time (MST) of 11.5 days compared to 27.5 days of those rats who received the drug at a low dose for two weeks. The latter compared favourably with the pre-transplant regimen while the post-operative administration of the drug had a MST of 23 days. No side effects were experienced by any of the experimental animals.

As FTY720 had proven its efficacy in rat skin and liver allografts, Hoshino *et al.* (1996) tested the drug on graft survival after heterotopic cardiac allotransplantation. They transplanted hearts from WKAH rats into ACI rats and found that at a dose of 0.1mg/kg or more, FTY720 significantly prolonged the cardiac allografts. They reported that FTY720 at 10mg/kg induced graft survival of over 100 days in three out of five rats which indicated that a short course treatment of the drug at a high dose completely prevented allograft rejection to induce indefinite graft survival. In addition to the promising effect of FTY720 on graft survival in rat skin, liver and heart, Suzuki *et al.* (1998) found that a pre-transplant dose of FTY720 significantly prolonged the survival of pancreaticoduodenal allografts in rats.

The success of FTY720 as an immunosuppressant agent has not been limited to smaller animals. Suzuki *et al.* (1996) transplanted kidneys from mongrel dogs into the iliac fossa of beagle dogs which received 10mg/kg FTY720. The survival rate was three-fold that of the control dogs indicating that FTY720 was effective in a canine model although the drug was only effective at a higher dose. Nevertheless, no adverse reactions were experienced by any of the dogs. In later experiments, Suzuki *et al.* (1999) documented that dose-dependent prolongation was not obtained in this model and the discrepancy in dose-dependency may be due to differences in animal species and in the different organs tested.

2.5 The role of FTY720 in rejection

Allograft rejection is initiated by the infiltration and subsequent activation of T cells by Th1-associated cytokines (IL-2, and IFN-γ), whilst Th 2 associated cytokines (IL-4 and IL10) are essential for xenograft rejection (Josein *et al.*, 1995). Adhesion molecules related to T-cell trafficking are enhanced in allografts and monoclonal antibodies to these adhesion molecules inhibit rejection. Yanagawa *et al.* (1998), using a MHC-incompatible rat skin allograft model, reported that after transplantation there was an enhanced expression of Th-1 associated cytokines and CD3 messenger RNA (mRNA) whereas Th-2 cytokines were undetectable. When FTY720 was administered there was a decrease of T lymphocytes, not only in circulation but also in the allografts yet no effect was seen on IL-2 and IFN-γ mRNA. On administration of a low dose of CsA, there was an inhibition of IL-2 and IFN-γ mRNA in the allografts, but CsA did not affect CD3 mRNA. Therefore, the combination of FTY720, which is effective at inhibiting T cell infiltration into allografts, and CsA, which reduced IL-2 and IFN-γ expression, proved to be synergistic and significantly prolonged allograft survival.

T-cell infiltration into transplanted allografts is essential for rejection. Yanagawa *et al.* (1999) assessed the influence of FTY720 on T-cell subsets of T-cell infiltration into grafts and noted that the effect was not selective to T-cell subsets including CD4+, CD8+, CD25+CD4+ and CD25+ CD8+ cells. The authors proposed that in allograft rejection, cytotoxic differentiation of infiltrated CD8+ T-cells requires intragraft Th-1 associated cytokines which are produced by infiltrated CD4+ T-cells.

Therefore, because FTY720 inhibited infiltration of CD4+ and CD8+ T-cells into the graft, allograft survival was prolonged. This was supported by Kunikata *et al.*, (1999) who reported that acquired immune tolerance by FTY720 treatment had been described in rodents who received anti-CD4 and anti-CD8 antibodies.

Kunikata *et al.* (1999) suggested that the improvement of renal allograft survival might require lymphocytopenia before grafting. The authors reported that FTY720 had a preventative rather than a therapeutic effect on graft rejection as, a pre-transplant administration of the drug caused a significant prolongation of graft survival. Anti-lymphocyte serum and monoclonal antibodies to decrease peripheral lymphocytes have been used for reversing ongoing acute rejection in organ transplantation. Since a single dose of FTY720 decreased peripheral lymphocytes within hours, the authors suggested that the lymphocytopenia contributed to graft survival.

The effect of FTY720 as a rescue drug for acute rejection was studied by Yuzawa *et al.* (1998), who administered a subcutaneous injection of FTY720 to five dogs with acute rejection following a mismatched renal transplant. Three dogs were rescued and biopsies taken revealed that the FTY720 reduced cell infiltration into the parenchyma of the rejected grafts.

Another crucial process in organ rejection is the upregulation of expression of adhesion molecule on leucocytes and endothelial cells by cytokines such asTNF-α. Li *et al.* (1997) tested the effect of FTY720 on the expression pattern of cell adhesion

molecules and found that in the absence of TNF-α, FTY720 had no effect on the expression of adhesion molecules including ICAM-1, VCAM-1 and E-selectin. However, in TNF-α-treated human umbilical vein endothelial cells (HUVECs), FTY720 enhanced the upregulation of ICAM-1 which plays an essential role in the capture of lymphocytes from the circulation. CsA and FK506 do not affect cell adhesion or migration. Therefore, FTY720 must possess a unique immunosuppressive mechanism of action distinct from CsA and FK506 and thus, have a synergistic effect on allograft survival when combined with these drugs.

2.6 FTY720 in combination therapies

CsA-based combination therapies are widely used in clinical organ transplantation to reduce the side effects of individual drugs. In order to compare the immunopharmacological profile of FTY720 with the well-documented profile of CsA, both drugs were administered either alone or as combination therapy in a rat skin allograft model (Chiba *et al.*, 1996). The results showed that the immunosuppressive activity of FTY720 was 30-fold more potent than CsA but that the combination of the two drugs was remarkably more effective than the use of either drug alone. There was no difference in the blood levels of the drugs when administered alone or given simultaneously suggesting that combination therapy did not affect trough levels of each drug in the blood. In combination therapy CsA could be used at a sub-therapeutic dose which would minimise the adverse effects of the drug. In clinical organ transplantation, both Azathioprine and Mizoribine have been effective in combination with sub-therapeutic doses of CsA in prolonging graft survival but both drugs cause adverse side effects. Unlike CsA, the administration of FTY720 in experimental models has showed no severe side-effects even at high doses.

CsA was reported as being more effective in a rat cardiac allograft model than skin model (Ochiai *et al.*, 1987). Hoshino *et al.* (1996) tested the effect of FTY720 in combination with CsA on graft survival in rats after a heterotopic cardiac allotransplantation. They reported that a sub-therapeutic dose of CsA was ineffectual as a monotherapy but when administered simultaneously with 3mg/kg FTY720 there was an indefinite graft survival of six out of seven recipients. However, they reported

that the immunosuppressive effect of FTY720 was better in hepatic allotransplants than in cardiac transplants which could be attributed to size or antigenicity of the two organs.

Although the administration of FTY720 or CsA alone did not prolong graft survival in canine kidney allotransplants (Suzuki *et al.*, 1996), combination therapy considerably improved survival from 11 days with CsA alone to 74 days with the combination regimen of 5 mg/kg FTY720 and 10 mg/kg CsA. The synergistic effect of these two drugs appeared not only to prevent acute rejection but also to reverse ongoing rejection of canine renal allografts (Kawaguchi *et al.*, 1996).

Troncoso *et al.* (1999) tested the efficacy of FTY720 in renal transplants in non-human primates. Cynomolgus monkeys were transplanted with kidney allografts that were incompatible in mixed lymphocyte culture reactions. FTY720 was administered at 0.1, 0.3 or 1mg/kg/day in combination with CsA using doses that maintained whole blood concentrations at sub-therapeutic values between 40-200ng/ml. The control group, which received CsA alone, rejected their grafts within 8 days. FTY720 at doses of 0.1 and 0.3mg/kg significantly prolonged graft survival with a MST of 71 and 63 days respectively. However, biopsies revealed mild rejection in the 0.1mg/kg/day group. Those monkeys that received 1mg/kg/day had a MST of 48 days but two of the five monkeys died within approximately two weeks which could be due to complications caused by over-immunosuppression. These results suggest that a combination therapy of approximately 0.3 mg/kg FTY720 and a sub-therapeutic dose of CsA may have a beneficial effect in other primate species including human beings.

FTY720 is a poor inhibitor of T-cell proliferation as shown by Wang *et al.* (1998) who examined the effect of FTY720 on human peripheral blood lymphocytes (PBL) stimulated by PHA or OKT3 monoclonal antibodies. Although FTY720 was not effective *in vitro* to block the proliferative responses of mitogen-stimulated human PBL, the drug had synergistic effects when added to CsA or Rapa *in vivo*. This was also observed in a rat cardiac allograft model where Rapa, unlike CsA, did not induce nephrotoxicity but the combination of these two drugs did have overlapping lipidogenic toxicities. The combination of FTY720 with low dose CsA/Rapa regimen prevented allograft rejection indefinitely in 4/6 rats (Wang *et al.*, 1998).

Strepkowski *et al.* (1998) used the median effect analysis method to calculate the dose reduction index values for the synergistic interaction between FTY720 and CsA and FTY720 and Rapa in a rat cardiac allograft model. They found that the dose of FTY720 could be reduced by 14-22 fold and that of CsA by 3-7 fold when administered as combined therapy. When combined with Rapa, the dose of FTY720 could be reduced by 2-4 fold and Rapa by 1-3 fold. Therefore, FTY720 combined with a low dose of either CsA and/or Rapa may improve the efficacy of a CsA/Rapa based immunosuppressive regimens as well as negate the adverse effects caused by CsA alone or in combination with Rapa.

Thompson (1989) reported that FK506 had a more potent immunosuppressive effect than CsA in liver, kidney and cardiac animal experimental allograft models. FK506 has shown a lower incidence of acute rejection than CsA in patients undergoing liver and kidney transplants (Pirsch *et al.*, 1997). Hoshino *et al.* (1999) showed that

combination therapy of FTY720 and a sub-therapeutic dose of FK506 significantly prolonged skin and heart allografts in rats. In order to determine whether immunological tolerance would be induced the authors transplanted skin from either WKAH or LEW rats into ACI recipients with long-term surviving WKAH hearts. The WKAH skin allografts survived indefinitely whereas the LEW skin allografts were rejected within ten days suggesting that donor-specific tolerance was established.

Yamashita *et al.* (1999) reported that FTY720 significantly prolonged heart and liver transplants in incompatible combination rat strains in a dose dependent manner and that the drug had a wide therapeutic window (0.05-10mg/kg). When used in combination therapy with both CsA and FK506, survival was enhanced. Xu *et al.* (1997) tested the combination of pre-transplant FTY720 and post-transplant FK506 and reported that this regimen clearly enhanced rat cardiac allograft survival. More promising however, was that no adverse effects of FTY720 were experienced by either the rat or canine models, and by combining FTY720 with sub-therapeutic doses of other immunosuppressive drugs the adverse effects seen with those drugs could be reduced or prevented altogether.

Miyata *et al.* (1998) tested the effect of FTY720 on xenotransplants and found that in the hamster-to-rat model FTY720 combined with FK506 significantly prolonged skin and liver allografts but was not effective on heart allografts. They reported that skin and liver are resistant to humoral rejection (B-cell) and are less dependent on anti-proliferative drugs and that combination therapy of FTY720 and FK506 probably

inhibits xenoreactive T-cell-mediated rejection, but does not sufficiently inhibit B cell proliferation.

In order to minimise the adverse side effects of the immunosuppressive drugs presently available, the concentration of the drugs needs to be reduced without sacrificing the efficacy of immunosuppressive prophylaxis. CsA and Rapa display a synergistic interaction not only in rats and mice, but also in man (Strepkowski *et al.*, 1996, Tu *et al.*, 1995, Watson *et al.*, 1999). However, although Rapa is not nephrotxic the combination therapy of CsA/Rapa has lipidogenic toxicities. Thus FTY720 may offer an alternative, as it appears to be synergistic in dual as well as triple-drug combinations. Immunosuppressive drugs with different mechanisms of action but without overlapping toxic side-effects offer the best possible protocols for combined synergistic anti-rejection therapy.

The mechanism with which FTY720 exerts its immunosuppressive property has not been elucidated although the effect of the drug on decreasing peripheral lymphocytes is unquestionable. Two theories exist; homing of peripheral lymphocytes to other lymphoid tissues and/ or apoptosis, both of which are discussed below.

2.7 Mode of Action: Lymphocyte Homing

Immunologically mature lymphocytes continuously circulate between peripheral blood (PB) and lymphoid tissues. This consists of lymphocytes trafficking from PB to peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN) and Peyer's Patches (PP) and returning to the PB via lymphatic vessels and thoracic duct lymph (TDL) (Butcher and Picker, 1996). As previously mentioned, oral administration of FTY720 at 1 mg/kg decreased the number of lymphocytes in PB and TDL by approximately 80% of the original number within 24 hours. No effect of the drug was seen in the thymus or bone marrow. Similar lymphopenia has been induced by treatment with steroids and Cyclophosphamide however, the decrease in lymphocytes was seen in the thymus and bone marrow as well as in the PB. In addition, other white blood cells are affected by steroids. The decrease seen in the number of PB lymphocytes after FTY720 treatment is not due to the inhibition of intrathymic differentiation of T-cells or the suppression of the bone marrow. Rather, FTY720 is selective for mature lymphocytes capable of trafficking between the bloodstream and lymphoid tissues. Butcher and Picker (1996) reported that the integration and control of systemic T and B-cell immune responses depended on regulated lymphocyte homing. It is possible that the effect of FTY720 could be seen inducing both cell-mediated and humoral immune responses.

Chiba *et al.* (1997) reported that the administration of doses of 0.1 and 1mg/kg FTY720 to rats caused a decrease in total lymphocytes in the blood and TDL to less than 10% of the control and the splencic lymphocytes by 40-80% within 3-24 hours.

By contrast, the number of lymphocytes increased in a dose dependent manner in the PLN, MLN and PP. The authors argued that because 4μM FTY720 was needed to induce apoptosis *in vitro*, and the trough level measured in dogs administered 5mg/kg FTY720 was less than 200ng/ml, it was impossible for FTY720 to induce apoptotic death at doses of 0.1-1mg/kg. In order to establish whether the lymphocytes were trafficking between the blood and lymphoid tissues calcein-labelled lymphocytes were infused into F344 rats. The authors documented that the labelled cells were found in PLN, MLN and PP within 3 hours of administration of FTY720 and no cell death was evident. They concluded that after FTY720 treatment, lymphocytes homed from the PB and TDL to lymphoid tissues.

The homing of circulated lymphocytes to lymphoid tissues is reported to be mediated by the attachment of lymphocytes to high endothelial venules in these tissues (Butcher and Picker, 1996). This occurs via the lymphocyte homing receptors L-selectin (CD62L), CD49d/β_7 integrin and CD11a/CD18. The involvement of these homing receptors was tested by Chiba *et al.* (1997) who pre-treated calcein-labelled lymphocytes with monoclonal antibodies to these receptors. After the administration of FTY720, the lymphocytes were completely blocked from homing to the lymphoid tissues which suggested that FTY720-induced lymphopenia in the PB, TDL and spleen was due to sequestration of lymphocytes in PLN, MLN and PP and not by cytotoxicity.

To elucidate the relationship between the acceleration of lymphocyte homing and the expression of lymphocyte homing receptors, Yanagawa *et al.* (1998) analysed these

receptors on T-cells in FTY720 treated rats. FTY720 markedly decreased the number of peripheral blood T-cells but had no effect on CD62L, CD49d and CD11a expression in PB, PLN and MLN. However, there was an increased of CD62L-positive T-cells and an increased intensity of CD62L expression in the PP of the FTY720-treated rats. This indicated that FTY720 increases the frequency of CD62L-positive T-cells by accelerating CD62L-predominant lymphocyte homing in PP.

Pre-incubation of FTY720-treated lymphocytes and anti-α4-integrin (VLA-4) monoclonal antibody prevented lymphodepletion suggesting that FTY720 increases the expression, affinity or avidity of VLA-4 expression (Troncoso and Kahan, 1998). Altered VLA-4 expression affects the circulation patterns of lymphocytes between the blood stream and lymphoid organs and may home lymphocytes towards high endothelial venules in lymph nodes and PP.

To clarify the mechanism of the decrease in the number of lymphocytes by FTY720 Chiba *et al.* (1999) analysed the tissue distribution of lymphocytes in peripheral blood, spleen and mesenteric lymph nodes after a single oral administration of FTY720. PBL decreased to less than 10% of the control within 24 hours and splenic lymphocytes were reduced to approximately 60%. In contrast, the number of lymphocytes in the MLN increased in a dose-dependent manner and returned to that of the control within 7 days. They proposed that the decrease in peripheral lymphocytes caused by FTY720 was due to the modulation of redistribution of circulating lymphocytes to secondary lymphoid tissues. To determine the effect of FTY720 on this proposed trafficking, lymphocytes from male rats were transfused

into strain-matched female rats which had received a single dose of FTY720. After thirty minutes the female rats were sacrificed and the tissue distribution of male lymphocytes was determined by polymerised chain reaction (PCR) method. The FTY720-treated female rats had increased male DNA in the PLN, MLN and PP in a dose-dependent manner which reflects the redistribution of male lymphocytes into these lymphoid tissues. There was decreased male DNA in the spleen but no changes occurred in the lungs and liver. They concluded that FTY720 sequesters circulating mature lymphocytes into PLN, MLN and PP by acceleration of lymphocyte homing which results in a decrease in the number of lymphocytes in the PB and spleen.

2.8 Mode of Action: Apoptosis

In order to elucidate the mode of action of FTY720, Suzuki *et al.* (1996) tested the drug *in vitro* and *in vivo*. They found that a single dose of 10mg/kg FTY720 administered to ACI rats resulted in a rapid decrease in peripheral lymphocytes which returned to pre-drug values within two weeks. When they incubated spleen cells, isolated from the ACI rats, with 10^{-5}M FTY720, at different time intervals, they noted that as the incubation time increased, there was an increase in dead cells and in the proportion of small cells, which appeared indicative of apoptosis. Electron microscopy of these cells demonstrated the characteristics of apoptosis including the absence of surface microvilli, chromatin condensation and formation of apoptotic bodies. Furthermore, spleen cells incubated for 4 hours with concentration higher than $4+10^{-6}$M FTY720 exhibited fragmentation of chromosomal DNA as revealed by agarose gel electrophoresis. They concluded that the lymphocytes were removed from the system by apoptosis.

Enosawa *et al.* (1996) reported that the mechanism of decrease in circulating lymphocytes was either due to cell death or migration of lymphocytes to other lymphoid organs. They confirmed that the total number of PB lymphocytes was rapidly reduced after a single administration of FTY720 in rats. In addition, there was a decrease of T-cells in the spleen and liver but no effect of the drug was seen in the thymus, lymph nodes and bone marrow. They reported that CD4+ T-cells were more sensitive to FTY720 than CD8+ T-cells and B-cells *in vivo* and *in vitro* and that FTY720 induced cell death selectively in mature T-lymphocytes.

Several pathways have been reported for inducing lymphocyte apoptosis including Fas-antigens, which are abundantly expressed in the thymus, and on activated or transformed T-lymphocytes (Itoh *et al.*, 1991). The apoptosis of immature thymocytes plays a major role in the negative selection that occurs during thymic development. Fas-antigens are not expressed in *lpr* mice due to mutation of the *fas*-gene. In order to establish whether FTY720-induced cell death was related to fas-antigens, Suzuki *et al.* (1996) incubated FTY720 with thymocytes isolated from *lpr* and wild-type mice. They observed that cell death occurred in a dose-dependent manner in cells from both the *lpr* mice and the wild-type mice. The addition of anti-Fas antibodies to the thymocytes induced a dose-dependent cell death in the wild-type mice but not in the *lpr* mice suggesting that there is no relationship between Fas-antigen-mediated cell death and FTY720-induced cell death. The authors then tested the *in vivo* effect of FTY720 on *lpr* mice who spontaneously develop auto-immune disease with massive lymphoadenopathy due to their mutation (Suzuki *et al.*, 1997). They found that treated mice had markedly reduced weights of their spleen and lymph nodes compared to the untreated mice. Apoptotic cells were detected in all the lymphoid organs by *in situ* DNA nick-end labelling. This suggested that FTY720 therapy induces apoptosis in abnormally expanding lymphocytes of Fas-mutant mice.

Although the *fas*-gene was not involved in FTY720-mediated cell death, other apoptosis-related genes may be involved. The *bcl*-2 gene is a proto-oncogene that is known to inhibit apoptosis. Its gene product Bcl-2 protein was implicated in blocking a common final pathway to apoptotic cell death (Reed, 1994). Contrary to Bcl-2, Bax a family of Bcl-2-related proteins, promotes programmed cell death, and the ratio of

Bcl-2 to Bax may determine cell survival or cell death following apoptotic stimuli. (Oltvia *et al.*, 1994). To establish the intracellular role of FTY720 on the expression of Bcl-2 and Bax proteins, Sukuki *et al.* (1996) cultured human mononuclear cells and human *bcl-2* gene-transferred Jurkat lymphoma cells and their neo-type, with increasing concentrations of the drug (2-10μM). The human cells and Jurkat-neo cells showed characteristic apoptotic cell death, as determined by ladder formation of the DNA agarose gel electrophoresis, in a dose-dependent manner. Unlike their neo-type, the Jurkat-*bcl-2* cells were resistant to FTY720 treatment. In addition, they found that one hour after FTY720 treatment, the human cells showed a decrease in Bcl-2 protein and an increase in Bax protein causing a reduced Bcl-2 to Bax ratio which resulted in cell death. The cells which were resistant to FTY720, displayed a similar ratio of Bcl-2 to Bax as that of the control cells suggesting that the drug displays *bcl-2*-associated apoptotic cell death in human mononuclear cells. Thus the authors proposed that FTY720 might be implicated in modulating a final common pathway of apoptotic cell death.

FTY720 has a similar structure to sphingosine which was reported to increase the intracellular Ca^{2+} ion concentration in the human promyelocytic HL-60 cells and consequently induced apoptosis (Okajima *et al.*, 1995). Shinomiya *et al.* (1997) studied cell death in HL-60 cells treated with 4-8μM FTY720 and found that the drug activated the phospholipase C-Ca^{2+} pathway which induced apoptosis in the HL-60 cell line. When they added a phospholipase C inhibitor, the increase in Ca^{2+} ions was ablated, as was the DNA fragmentation. They concluded that the DNA fragmentation and cell death were related to a sustained increase in the cytosolic Ca^{2+} concentration

and that FTY720 acts via intracellular Ca^{2+} mobilisation which might be the cause of the cell death seen in the HL-60 cells.

Allograft rejection is associated with cytokine release and infiltrating T-cells into the graft. Cytotoxic T lymphocytes (CTL) and natural killer cells (NK) cells have been implicated to cause apoptosis via two independent pathways namely perforin/granzyme-cell-mediated cytotoxicity and Fas ligand/Fas-based cytotoxicity (Nakajima *et al.*, 1998, Lowin *et al.*, 1994). These cytotoxic lymphocytes produce apoptotic molecules including perforin, granzyme B and FasL which are the effector molecules in apoptosis. Nakajima *et al.* (1998) studied the effect of FTY720 on these molecules in a rat cardiac allograft model. They focussed on the killer function of CTL and NK cells and their apoptotic molecules. The untreated control group showed that, during acute rejection, there was an upregulation of perforin, granzyme B and FasL. The rats treated with increasing concentrations of FTY720, showed an inhibition of these cytotoxic molecules in a dose dependent manner, which led to a prolonged survival of the graft. They postulated that FTY720 either caused apoptosis of activated lymphocytes in the graft causing a reduction of perforin, granzyme B and FasL, or that the drug selectively inhibited the transcription of these apoptotic effector molecules in lymphocytes which prevented acute rejection.

Matsuda *et al.* (1998) studied the role of caspases in FTY720-mediated apoptosis as caspases and their inhibitors have been implicated in programmed cell death (Henkart, 1996). Mouse lymphoma WR19L cells treated with FTY720 demonstrated an upregulation of caspase-1 and subsequently caspase-3 within two hours. The drug

induced both nuclear damage and cell lysis on the WR19L cells. No inhibition of lysis was observed when caspase inhibitors were added to the cultures. However, the nuclear damage was completely blocked suggesting that caspases are not required for cell lysis in FTY720-mediated apoptosis but nuclear damage is involved in the caspase activation.

Shimizu *et al.* (1998) reported that an *in vivo* administration of FTY720 enhanced antigen-induced T-cell death of mature cells but inhibited that of immature thymocytes. They suggested that two different apoptotic pathways were responsible for T-cell death depending on the maturity of the cell. Both T and B-cells in the spleen expressed DNA fragmentation *in vitro* after 10 μM FTY720 treatment which was indicative of apoptosis, whereas FTY720-induced apoptosis *in vivo* (10mg/kg) was preferentially detected in peripheral lymphocytes. They proposed that the immunosuppressive effect of FTY720 could be an increased susceptibility of T-cells to antigen-induced apoptosis. They found that FTY720 induced apoptotic cell death of thymocytes, splenic cells and peripheral lymphocytes *in vitro*, but caused apoptosis on peripheral lymphocytes and not thymocytes or splenic cell *in vivo*. The authors concluded that FTY720 is not myelotoxic but specifically attacks peripheral lymphocytes, is cytotoxic to both T and B-cells *in vitro*, and the apoptosis-inducing effect is higher in T- than B-lymphocytes.

2.9 FTY720 in other roles

FTY720 induced long-lasting unresponsiveness with a low dose of 0.1mg/kg in a lethal graft-versus-host -reaction (GvHR) system indicating its effectiveness in preventing graft versus host disease (Masubuchi *et al.*, 1996).

Suzuki *et al.* (1998) reported that although diabetes mellitus is associated with lymphopenia, FTY720-induced lymphopenia did not induce autoimmune diabetes in the diabetes-prone bio-breeding rat model. They proposed that FTY720 appeared to keep the balance between regulatory cells and autoimmune effector cells.

Immunosuppression tolerance is the ultimate goal following solid organ transplantation. Induction of tolerance by a donor-specific blood transfusion (DST) prior to transplantation has been used in experimental transplantation models (Jenkins and Woodruff, 1971). Antoniou *et al.* (1998) reported that FTY720 administered as a pre-transplant dose significantly prolonged allograft survival beyond that of DST. However, no further enhancement of graft survival was observed when DST was given in the presence of FTY720.

Graft coronary artery disease is a major problem in clinical cardiac transplantation because approximately 50% of patients who survive five years show significant atherosclerosis on routine angiography (Schroeder *et al.*, 1992). Hwang *et al.* (1999) studied the clinical potential of FTY720 as an immunosuppressant in a murine cardiac model using mice that shared major but not minor histocompatibility antigens.

Recipients, who do not receive immunosuppressants but survive allotransplantation, develop graft atherosclerosis (Hirozane *et al.*, 1995). The authors found that administration of FTY720 suppressed neointimal proliferation and preservation of the luminal area. Furthermore, when combined with CsA, the drugs prevented atherosclerosis altogether. Together with its ability to suppress acute rejection, coronary vasculitis was attenuated by FTY720 treatment.

3. AIM

FTY720 has proven to be active as an immunosuppressant drug in rodent and dog transplantation models and has not exhibited any major adverse side effects. Baboons are the species of choice in pig to non-human primate xenotransplantation, primarily because of their similarity in size. This is especially true for orthotopic cardiac transplants and for studies that require large blood volumes. The aim of this project was to evaluate the susceptibility of baboons to FTY720, with respect to lymphocyte functions and numbers, in an introductory study to xenotransplantation protocols.

4. MATERIAL AND METHODS

4.1. In vivo Experiments

4.1.1 Drug application

Chacma baboons (Papio Ursinus: body weight range 14-20kg, matched per group of three) were obtained from The Medical Research Centre (MRC) Delft Animal Unit, Cape Town, South Africa. The baboons were brought to the University of Cape Town (UCT) Animal Unit approximately one week prior to the commencement of the experiment in order for them to acclimatise to their new environment. They were housed in separate cages at the UCT Animal Unit.

FTY720 (Novartis Pharma, Basel) was dissolved in sterile distilled water and administered at dosages of 0.3, 0.1 or 0.03mg/kg. The drug was given in a volume of 2ml/kg when administered by gastric intubation or 1mg/kg if given as a ball of food. Three doses of FTY720 were administered. The first two groups of baboons (n=3) received 0.3 or 0.1mg/kg/day respectively for three consecutive days (day 0, 1, 2) using a gastric catheter under ketamine hydrochloride anaesthesia (10mg/kg i.m. Brevinaze, Intramed LTD, Port Elizabeth, SA). The third group (n=3) received 0.03mg/kg for 10 consecutive days. For this group the FTY720 was administered by gastric gavage only on the days of blood sampling (day 0, 1, 2, 7 9, 12, 15 and 22); on the remaining days the drug was incorporated in golf-size balls of maize flour and banana. This provided an alternative to gastric catheterisation which would have required anaesthesia for ten consecutive days, impairing the feeding process of the baboons. To ensure that the food balls containing the drug were eaten, the baboons

were starved overnight and the food balls were provided the following morning. The administration of the drug in this manner was validated in an earlier experiment; three baboons received a single dose of 0.3mg/kg in food and the FTY720 blood levels were measured and compared to the trough levels of the drug in baboons receiving the same dose by gastric intubation. The drug levels of FTY720 were 2.7 ± 0.4ng/ml in the baboons receiving the ball of food compared to 2.6 ± 0.3 ng/ml after gastric gavage.

In order to determine the baseline variability and to exclude the possibility of side effects to either the anaesthetic or the vehicle, the first group of baboons was anaesthetised and received the appropriate volume of distilled water instead of FTY720 (Table 1). This was repeated twice, the baboons monitored and blood samples analysed in the same manner as if they were receiving the drug.

4.1.2 Blood sampling

In all experiments blood sampling was done when the baboons arrived at the Animal House to establish baseline values. The blood was collected under sterile conditions in vacutainers containing either ethylenediaminetetraacetic acid (EDTA) K_3 for haematological assessment and the determination of drug levels, or lithium heparin for cell culture and flow cytometry. In the group of baboons receiving 0.3mg/kg FTY720, blood was taken before the administration of the drug or vehicle and again on day 4, 7, 9, 12, 14 and 21 (Table 1). For later experiments the number of days on

which blood sampling was performed were reduced to prevent the possibility of anaemia and to minimise stress caused to the baboons (Tables 2 and 3).

Table 1

PROTOCOL FOR BABOONS RECEIVING 0.3mg/kg FTY720 BY GASTRIC GAVAGE

DAY	DOSING	FBC/DIFF	FACS	WB PROLIF	DRUG LEVELS
-14	ENTRY	+	+	+	–
-7	VEHICLE	+	+	+	–
-6	VEHICLE	+	+	+	–
-5	VEHICLE	+	+	+	–
0	FTY720 0.3mg/kg	+ Pre-dose	+ Pre-dose	+ Pre-dose	+ Pre-dose
1	FTY720 0.3mg/kg	+	+	+	+
2	FTY720 0.3mg/kg	+	+	+	+
4	–	+	–	–	+
7	–	+	+	+	+
9	–	+	+	+	–
12	–	+	+	+	–
14	–	+	+	+	–
21	–	+	+	+	–

+: analysis performed

-: no analysis performed

Table 2

PROTOCOL FOR BABOONS RECEIVING

0.1mg/kg FTY720 BY GASTRIC GAVAGE

DAY	DOSING	FBC/DIFF	FACS	WB PROLIF	DRUG LEVELS
-7	ENTRY	+	+	+	–
0	FTY720 0.1mg/kg	+	+ Pre-dose	+ Pre-dose	+ Pre-dose
1	FTY720 0.1mg/kg	+	+	+	+
2	FTY720 0.1mg/kg	+	+	+	+
4	–	+	+	+	+
7	–	+	+	+	+
14	–	+	+	+	–
21	–	+	+	+	–
28	–	+	+	+	–

Table 3

PROTOCOL FOR BABOONS RECEIVING

0.03mg/kg FTY720 IN FOOD OR BY GASTRIC GAVAGE

DAY	DOSING	FBC/DIFF	FACS	WB PROLIF	DRUG LEVELS
-9	ENTRY	+	+	+	-
0	FTY720 0.03mg/kg	+	+	+	+
1	FTY720 0.03mg/kg	+	+	+	+
2	FTY720 0.03mg/kg	+	+	+	+
7	FTY720 0.03mg/kg	+	+	+	+
9	FTY720 0.03mg/kg	+	+	+	+
12	_	+	+	+	+
15	_	+	+	+	+
22	_	+	+	+	_

Days 3, 4, 5, 6, and 8: 0.03mg/kg FTY720 was incorporated in food

4.1.3 Pharmacodynamic study

A pharmacodynamic study was performed on three baboons to assess the efficacy of 0.3mg/kg of FTY720 at 0, 1, 2, 4, 8, 24 and 48 hours. Anaesthesia was induced with ketamine hydrochloride (7-14mg/kg i.m.) and maintained for eight hours using 0.5% hallothane in 30% O_2 and 70% N_2O administered intra-tracheally to secure the airways during the intravenous infusion of 0.09% saline. FTY720 was substituted with distilled water in a later experiment to exclude possible side effects from either the anaesthetic or repeated blood sampling. At each time point 10ml of blood was taken and analysed for the same parameters as in the experiments with the different doses of FTY720.

4.1.4 Neutralisation Studies: FTY+αGal-Antibody response

α-Galactosyl (α-Gal) antibodies are found in high titres in humans and Old World primates and play a significant role in hyperacute rejection (Cooper *et al.*, 1993). Since FTY720 is believed to cause a dramatic decrease in both T and B lymphocytes and the αGal response is considered to be "T-cell independent", it was decided to test the potential of FTY720 on a "T-cell independent" B-cell response which is relevant for xenotransplantation. Three baboons were treated for five weeks with either 0.1mg or 0.3mg/kg/day FTY720 in maize food balls as previously described, or by gastric intubation when anaesthesia was performed (day -1, 2, 4, 5, 7, 8, 11, 14, 21, 28, 35, 42 and 52). The drug was made up weekly, dissolved in distilled water as a stock solution, and then aliquoted for each application and stored at 4°C until use. Alpha-

galactosyl polymers (Novartis Pharma, Basel) were dissolved in normal saline, aliquoted and frozen at -20°C. αGal was administered under anaesthesia (ketamine, 10mg/kg i.m) via the saphenous vein at 1mg/kg. The concentration of αGal at 1mg/ml was given at a rate of approximately 2ml per minute using a syringe driver (Vial Medical SE 200). The antibodies were administered with the drug on days 1, 4 and 7 after the commencement of FTY720 (Table 4).

4.1.5 FTY720 in mice

C57Bl/6+129SV mice deficient in lymphotoxin-alpha (LT-α-/-)were obtained from Theresa Banks of the University of Tennessee, Knoxville 37996, USA.
C57Bl/6+129SV mice were supplied by the UCT Animal House.
C57Bl/6+129SV mice deficient in L-selectin (L-selectin-/-) were obtained from Thomas Tedder of Duke University Medical Centre, North Carolina, 27710, USA.

1mg/kg FTY720 was administered intraperitoneally to four LT-α-/- mice, four C57Bl/6+129SV mice and 2 L-selectin-/- mice. One mouse from the first two groups received the same volume of distilled water as controls. Blood was obtained from the orbital sinus prior to the commencement of the drug and 24 hours after the administration of FTY720. The white blood cell count and lymphocyte counts were determined using a Technikon H1 blood counter.

Table 4

PROTOCOL FOR BABOONS RECEIVING

FTY720 and 1mg/kg α-GAL POLYMER

DAY	DOSING	FBC/DIFF/ FACS	IgM, IgG, C^1	WB PROLIF	DRUG LEVELS
-14	ENTRY	+	+	+	-
-5	FTY720	+	+	+	+
-3	FTY720	+	+	+	+
1	FTY720 + α-GAL	+	+	+	+
2	FTY720	+	+	+	+
4	FTY720 + α-GAL	+	+	+	+
5	FTY720	+	+	+	+
7	FTY720 + α-GAL	+	+	+	+
8	FTY720	+	+	+	+
11	FTY720	+	+	+	+
14	FTY720	+	+	+	+
21	FTY720	+	+	+	+
28	-	+	+	+	+
35	-	+	+	+	+
42	-	+	+	+	+
52	-	+	+	+	+

0.1mg/kg FTY720 was administered from day –5 to day 10 and 0.3mg/kg FTY720 was given from day 11 to day 27.

4.2 Ex Vivo Experiments

4.2.1 Haematology

5ml of blood was collected into tubes containing EDTA K_3. The peripheral blood parameters and differential were determined using a Technicon H1 blood counter (Bayer, Munich, Germany).

4.2.2 Immunophenotyping

Whole blood was tested for cell surface expression of CD3, CD20, CD4 and CD8 positive cells by flow cytometry using dual colour analysis. The antibodies used were as follows:

- mouse anti-monkey CD3 antibody coupled to FITC, clone FN18 (Biosource, International, Fleurus, Belgium)
- mouse anti-human CD4 antibody coupled to phycoerythrin (P.E.), clone SFC/12T4D11 (T4-RD1; Coulter Coporation, Miami, Florida)
- mouse anti-human CD8 antibody coupled to FITC, clone B9-11 (Immunotech, Marseille, France)
-mouse anti-human CD20 antibody coupled to PE, clone H299 (B1-RD1; Coulter Coporation, Miami, Florida)

The isotypic controls were:

- mouse IgG1 coupled either to FITC or PE (MsIgG1-FITC; or MsIgG1-RD1; Coulter Coporation, Miami, Florida).

Anti CD3 and 8 were undiluted, whilst anti-CD4, CD20 and the isotypic controls were made up using 5µl of the antibody added to 20µl of FACs buffer (Appendix A).

For the experiment, 40µl of whole blood was added to 10µl of each antibody (or antibody dilution) and incubated on ice in the dark for 30 minutes. The red blood cells were then lysed using 2mls of a 1:10 lysis buffer (FACS™ Lysing solution, Becton Dickinson, Mountain View, California) for 10 minutes. After two washes in FACs buffer the cells were suspended in 0.5ml of the buffer for flow cytometry analysis using a Becton Dickinson FACScan with Cell Quest software. Negative controls were set up using isotypic controls of the appropriate antibodies coupled with either FITC or PE.

4.2.3 Whole blood proliferation assay

Whole blood was diluted 1:20 with RPMI-1640 (Bio Whittaker, Walkersville, Maryland). 100µl of the diluted blood was stimulated by 4µg/ml Concanavalin A (Con A; Sigma Chemical Company, St. Louis, USA). The cultures were performed in 96 well U-bottomed plates (Corning Corporation, Corning, New York) and incubated for 5 days at 37°C in a humidified atmosphere containing 5% CO_2. After 4 days, 1µCi/well of ^{3}H Thymidine (^{3}H Tdr 6.7Ci/mmol, ICN Pharmaceuticals, Inc., Irvine, California) was added to each test well and cell proliferation was measured 18 hours later by ^{3}H-Tdr incorporation using a Beckman LS3800 β counter. Negative controls were performed in the absence of Con A.

4.2.4 FTY720 whole blood levels

Whole blood from each time point was collected in a vacutainer containing EDTA K_3 and kept frozen until the completion of each group study. Blood levels of FTY720 were determined by LC/MS on a HP Series 1100 HPLC system interfaced with a Finnigan Mat TSQ 7000 mass spectrometer. The analytical performance was estimated from the calibration curves and from the results obtained with the quality control samples, which were analysed simultaneously. The limit of quantification (LOQ), defined by the lowest concentration of calibration samples that could be quantified with an accuracy of 100% ± 20%, was 0.38ng/ml for the 0.3 and 0.1mg/kg FTY720 and 0.072ng/ml for the 0.03mg/kg FTY720. All samples were tested in parallel to ensure accuracy. The drug monitoring was performed at Novartis in Basel.

4.3 In vitro Experiments

4.3.1 Immunosuppressive drugs

A. Peripheral blood mononuclear cells (PBMCs)

PBMC were isolated as follows: heparinised whole blood was diluted 1:4 in phosphate buffered saline (PBS, see Appendix A). Ten millilitres (mls) of diluted blood was layered onto 4mls of histopaque^{R}-1077 (Sigma Chemical Company, St. Louis, USA) and centrifuged at 1800rpm for 20 minutes. The cells at the interphase were harvested, washed twice in PBS, then resuspended in RPMI supplemented with 10% foetal calf serum (FCS, Delta Bioproducts, Kempton Park, South Africa) and 200μg/ml gentamycin (Biowhittaker, Walkersville, Maryland). $1x10^5$ PBMC/ml from two mismatched baboons were incubated together with 0-10μM of an immunosuppressive drug in order to evaluate the concentration that inhibits 50% of proliferation (IC50). The drugs tested were Cyclosporine A, (CsA), Tacrolimus (FK506), SDZ RAD and FTY720 (all drugs from Novartis Pharma, Basel). The assay was performed in 96 well U bottomed plates which were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 5 days the cultures were pulsed with 1μCi/well ^{3}H-Tdr and eighteen hours later the cells were harvested and ^{3}H-Tdr incorporation was assessed using a Beckman LS3800 β counter.

Controls were set up simultaneously where the drug was substituted with the vehicle that they were made up in i.e. either alcohol for CsA, FK506 and SDZ RAD or distilled water for FTY720.

B. Whole Blood

Baboon whole blood was diluted 1:20 and 100µl of diluted blood was incubated with 50µl of Con A (at a final concentration of 4µl/ml) and 50µl of each drug at varying concentrations (0-10µM) as described in the previous assay. The cultures were incubated for 4 days, then pulsed with ^{3}H-Tdr and treated as for the PBMC.

4.3.2 Apoptosis

Apoptosis was measured using the Annexin-V-Fluos staining kit (Boehringer Mannheim, Mannheim, Germany). The experiment was set up using both human and baboon PBMC, as it had not been determined whether the reagents in the kit were cross reactive with baboon cells. $1x10^{6}$ PBMC/ml, isolated as previously described, were incubated with 0, 3, 6 and 10µM FTY720 for 4 hours at 37°C in a humidified atmosphere containing 5% CO_2. After incubation, the cells were washed once in PBS to remove the drug, then 100µl of the labelling solution (which included propidium iodide to assess dead cells) was added and the tubes were left on ice for 15 minutes. 400µl of the buffer (Appendix B) was added and cell death was assessed using a Becton Dickinson FACScan.

5. RESULTS: *Ex vivo:*

5.1 FTY720 at 0.3mg/kg

5.1.1 Effect of 0.3mg/kg FTY720 on the haematological parameters

Figure 2A shows the decrease in lymphocyte counts in three baboons that received 0.3mg/kg FTY720 (day 0, 1, 2), and the recovery phase over 4 weeks. There was no systemic effect of the procedure i.e. ketamine anaesthesia, gastric intubation or stress induced as shown by the lymphocyte counts during the administration of the vehicle (day –7, -6, -5); the lymphocyte count variability was ± 20% with vehicle only. Within 24 hours of FTY720 treatment there was a 59 ± 6% decrease in circulating lymphocytes which was further reduced to 70 ± 7% 24 hours after the second dose. In all three baboons the lymphocyte counts started to recover within 48 hours after the last FTY720 administration although the recovery rate was different for each baboon as seen in figure 2B. There was no significant effect of the FTY720 on the other haematological parameters although there was an intrinsic variation of neutrophil and monocyte counts namely –58 to +24% for neutrophils and –22 to +168% for monocyte counts (Figures 2C and 2D). These counts however, remained within the normal parameters for each cell type. There was a marked overshooting of the neutrophil counts 5-8 days after the treatment which was transient and is unexplained. It may however, be attributed to stress due to the procedure. There was no effect on red blood cell parameters or platelet counts.

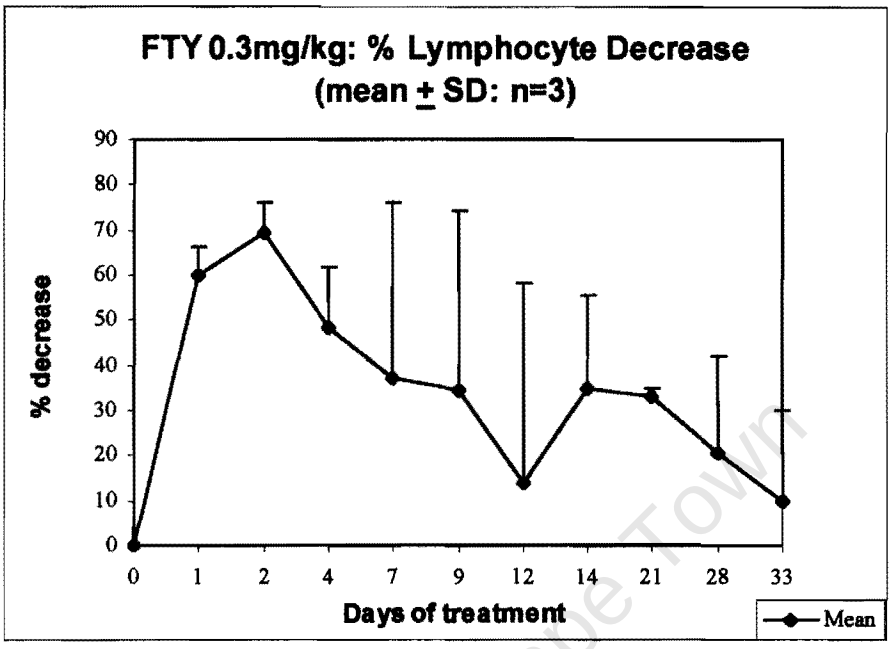


Figure 2A: % Decrease of lymphocytes in three baboons treated with 0.3mg/kg FTY720

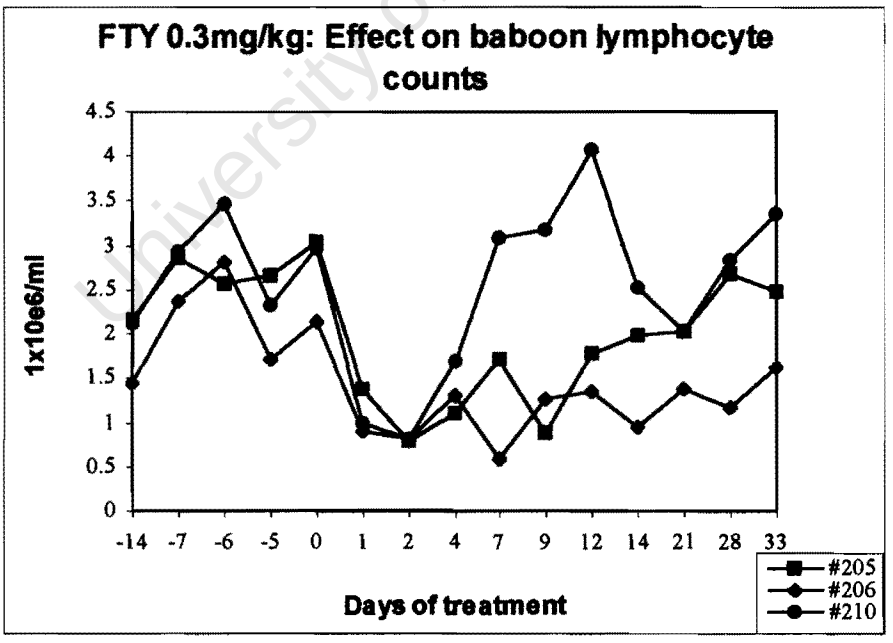


Figure 2B: Effect of 0.3mg/kg FTY720 on the lymphocyte counts in three baboons

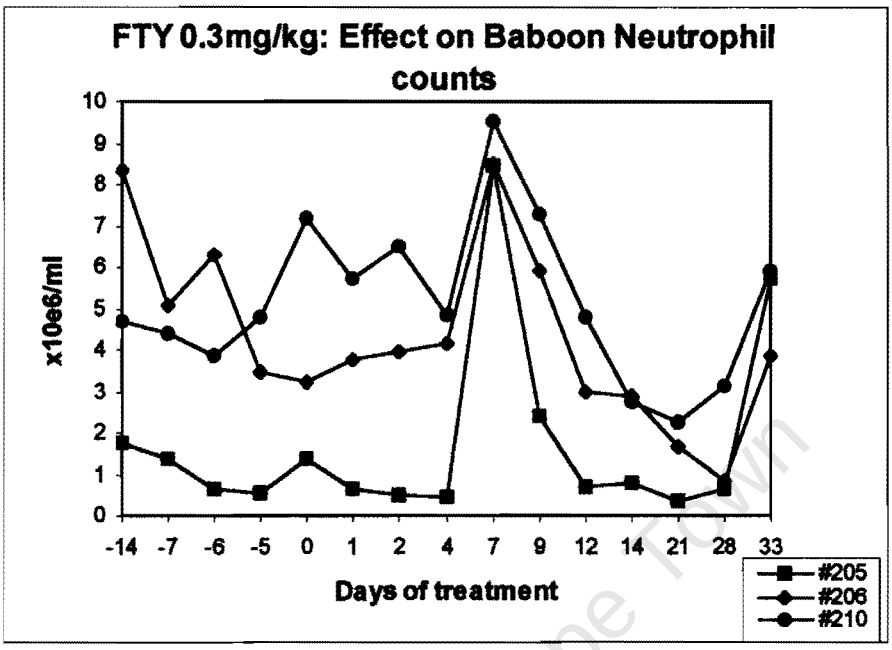


Figure 2C: Effect of 0.3mg/kg FTY720 on neutrophil counts in three baboons

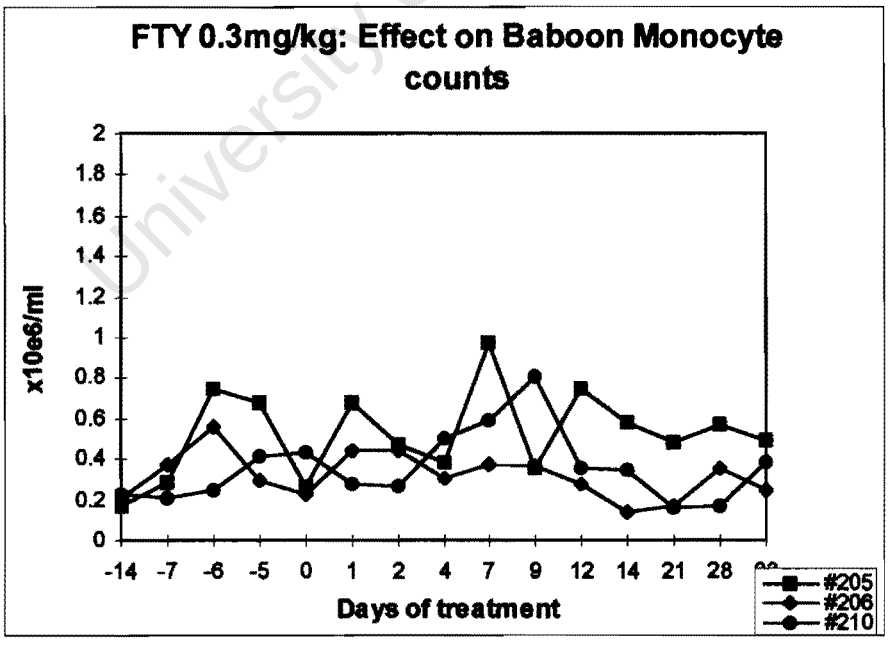


Figure 2D: Effect of 0.3mg/kg FTY720 on monocyte counts in three baboons

5.1.2 Effect of 0.3mg/kg FTY720 on T and B lymphocytes

FTY720 had an effect on both T and B peripheral lymphocytes, the effect being slightly more pronounced on T-cells than on B-cells as seen in Figure 3A. Within 24 hours of FTY720 administration CD3+ T-cells decreased by 64 ± 8% in the three baboons whereas CD20+ B-cells decreased by 39 ± 6%. After two days of treatment CD3+ T-cells were reduced by 75 ± 7% and CD20+ B-cells by 51 ± 9%. CD3+ T-cells recovered quicker than CD20+ B-cells, which only returned to pre-treatment values three weeks post FTY720 administration.

There was no systemic effect of the mock vehicle treatment on T or B cells, however the variability was –18%-28% for CD3+ cells and –22%-20% for B cells.

5.1.3 Effect of 0.3mg/kg FTY720 on T-cell subsets

Figure 3B demonstrates the effect of FTY720 on T-cell subsets. CD4+ cells were reduced by 73 ± 6% and CD8+ cells by 57 ± 10% within 24 hours and by 84 ± 7% and 66 ± 10% respectively after two days of treatment. The recovery of CD8+ cells was substantially quicker than CD4+ T cells. Within four weeks the pre-treatment values of CD4+ and CD8+ cells were recovered.

A small percentage of double positive CD4/CD8 cells (1.3%, 3.4%, 6.9%) were seen in circulation and in the post treatment phase two of the three baboons showed a marked increase of these cells (11.1% and 10.5%).

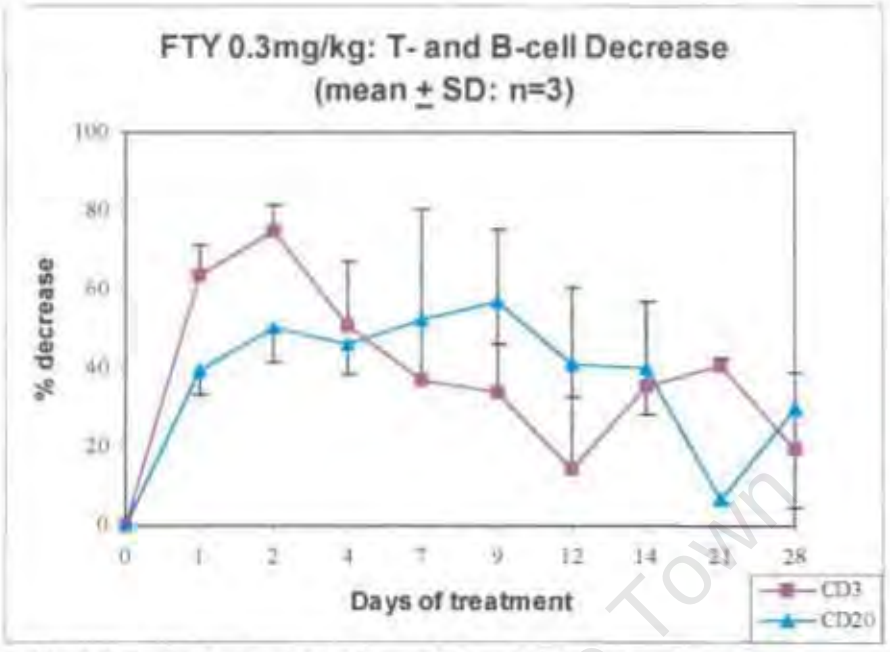


Figure 3A: Effect of 0.3mg/kg FTY720 on CD3+ and CD20+ lymphocytes

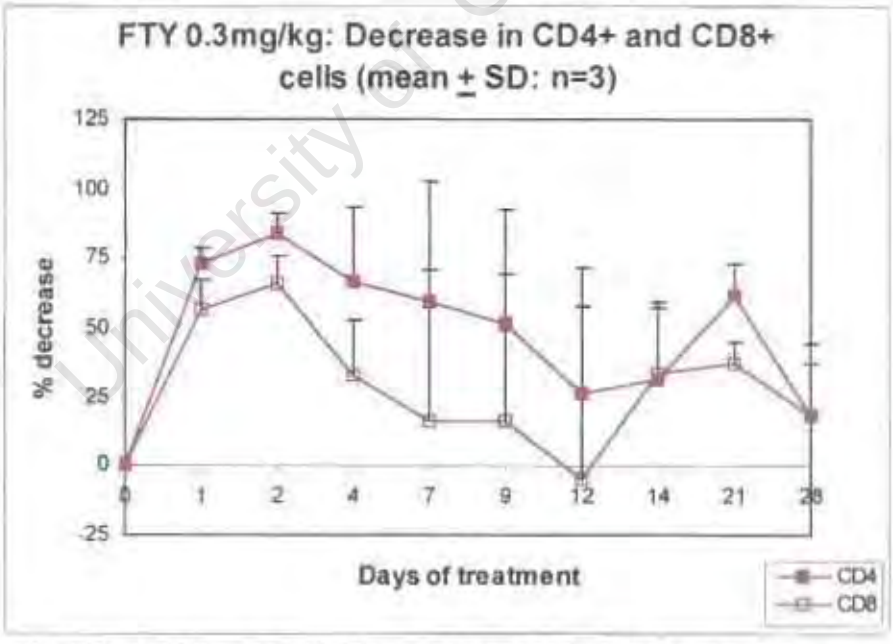


Figure 3B: Effect of 0.3mg/kg FTY720 on CD4+ and CD8+ lymphocytes. CD4+ T-cells are shown with closed squares and CD8+ cells are a shown with open squares

5.1.4 Effect of 0.3mg/kg FTY720 on *ex vivo* whole blood proliferation

Figure 4A shows the response of whole blood to the mitogen Con A before and after treatment with FTY720. The background, in the absence of stimulus, and the response to Con A was stable prior to drug initiation i.e. day -14, -7, -6, -5 and 0. The stimulation index for these time points was between 40-300 in response to Con A. There was a dramatic decrease in Con A stimulation after the drug was administered viz. pre drug values of 11000-27000 were reduced to 100-700 cpm. In a whole blood assay this decrease in proliferation almost certainly could be due to a decreased lymphocyte count however, figure 4B shows when proliferation (i.e. response to Con A) was corrected by lymphocyte counts (i.e. cpm/1x10^6 lymphocytes) there was a decrease of up to two logs.

Since there is no linearity between cell concentration and proliferative response to Con A in the whole blood proliferation assay, the unresponsiveness is difficult to interpret. However, the proliferative response in two out of the three baboons was at the lowest on day 4 when the lymphocyte counts were already starting to recover, so one can assume that FTY720 does have an affect on the function of peripheral blood lymphocytes.

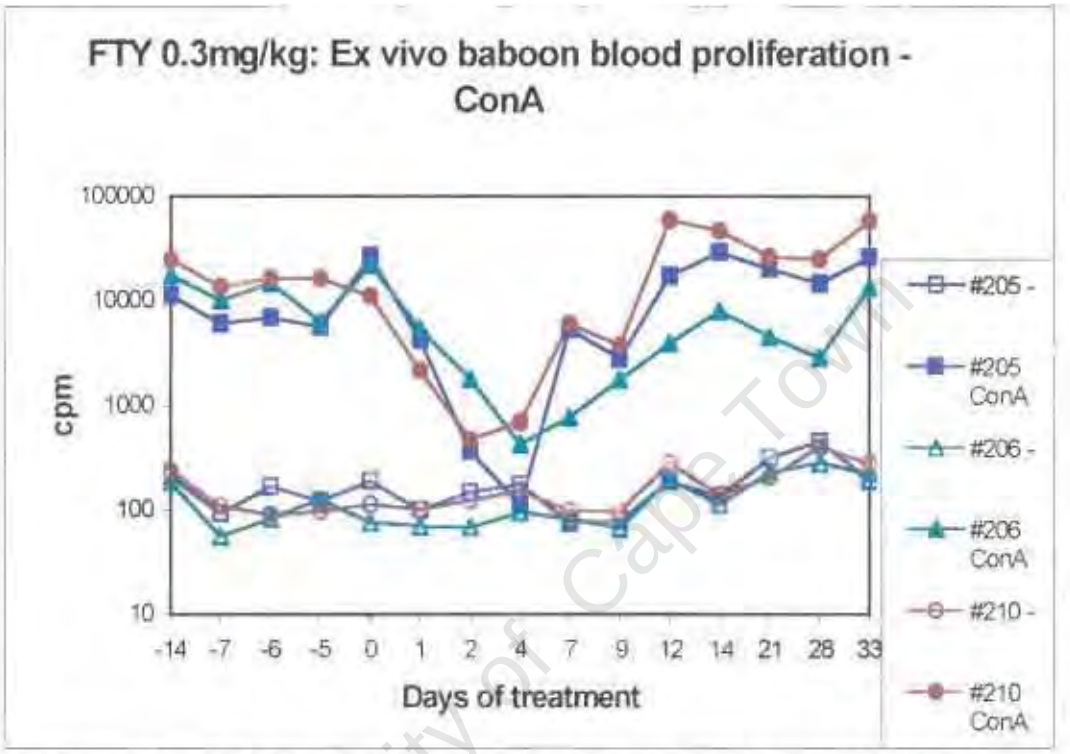
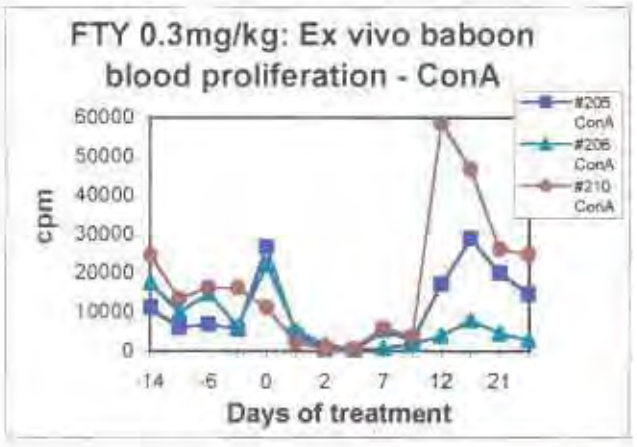
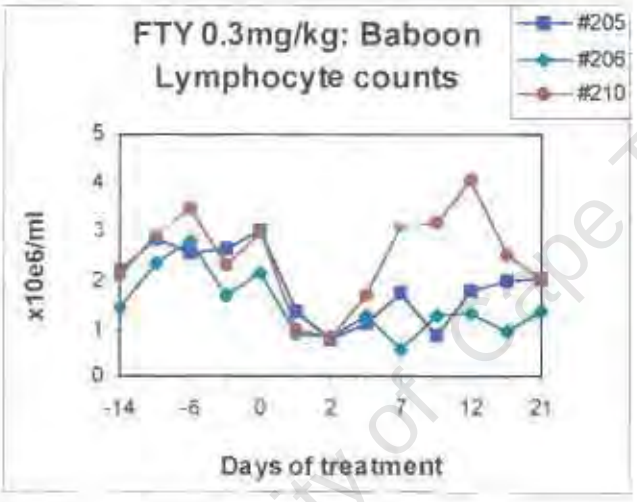


Figure 4A: Whole blood proliferation in response to no stimulus (open symbols) or stimulated with Con A before and after treatment with 0.3mg/kg FTY720 (closed symbols)





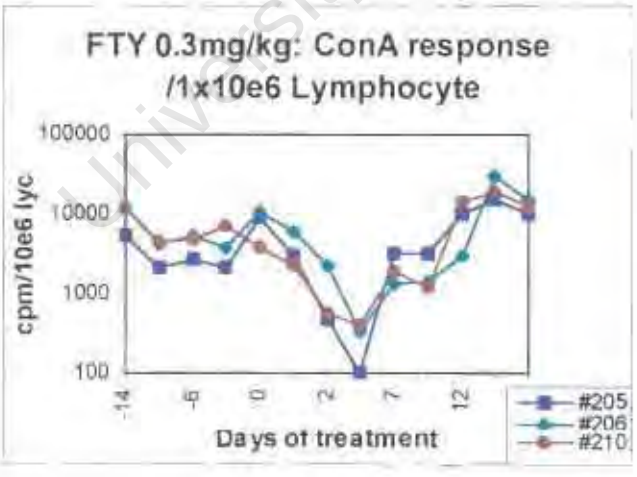


Figure 4B: Whole blood proliferation in response to Con A, corrected by lymphocyte counts ($1x10^6$ cells)

5.1.5 Conclusion

0.3mg/kg FTY720 on three successive days caused a rapid, dramatic effect on the number of peripheral lymphocytes within 24 hours and this effect was apparent on both T and B-cells. The proliferative function of circulating lymphocytes was also affected as seen in the whole blood proliferation assay. No obvious side effects of the drug e.g. vomiting or respiratory problems were encountered by any of the three baboons tested although there was a transient loss in body weight in one of the three baboons (Table 5). This was likely due to an impaired feeding after the anaesthetic. Although the body weight was back to pre-drug values within two days after the last FTY720 treatment, it was decided that frequent bleedings, which require anaesthesia, were responsible for this weight loss and further experiments would be monitored at less time points. It was also noted that the time required for the animals to fall asleep after administration of the ketamine was markedly accelerated on the third day of treatment.

FTY720 had no effect on any other parameters tested.

Table 5 **Body weight of baboons**

Day	#205	#206	#210
-7	14.0 kg	14.5kg	14.0kg
0	14.0kg	14.5kg	14.0kg
2	13.5kg	14.0kg	12.0kg
7	13.5kg	13.5kg	15.0kg
9	13.5kg	15.0kg	16.0kg
12	14.5kg	15.0kg	15.5kg
14	15.0kg	15.5kg	16.0kg

5.2 Pharmacodynamic study (PD) of 0.3mg/kg FTY720

5.2.1 Kinetic effect of 0.3mg/kg FTY720 on lymphocyte counts

The lymphocyte counts of all three baboons were decreased within 1-2 hours of FTY720 treatment. A maximum decrease was seen after 24 hours when there were 77 ± 3% of the pre-drug lymphocyte values. This decrease was still observed 24 hours later i.e. 48 hours after the first administration of FTY720 (Figure 5A).

5.2.2 Kinetic effect of 0.3mg/kg FTY720 on T and B-cells

Figure 5B shows a rapid and steady reduction in both CD3+ T-cells and CD20+ B-cells. Within 24 hours CD3+ cells decreased by 83 ± 5% and CD20+ cells showed a less pronounced decrease of 52 ± 4%. These results compared favourably with the data of the 0.3mg/kg FTY720 over 3 days.

5.2.3 Kinetic effect of 0.3mg/kg FTY720 on T-cell subsets

CD4+ T-cells were only slightly more affected than CD8+ cells as shown by figure 5C. CD4+ cells were reduced by 89 ± 1% and CD8+ cells were reduced by 82 ± 1%.

There was an increase of CD4/CD8 double positive cells in two out of the three baboons viz. 1.7% up to 3.7% and 6.7% up to 9.7%.

5.2.4 Kinetic effect of 0.3mg/kg FTY720 on whole blood proliferation

Within 4 hours there was a decrease in response to Con A which was at its maximum 24-48 hours after the treatment (Figure 5D). This followed the decrease in lymphocyte counts. When the proliferation was corrected by the lymphocyte counts (i.e. cpm/1+10^6 lymphocytes) the stimulation index dropped from 137 ± 6% at 0 hours to 10 ± 6 at 48 hours (Figure 5E).

5.2.5 Kinetic effect of 0.3mg/kg FTY720 on cell death

Annex-V staining was applied to the PBMC collected at each time point during the PK study. No apoptosis was observed in the baboon blood at any time point although in control experiments, using the same detection system, apoptosis was induced in human PBMC after 4 hours in the presence of 3μmol FTY720 (Figure 5F- apoptotic cells are seen in the lower right quadrant whilst necrotic cells are found in the upper right quadrant).

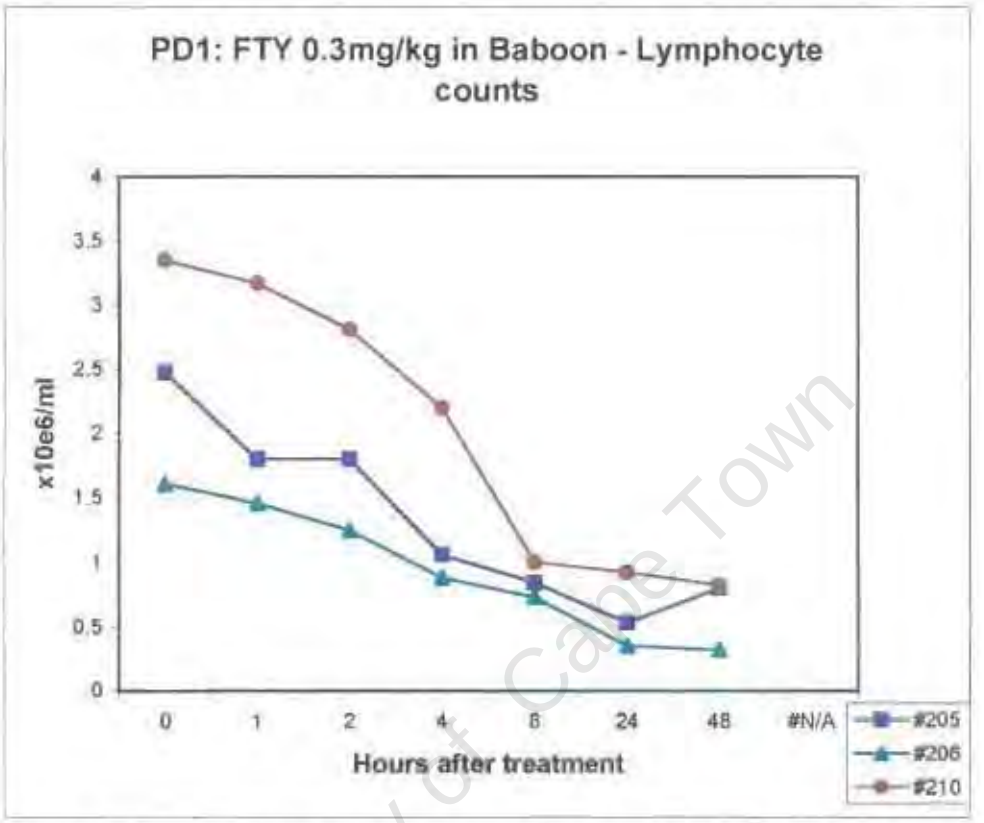


Figure 5A Decrease in lymphocyte counts over 48 hours, after 0.3mg/kg FTY720 treatment

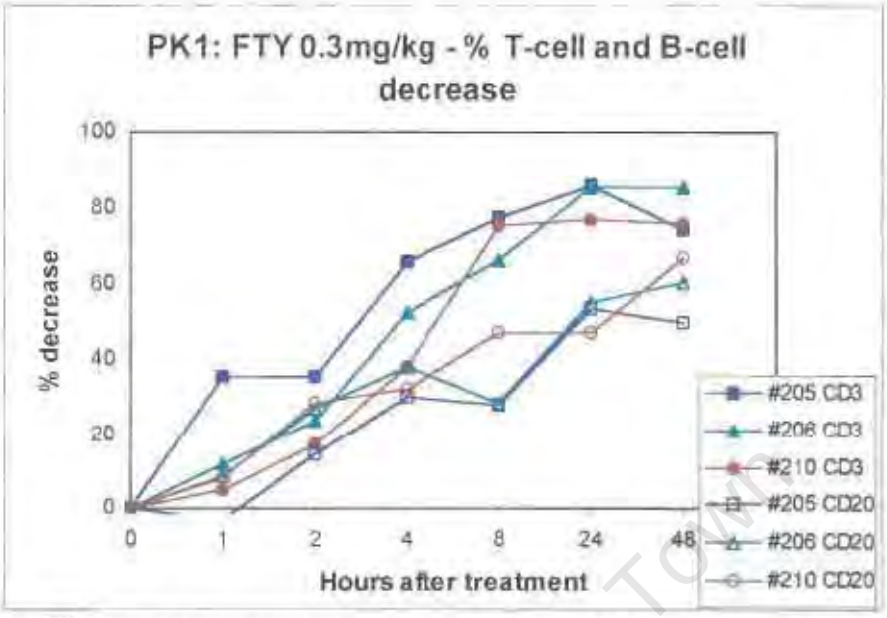


Figure 5B: % T and B lymphocyte decrease over 48 hours. CD3+ T-cells are shown with closed symbols and CD20+ B-cells are shown with open symbols.

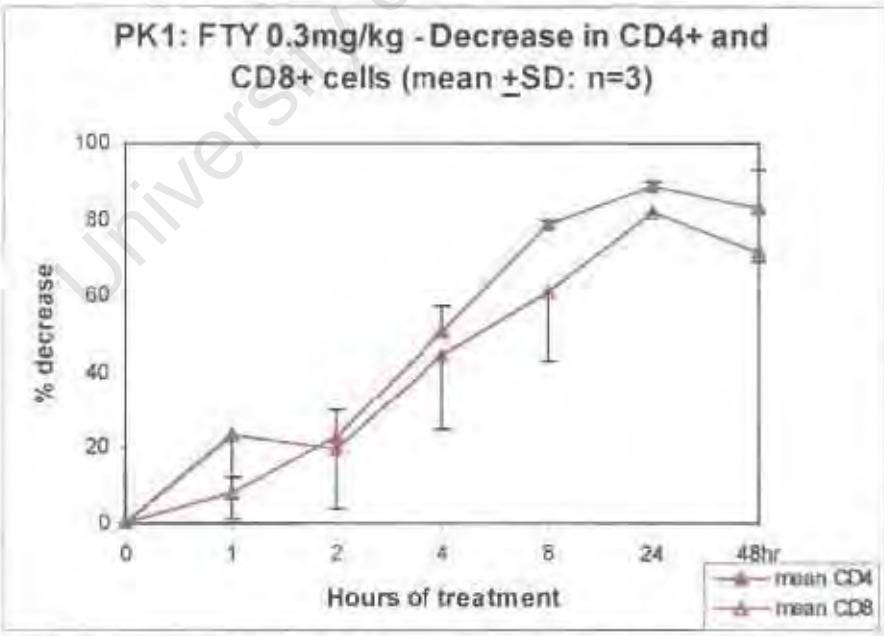


Figure 5C: % Decrease of CD4+ and CD8+ lymphocyte over 48 hours. CD4+ cells are represented by a closed symbol and CD8+ cells by an open symbol.

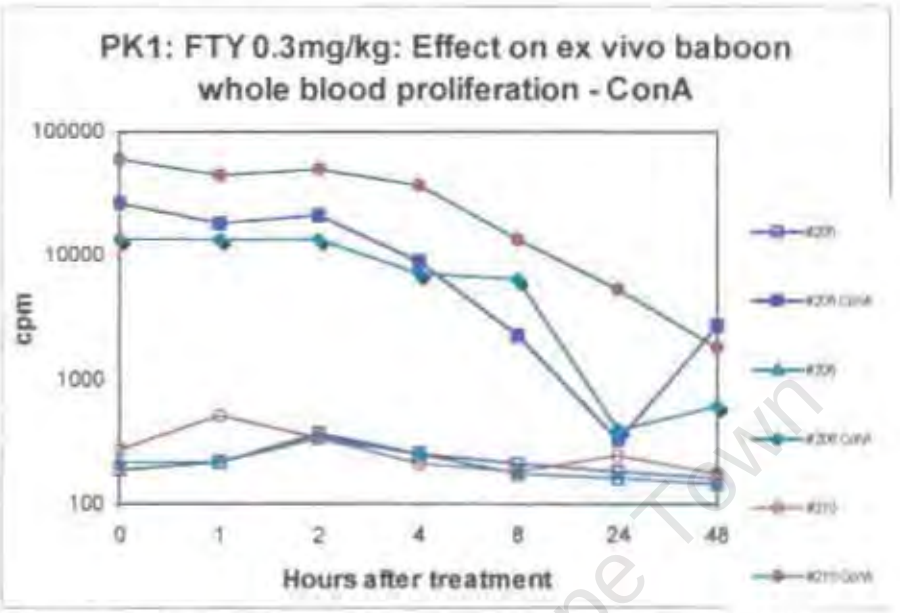


Figure 5D: Whole blood proliferation in response to no stimulus (open symbols) or stimulated with Con A (closed symbols).

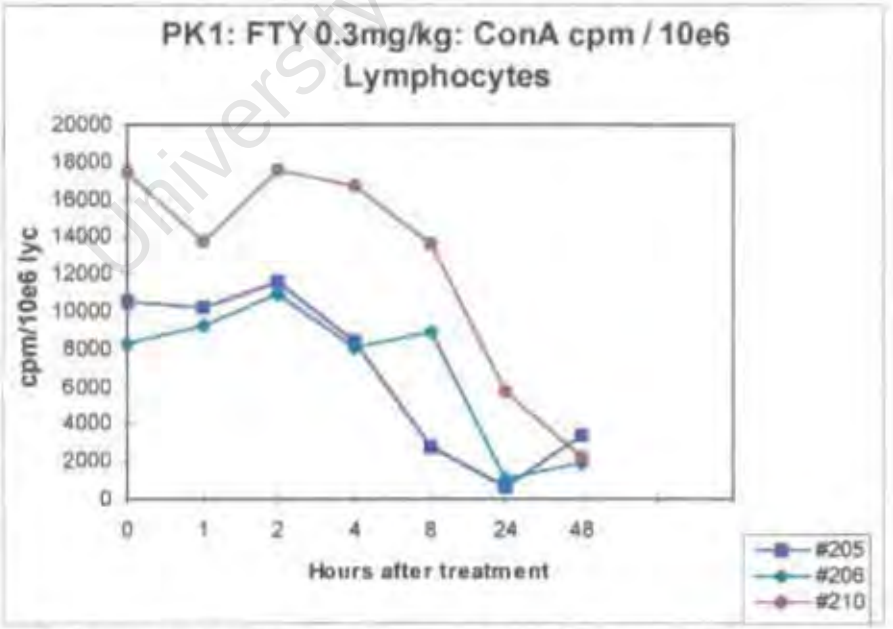


Figure 5E: Whole blood proliferation in response to Con A corrected by lymphocyte counts (i.e. cpm/1x10^6 lymphocytes).

5.3 FTY720 at 0.1mg/kg

5.3.1 Effect of 0.1mg/ml FTY720 on the haematological parameters

Figure 6A shows the effect of 0.1mg/kg of FTY720 on the lymphocyte counts of three baboons during drug administration, and the recovery phase over two weeks. Within one day of treatment there was a slight decrease in circulating lymphocytes by 17 ± 12% which was further reduced to 49 ± 7% after the second dose. The lymphocyte counts continued to drop to 58 ± 7% (Figure 6B) two days after the last administration of FTY720 which suggests an accumulation of the drug. Pre-drug values were recovered three days later i.e. day 7.

There was no effect of the FTY720 treatment on any other haematological parameters.

5.3.2 Effect of 0.1mg/ml FTY720 on T and B lymphocytes

The effect of 0.1mg/kg FTY720 on T and B-cells was similar to that seen with the higher dose of 0.3mg/kg FTY720. Figure 7A shows the effect of the drug on CD3+ and CD20+ cells. CD3+ T-cells were slightly more reduced than CD20+ B-cells viz. T-cells were reduced by 52 ± 8% and B-cells were reduced by 41 ± 11%. Two days after the last dose of FTY720 (i.e. day 4) CD3+ cells were reduced by 59 ± 11% and CD20+ cells were reduced by 64 ± 5%. The recovery of B-cells was once again slower than the T-cells that were back to the pre-drug levels within one week.

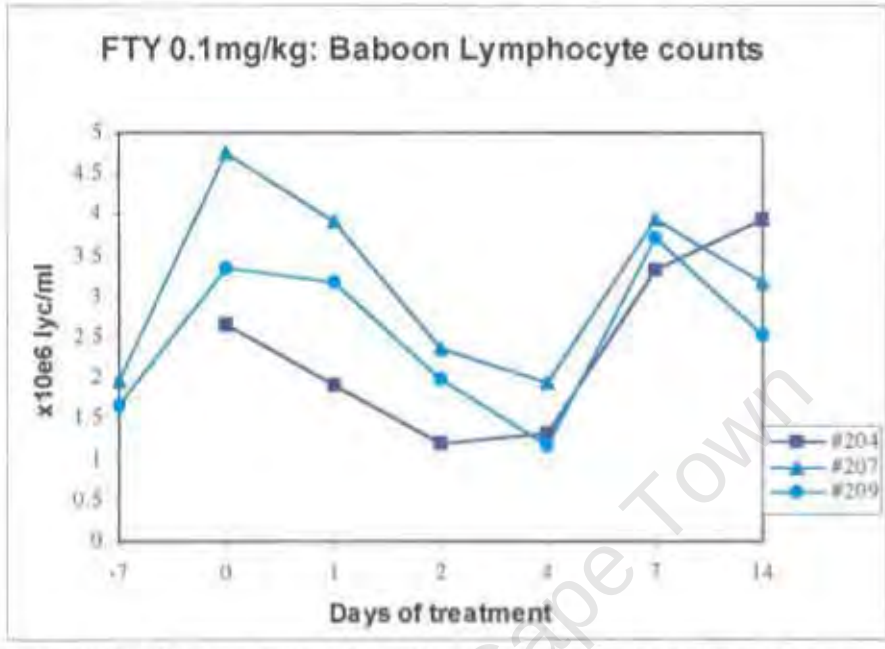


Figure 6A: Effect of 0.1mg/kg FTY720 on the lymphocyte counts in three baboons

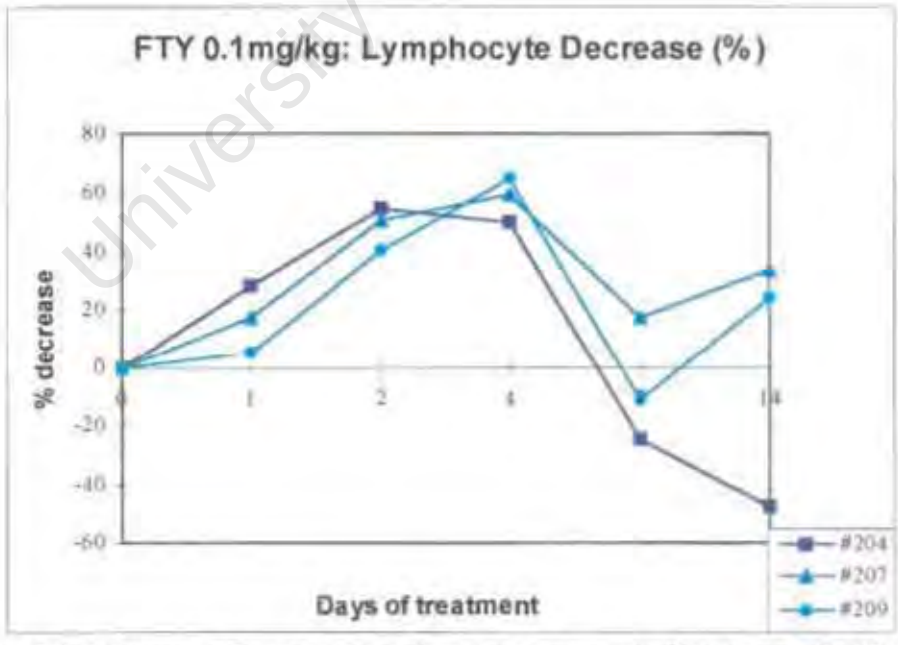


Figure 6B: % Decrease of lymphocytes in three baboons treated with 0.1mg/kg FTY720

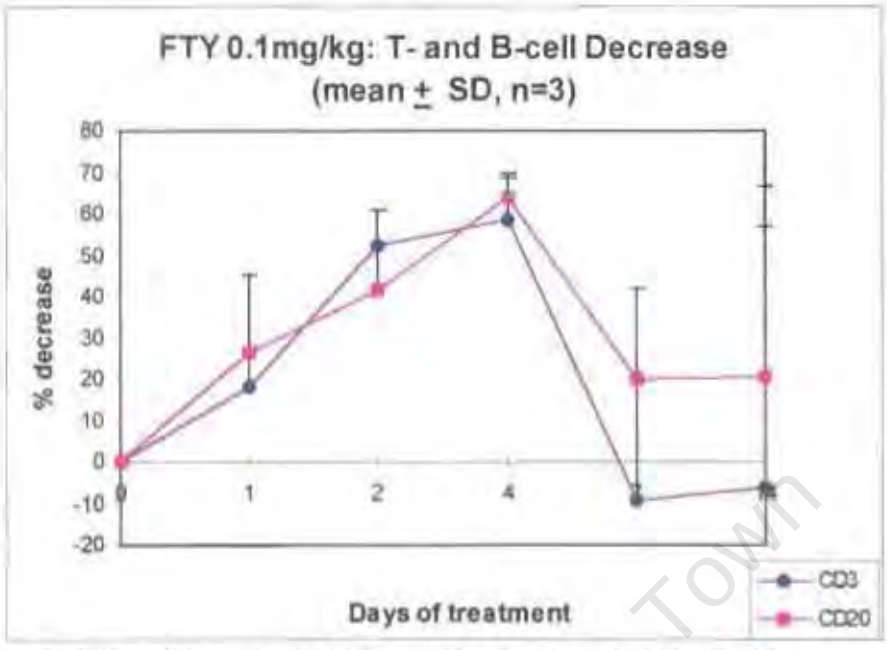


Figure 7A: Effect of 0.1mg/kg FTY720 on CD3+ T-cells and CD20+ B-cells

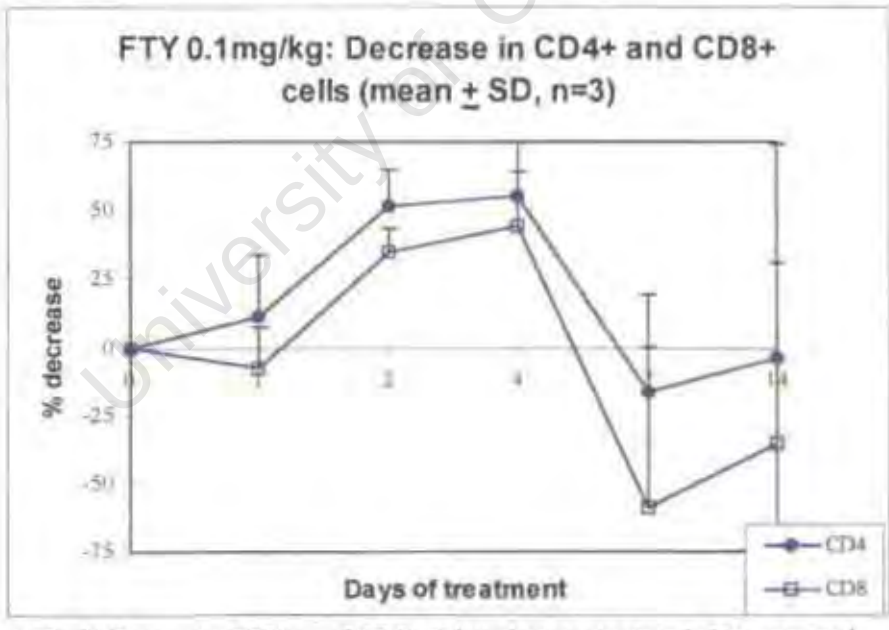


Figure 7B: % Decrease of CD4+ and CD8+ T lymphocytes in three baboons treated with 0.1mg/kg FTY720

5.3.3 Effect of 0.1mg/ml FTY720 on T-cell subsets

CD4+ T-cells were slightly more affected than CD8+ cells as seen in figure 7B. After two days of treatment CD4+ cells were reduced by 52 ± 13% and CD8+ cells by 30 ± 13%. On day 4 the reduction was 55 ± 9% and 44 ± 31% respectively. Both T-cell subsets were at least back to pre-drug values on day 7 although CD8+ T- cell counts showed an overshooting. As seen with the higher dose of FTY720 there were a small number of double positive CD4/CD8 cells that increased after FTY720 treatment i.e. 4.7 ± 1.5% to 9.8 ± 2.1%

5.3.4 Effect of 0.1mg/ml FTY720 on *ex vivo* whole blood proliferation

The response to Con A was decreased dramatically in two of the three baboons 24 hours after the first treatment. Baboon 209 showed a less potent but steady reduction in response to Con A. Two days after the cessation of the drug i.e. day 4, all three baboons had a decreased response to Con A although baboon 207 had already started to recover (Figure 8A). When proliferation was corrected by the lymphocyte counts (i.e. cpm/1+10^6 lymphocytes) a decrease of 1.5 logs was observed (Figure 8B). By day 7 all three baboons had recovered fully.

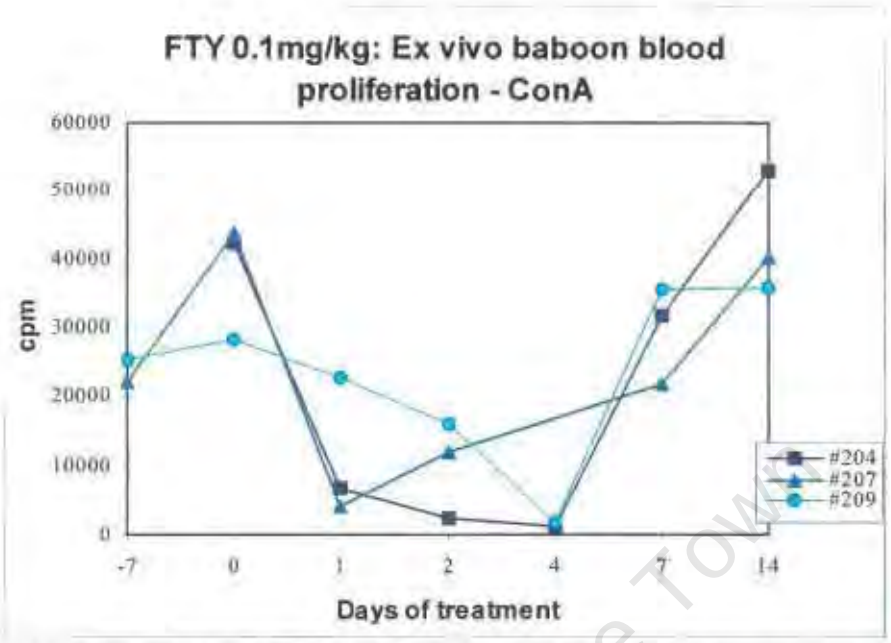


Figure 8A: Effect of 0.1mg/kg FTY720 on whole blood proliferation in response to Con A in three baboons

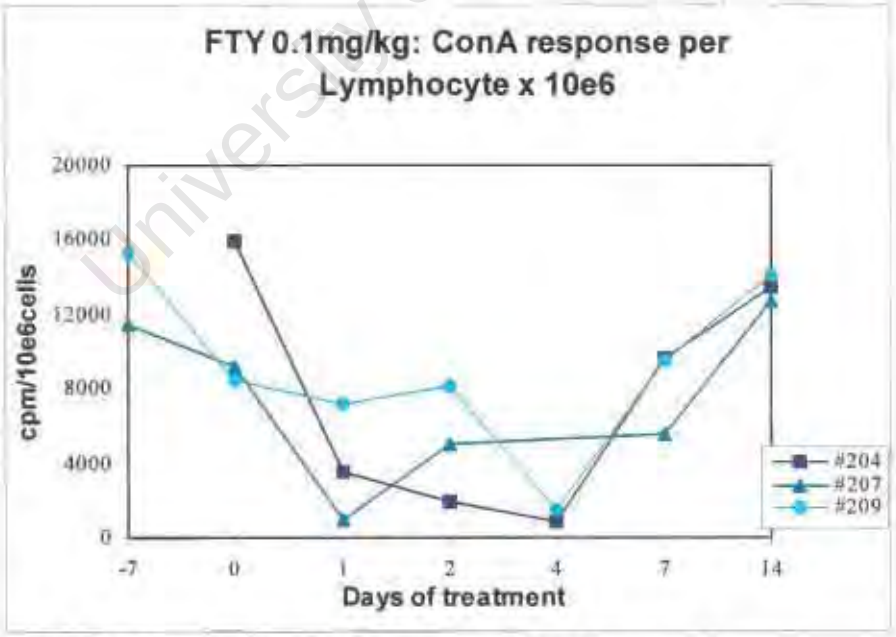


Figure 8B: Whole blood proliferation in response to Con A, corrected by lymphocyte counts (i.e. cpm/1x10^6 lymphocytes)

5.3.5 Conclusion

0.1mg/kg FTY720 on three successive days induced a less dramatic effect than the higher 0.3mg/kg dose. The decrease in circulating lymphocytes was very modest after the first dose of FTY720 however, two days after the last dose there was still a 58 ± 7% decrease. Five days after the cessation of the drug the lymphocyte counts recovered which demonstrates a transient partial lymphodepletion. The effect was seen on both T and B-cells. The proliferative function of circulating lymphocytes was also affected as seen in the whole blood proliferation assay.

No side effects of the drug were encountered by any of the three baboons tested however, as seen with the previous trial of 0.3mg/kg FTY720, the body weight fluctuated during the period whilst the drug was being administrated (Table 6).

Table 6 **Body weight of baboons**

Day	#204	#207	#209
-7	20.0 kg	17.0kg	18.0kg
0	20.0kg	13.5kg	17.0kg
1	17.5kg	13.0kg	15.0kg
2	18.0kg	12.5kg	15.0kg
4	18.0kg	14.5kg	17.0kg
14	19.5kg	15.5kg	18.0kg

5.4. FTY720 at 0.03mg/kg

5.4.1 Effect of 0.03mg/ml FTY720 on the haematological parameters

The lymphocyte counts were reduced in two of the three baboons in response to the low dose of 0.03mg/kg FTY720; the third baboon, #219, showed essentially no lymphocyte decrease throughout the ten-day administration of the drug. Figure 9A shows the lymphocyte depletion in the two baboons which was 42 and 56% within one day and reached 60-80% by day 7-9. The counts had recovered to pre-drug levels by day 22 (Figure 9B).

There was no effect of the drug on the other haematological parameters.

5.4.2 Effect of 0.03mg/ml FTY720 on T and B-lymphocytes

Two of the three baboons showed a reduction of both T- and B-cells within one day of FTY720 administration as seen in figure 10A. The CD3+ T-cells in baboons 235 and 263 were reduced by 60% and 47% respectively after the first dose and this was further reduced to 75% and 88% respectively by day 7. The lymphocyte counts had recovered to pre-drug levels by day 22.

CD20+ B-cells were less affected but nevertheless were reduced by 43% and 17% on day 1. These figures reached 74% and 56% by day 7. As seen in higher doses of FTY720, the recovery of T-cells was much quicker than B-cells. On day 22 when the experiment was terminated, baboon 235 had still not reached the pre-drug levels of CD20+ B-cells.

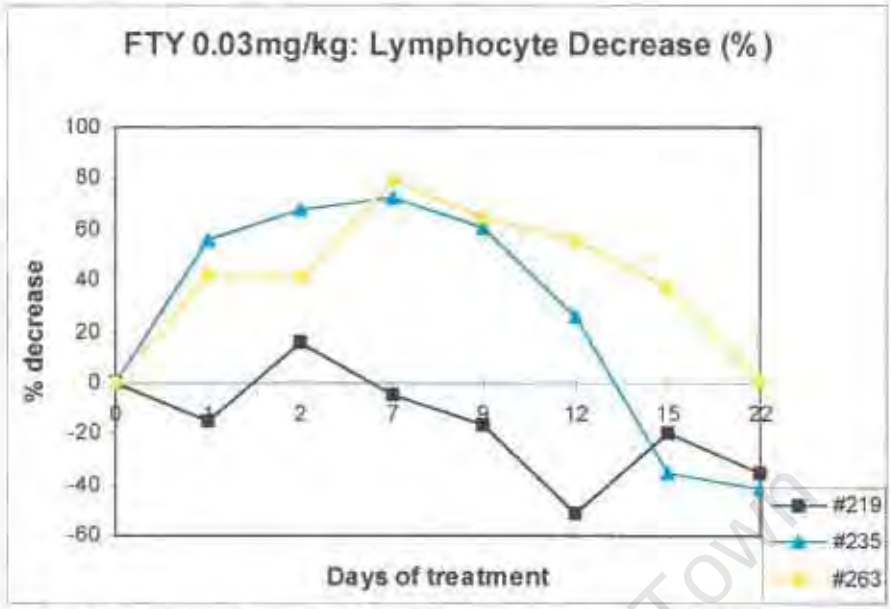


Figure 9A: % Decrease of lymphocytes in three baboons treated with 0.03mg/kg FTY720

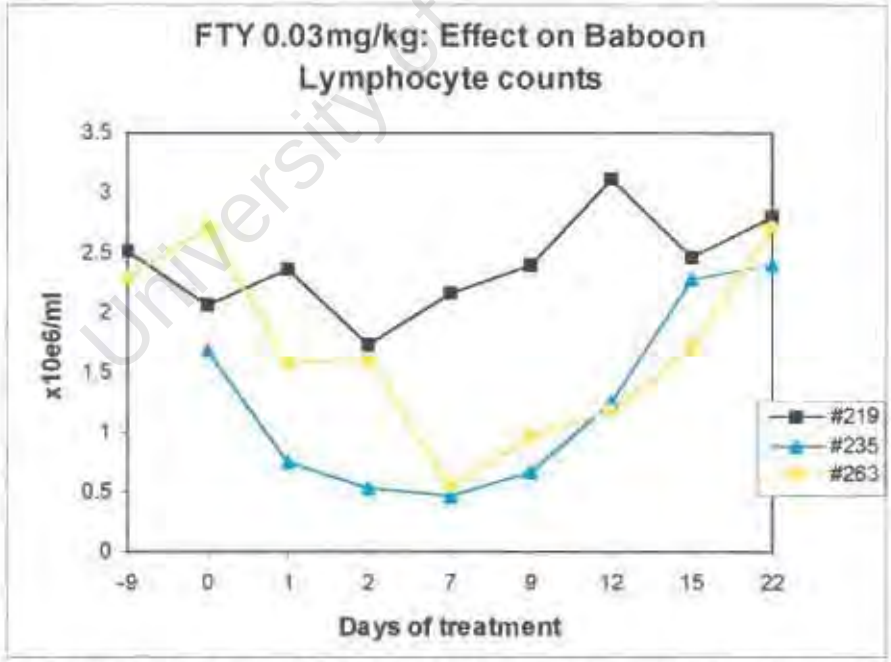


Figure 9B: Effect of 0.03mg/kg FTY720 on the lymphocyte counts in three baboons

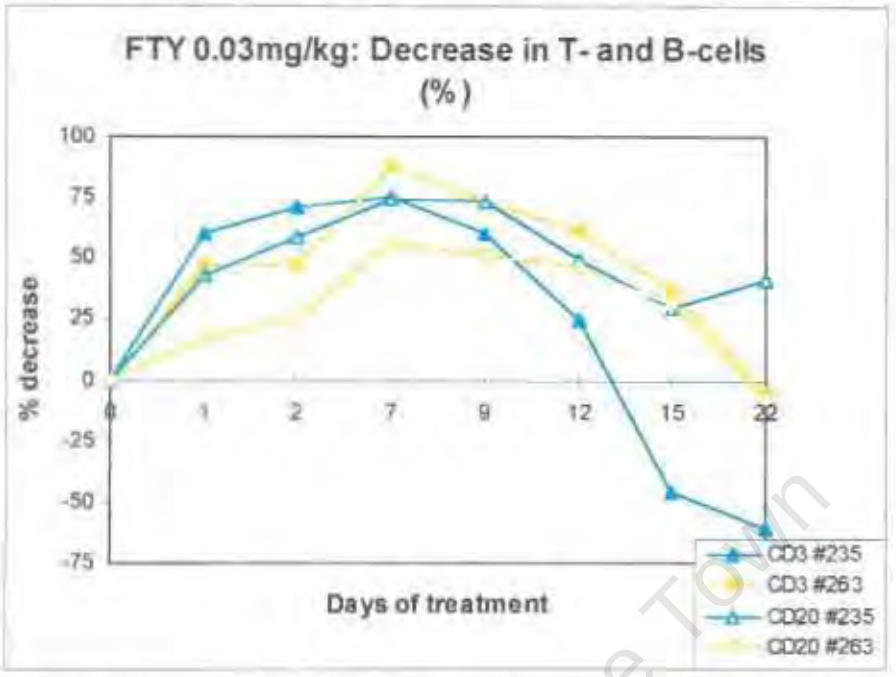


Figure 10A: % Decrease in T and B-cells in two baboons treated with 0.03mg/kg FTY720

5.4.3 Effect of 0.03mg/ml FTY720 on T-cell subsets

CD4+ T-cells were slightly more affected than CD8+ T-cells in the two responding baboons as seen in figure 10B. CD4+ cells were reduced by 65% and 43% in the two baboons and these figures increased to 88% and 93% on day 7. CD8+ T-cells decreased by 50% and 49% within 24 hours and these figures increased to 52% and 82% by day 7. The third baboon in this group (#219) which had no overall decrease in lymphocyte counts, nevertheless, had a strong imbalance of CD4+ and CD8+ T-cells. CD4+ T-cells decreased up to 40% by day 7 whereas CD8+ T-cell numbers more than doubled on day 7-12 (Figure 10C).

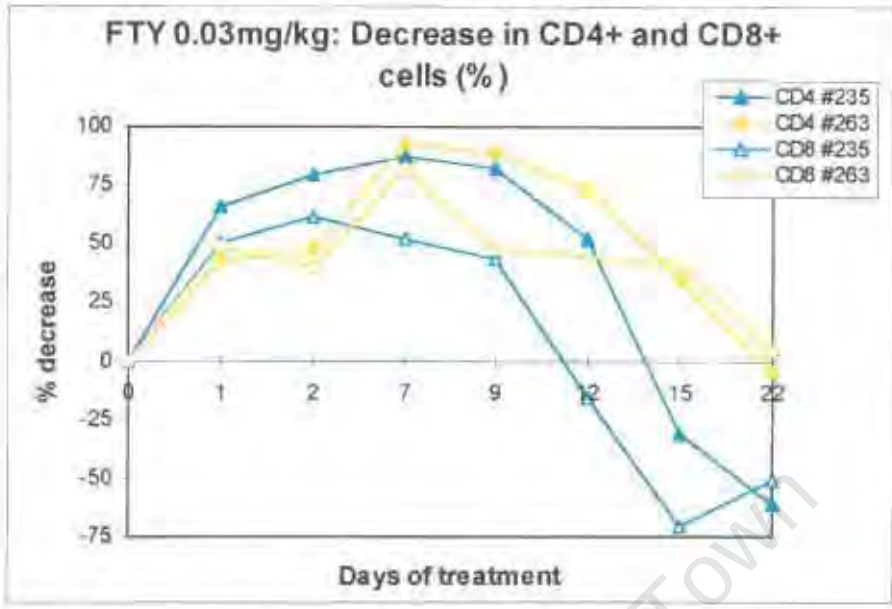


Figure 10B: % Decrease in CD4+ and CD8+ T-cells in two baboons treated with 0.03mg/kg FTY720

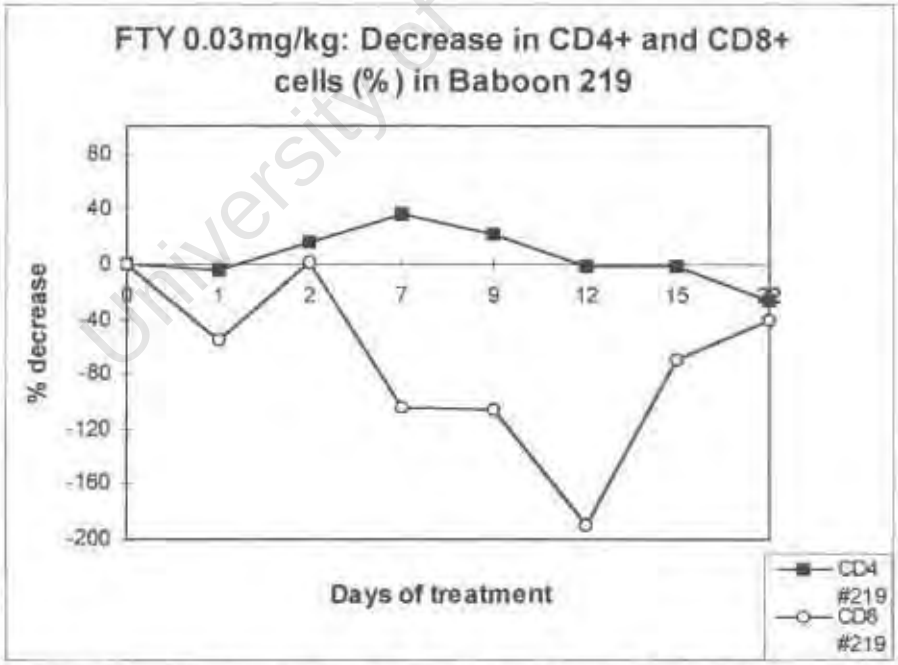


Figure 10C: Effect of 0.03mg/kg FTY720 on CD4+ and CD8+ T-cells in baboon 219

5.4.4 Effect of 0.03mg/ml FTY720 on *ex vivo* whole blood proliferation

Due to a technical error no results could be rescued for pre-drug, day 0 and day 1. However, by day 9 all three baboons had shown a decrease in response to Con A as compared to day 2 results. Although baboon 219 had shown no decrease in lymphocyte counts after treatment, there was a ten-fold reduction in response to Con A on day 12 compared with day 2. This suggests that lymphocytes are intrinsically less responsive to mitogens after FTY720 treatment. All three baboons had recovered fully by day 15 (Figure 11).

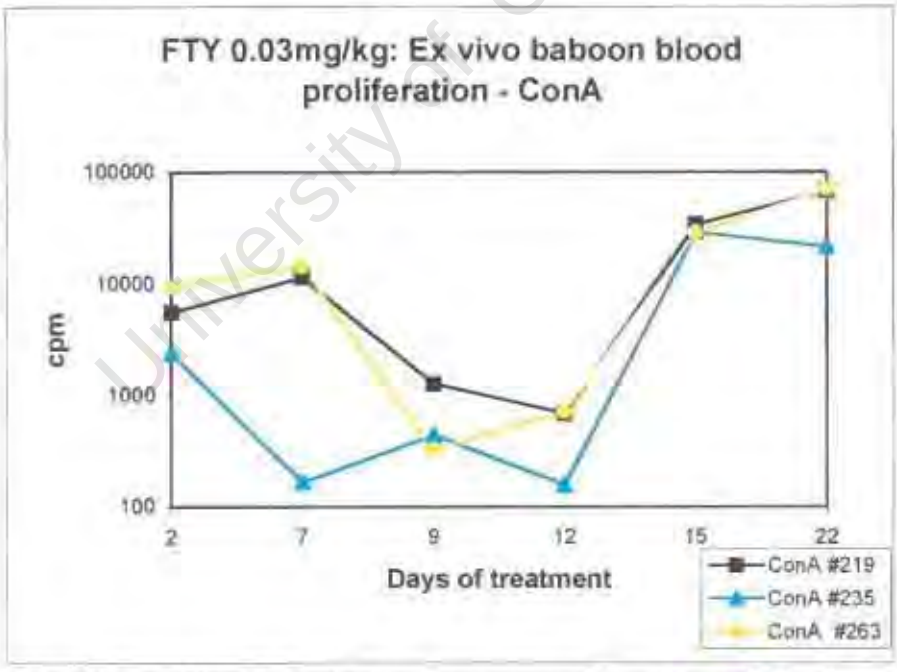


Figure 11: Effect of 0.03mg/kg FTY720 on the response of Con A in whole blood in three baboons

5.4.5 Conclusion

Two of the three baboons treated with 0.03mg/kg FTY720 showed a response to the drug. As seen with the higher doses of the drug, CD3+ lymphocytes were more reduced than CD20+ cells and CD4+ T-cells were more affected than CD8+ T-cells in the responding baboons. The lymphocyte counts in the other baboon (#219) were not affected at all. Although there were no pre-drug results for the whole blood proliferation assay, there was a significant decrease in response to Con A as the treatment progressed. This was also seen in baboon 219 although no drop in his lymphocyte count was observed. This could suggest that FTY720 does affect the function of lymphocytes even at a low dose. The effect of the drug on the sub-populations of T-cells in baboon 219 i.e. imbalance in CD4+ and CD8+ lymphocytes is unexplained.

No side effects of the drug were experienced by any of the baboons although the body weights fluctuated as seen in the other experiments using higher doses of FTY720. As the drug was administered in food and the baboons were starved overnight, this could be the cause of weight loss.

5.5 Pharmacokinetic Study of FTY720 blood levels

The concentration of FTY720 in the blood of the baboons after treatment was assessed in Basel, Switzerland. Whole blood was frozen at each time point in all the experiments. Figure 12A shows that 24 hours after the first dose of 0.3mg/kg/day FTY720 for three days, 2.6 ± 0.3 ng/ml of the drug was found in the blood circulation. The concentration rose to 4.6 ± 0.8ng/ml 24 hours after the second dose. This was followed by an elimination period with a mean half-life of 2.1 days. Although the concentration of FTY720 correlated with the lymphodepletion on day 1 and 2, there was no correlation between drug levels and a decrease in lymphocyte counts in the wash out period. On day 7 the level of FTY720 in the three baboons was 0.6, 1.3, and 0.8ng/ml which was ± 20% of the FTY720 level on day 2, whereas the lymphocyte counts decreased by 43, 73 and –4% of the pre-treatment values, respectively. There was a low inter-animal variability in the drug concentration profiles of the three baboons.

The blood levels of FYT 720 administered in a single 0.3mg/kg dose and observed over a 48-hour period showed peak concentrations of 2.16 ± 0.58 ng/ml between 2 and 24 hours (Figure 12B). Thereafter, the blood concentration of FTY720 decreased with a mean elimination half-life of 36 hours (CV32%) between 24 and 48 hours (Table 7).

Administration of FTY720 at 0.1mg/kg/day for three days resulted in a mean blood concentration of 0.6ng/ml after 24 hours and this range was maintained for 7 days

(Figure 12D). There was a relatively high inter-animal variability during the three days of treatment. This could be related to the fact that the concentrations measured were very low and those concentrations below the limit of quantification i.e. 0.38 ng/ml were set to zero. No elimination half-life could therefore be calculated.

Administration of 0.03mg/kg FTY720 for ten days resulted in a mean blood concentration of 0.161 ± 0.139 ng/ml after 24 hours, increasing to 0.317 ± 0.167 ng/ml after 2 days (Figure 12C). This reached a maximum of 0.718 ± 0.025 ng/ml after seven days of treatment and these concentrations remained in two out of the three baboons. There was a relatively low inter-animal variability especially during the 0-7 day period.

Table 7 CONCENTRATIONS OF FTY720 (in ng/ml)

TIME (hours)	BABOON 205	BABOON 206	BABOON 210	MEAN	CV(%)
0	0	0	0	0	N/A
1	0.51	0	0.55	0.35	87
2	1.52	0.55	1.63	1.23	48
4	1.20	0.84	2.25	1.43	51
8	1.09	0.76	2.65	1.50	67
24	1.48	2.32	2.34	2.05	24
48	0.71	1.54	1.61	1.29	39

Individual concentrations of FTY720 after a single 0.3mg/kg dose

Concentrations below the limit of quantification were set to zero

N/A: not applicable

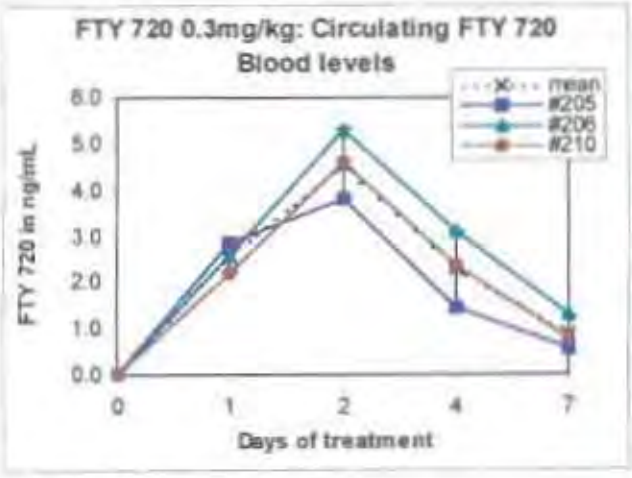


Figure 12A

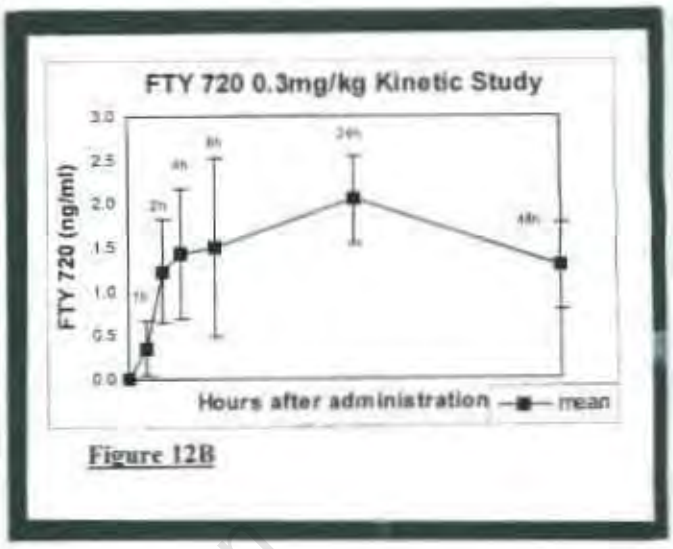


Figure 12B

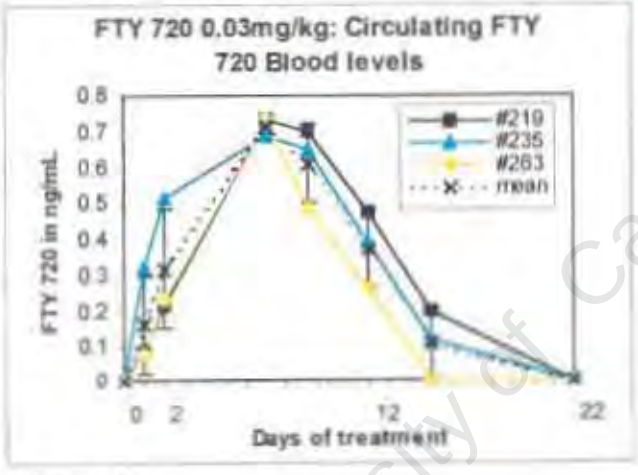


Figure 12C

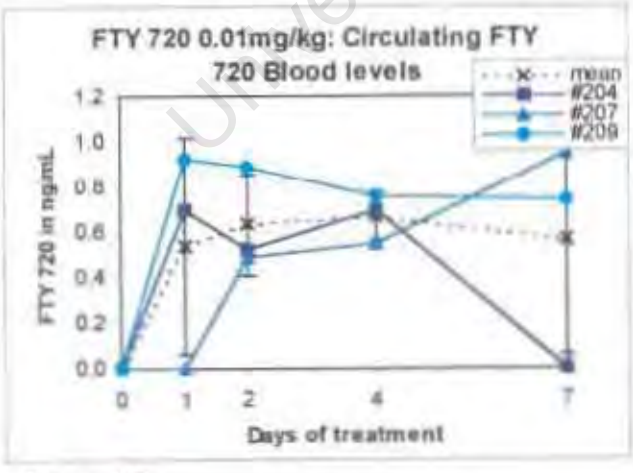


Figure 12D

Figure 12A, B, C, D: Blood concentrations of FTY720 after treatment

5.6 α-Gal polymer + FTY720 at 0.1 or 0.3mg/kg

In a previous collaborative study performed in Basel and in our laboratory three groups of three baboons per group were administered Alpha Galactosyl (αGal) polymers of different polymerisation degrees and one group received the vehicle i.e. saline. The rationale is that these polymers contain polylysine that has been shown to neutralise anti-αGal antibodies which are found in high titres in humans and Old World primates. These naturally occurring antibodies are known to play a significant role in hyperacute rejection. The results showed that after the infusion of the polymers there was a rapid and reproducible decrease of the natural anti αGal IgG and IgM. The titres of the antibodies were recovered by 21 days post administration of the polymers and the return of these antibodies was considered due to a *de novo* synthesis. The saline control had no effect as expected. Since FTY720 causes a dramatic decrease in both T and B lymphocytes and the αGal response is considered to be "T-cell independent", it was decided to test the potential of FTY720 on a "T-cell independent" B-cell response which is relevant for xenotransplantation.

Figure 13 shows the effect of FTY720 in the presence of αGal polymer on the lymphocyte counts in three baboons. All three baboons showed a drastic decrease in counts as seen with FTY720 alone (data shown in earlier experiment). Figure 14A and 14B shows the effect of FTY720 and αGal polymer on T and B-cells and CD4+/CD8+ T-cells respectively. These results correlate with the results seen when FTY720 was administered alone.

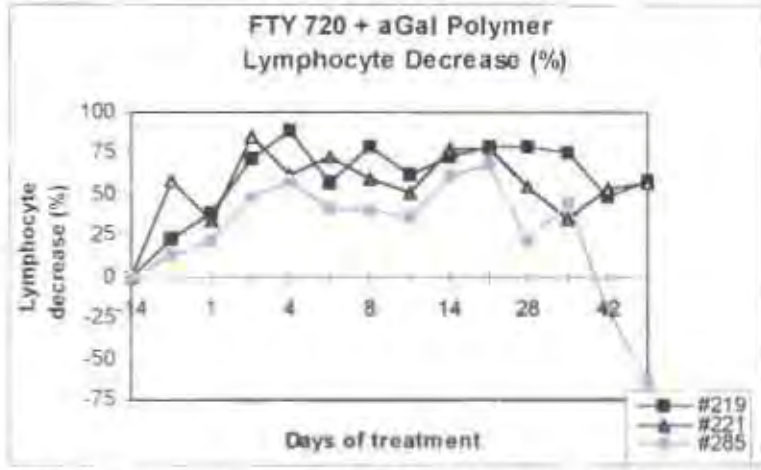


Figure 13: % Lymphocyte decrease after treatment with FTY + αGal Polymer

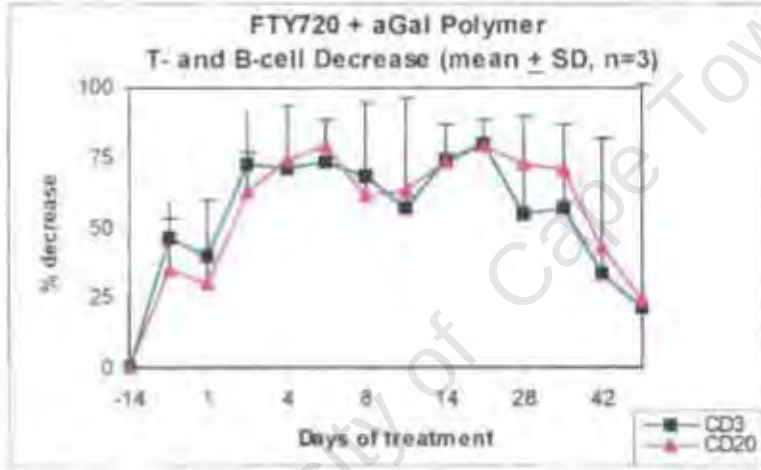


Figure 14A: % Decrease of T and B cells after FTY720 + αGal Polymer

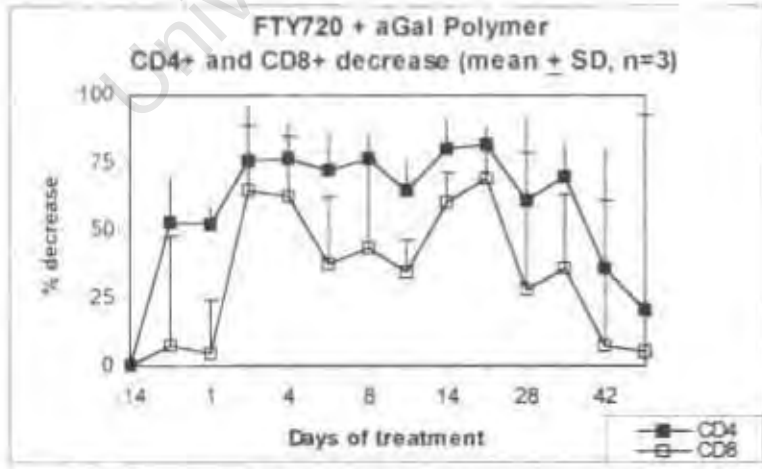


Figure 14B: % Decrease in CD4+ and CD8+ lymphocytes after treatment with FTY720 αGal Polymer

Figures 15A, 15B and 15C (results courtesy of Novartis Pharma, Basel) show the effect of the αGal Polymer injections (1mg/kg on days 1, 4 and 7) on anti-αGal and the haemolytic titres in the presence of FTY720 treatment. The results were compared to historical controls using the same αGal Polymer in the absence of FTY720 (earlier collaborative study; data not shown). The baboons responded to the injections with a marked decrease of anti-αGal IgMs and IgGs, and a corresponding drop of haemolytic activity to background levels throughout the treatment period. There was however, a rebound on day 7 which decreased after the last injection. Figure 15A shows that anti-αGal IgM recovered to levels slightly lower than the starting values on day 14-28 and remained at this level up to day 52. In contrast, the anti-αGal IgG titres observed on day 14-28 were higher than the initial values and continued to increase from day 35-52 (Figure 15B). The haemolytic titres were back to pre-treatment values on days 21-28 (Figure 15C). These results indicate that FTY720 treatment did not prevent the recovery of anti-αGal IgM, IgG and haemolytic titres after the injection of -αGal polymers.

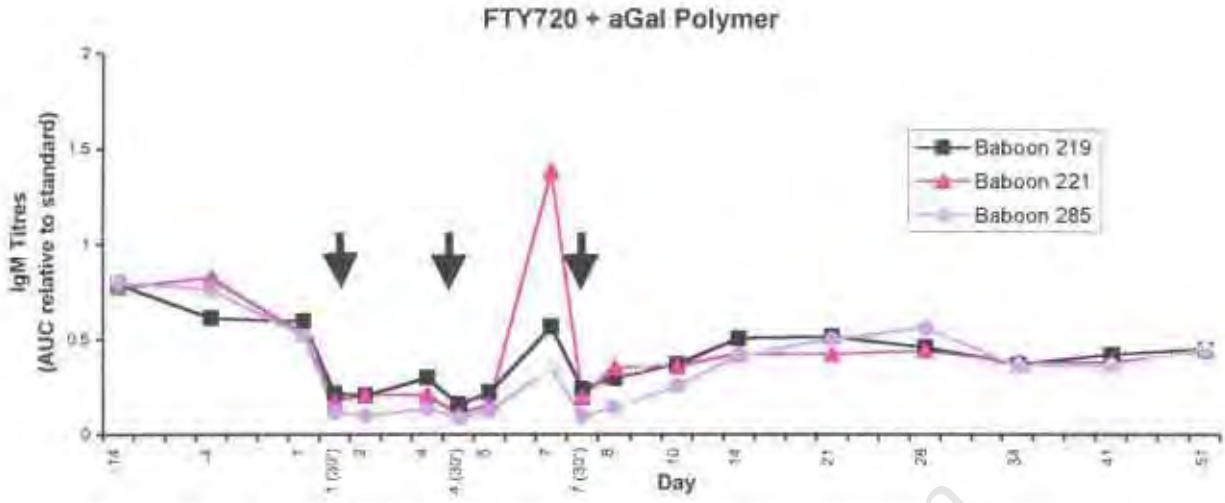


Figure 15A Effect of αGal Polymer on anti-αGal IgM titres in the presence of FTY720 in three baboons. The arrows indicate 1mg/kg injections of the αGal Polymer.

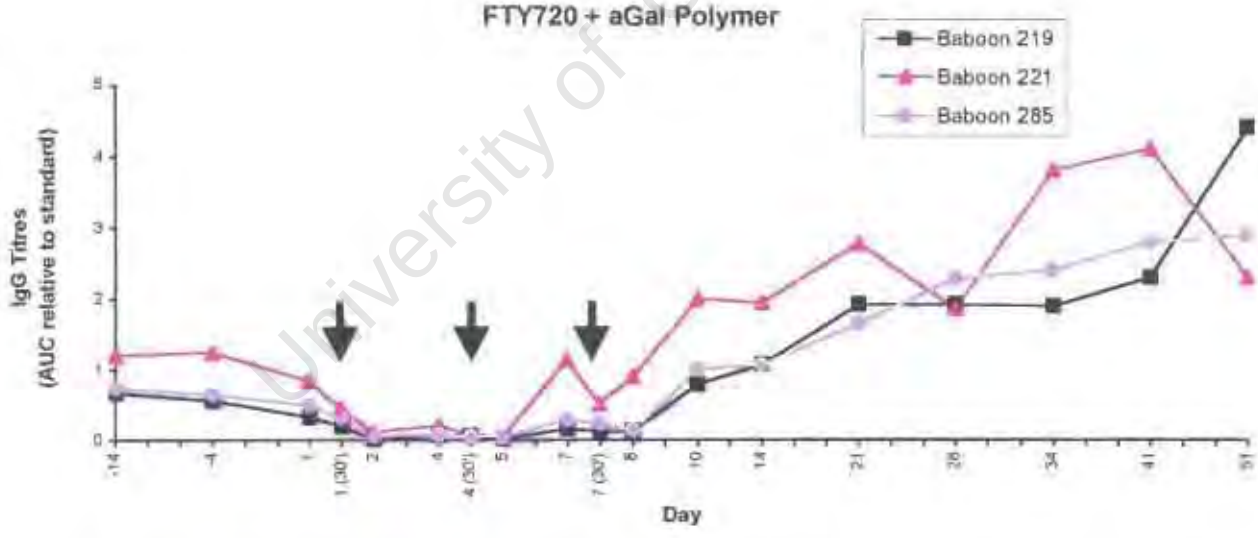


Figure 15B Effect of αGal Polymer on anti-αGal IgG titres in the presence of FTY720 in three baboons. The arrows indicate 1mg/kg injections of the αGal Polymer.

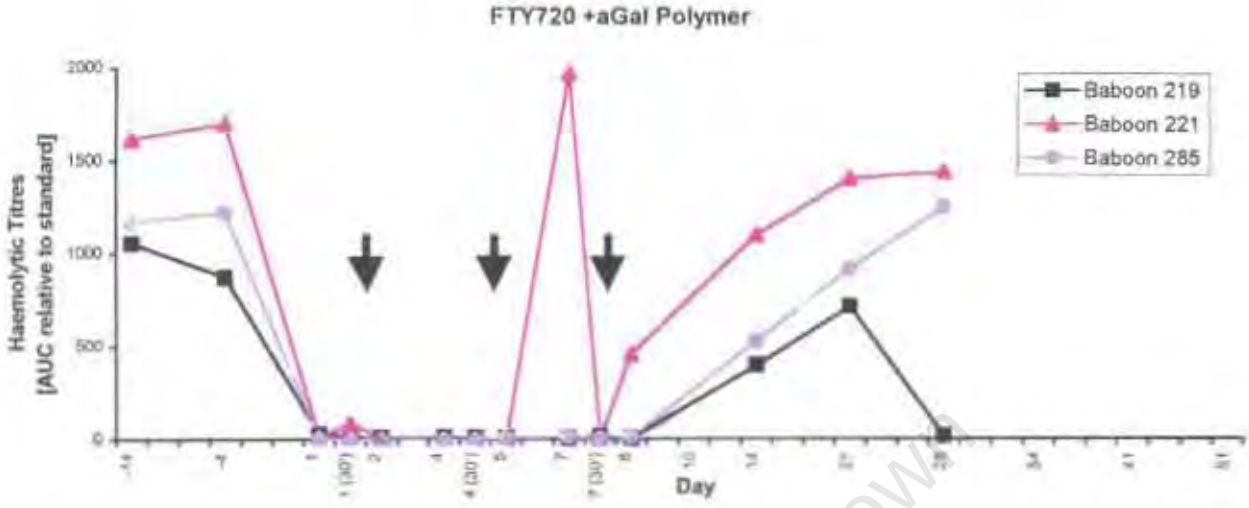


Figure 15C Effect of αGal Polymer on haemolytic titres in the presence of FTY720 in three baboons. The arrows indicate 1mg/kg injections of the αGal Polymer

5.7 FTY720 in mice

The real mechanism of action of FTY720 has not been established although it is believed to be either due to apoptosis of activated lymphocytes or homing to peripheral lymphoid tissue. In the latter, the circulating lymphocytes are believed to home to lymph nodes and Peyer's Patches through the interaction of lymph homing receptors including CD62L and interleukin-2 (IL-2). Table 8 shows the decrease in white blood cell counts (WBCC) 24 hours after 1mg/kg FTY720 was administered to four LT-α-/- mice, 4 wild-type C57Bl/6+129SV mice and 2 L-selectin-/- mice. One LT-α-/- mouse and one wild-type mouse received distilled water only as controls.

Table 8: Mouse white blood cell counts pre and post 1mg/kg FTY720

Mice	WBCC (Pre) $+10^9$/L	WBCC (Post) $+10^9$/L	% Decrease
LT-α-/- 1	45.1	5.52	87.8%
LT-α-/- 2	49.23	10.38	78.9%
LT-α-/- 3	45.31	3.25	92.8%
LT-α-/- 4	49.43	21.31	56.9%
C57Bl/6+129-1	13.04	3.43	73.7%
C57Bl/6+129-2	7.51	2.0	73.4%
C57Bl/6+129-3	9.47	1.94	79.5%
C57Bl/6+129-4	5.52	0.76	86.2%
L-selectin-/- 1	5.18	0.76	85.3%
L-selectin-/- 2	14.35	3.77	73.7%
LT-α-(control)	*24.07*	*21.20*	*11.9%*
C57Bl/6+129-(control)	*7.34*	*6.9*	*5.9%*

The white blood cell counts were significantly reduced in all of the LTα-/- mice that received FTY720 with a mean decrease of 79.1%. The wild-type C57Bl/6x129SV showed a mean decrease of 78.2% whereas the L-selectin-/- mice were reduced by 79.5%. The control LTα-/- mice that received the vehicle only showed an 11.9% decrease whereas the WBCC of the wild-type control mouse was reduced by 5.9%. These slight reductions were almost certainly due to stress or a drop in blood volume.

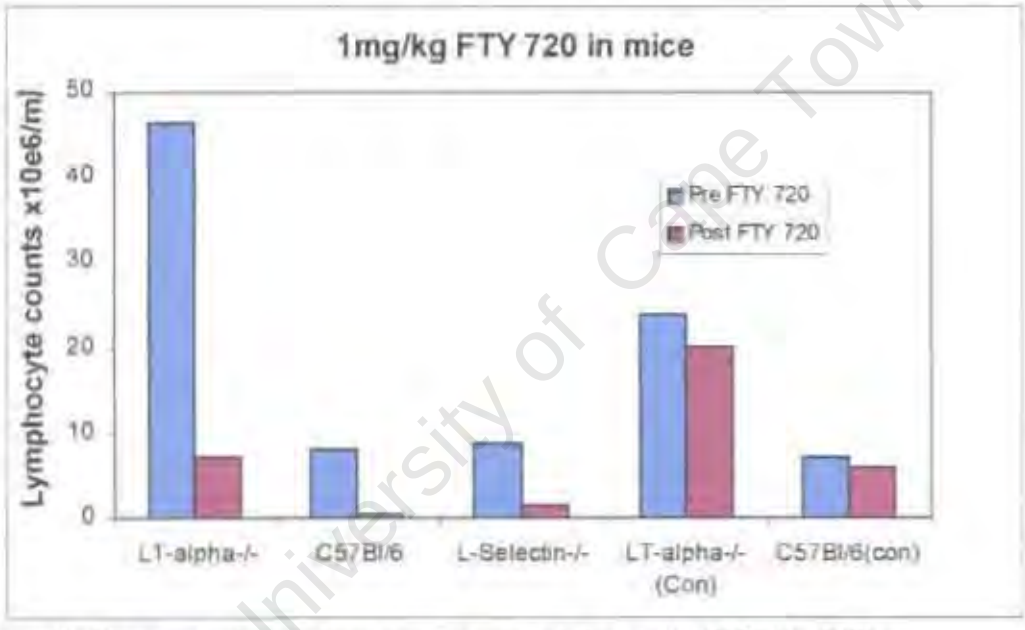


Figure 16: Decrease in absolute lymphocyte counts in LTα-/- mice, C57Bl/6x129SV mice and L-selectin-/- mice 24 hours after the administration of 1mg/kg FTY720. The control mice showed a very slight decrease in lymphocyte counts.

Figure 16 shows that 24 hours after 1mg/kg FTY720 was administered, the mean absolute lymphocyte count was reduced by 84.5% in LTα-/- mice, by 94% in the C57Bl/6x129SV mice and by 86.4% in the L-selectin-/- mice. The control LTα-/-

mouse showed a 16.7% decrease. The decrease in the absolute lymphocyte counts in the two control mice could be due to stress or loss of blood.

5. RESULTS: *in vitro:*

5.8 Effect of Immunosuppressive drugs on Baboon Mixed Lymphocyte Culture (MLC)

Figure 17A shows the response of mismatched baboons in a MLC, to various concentrations of CsA. The mean lymphocyte reaction (MLR) without any inhibitory agents was 22274 cpm with a stimulation index (SI) of 6.5. The reason for the low SI is that most of the baboons had a high auto-stimulation (background), the mean being 3791 cpm for each pair of baboons. All tests were performed in triplicates and the percentage of inhibition was calculated according to the formula:

$$\left(100 - \frac{\text{Test (cpm)- Background (cpm)}}{\text{Maximum stimulation (cpm) - Background (cpm)}} \right) \times 100$$

-where the background equals the sum of the each baboon PBMC + RPMI only, and maximum stimulation equals the MLR of two baboons without any inhibitory drugs.

Figure 17B and 17C show the response of baboon MLC which where inhibited by FK 506 and RAD respectively. The mean MLR was 17425cpm with an auto-stimulation of 3461 for experiments using FK 506, and 16268cpm with a background count of 3112 for RAD. The SI were 5.6 and 6.5 respectively. The high auto-stimulation could once again attribute to the low SI. Although each set of results did show some inconsistencies when compared against each other, the general trend of increasing drug concentration causing a correlating decrease in MLC response (i.e. inhibition) was seen in all experiments. Table 9 shows the IC50 of each drug which were determined by Graph Pad software using the package Graph Pad Prism Version 2. 01A. The IC50 are expressed in nMol concentrations.

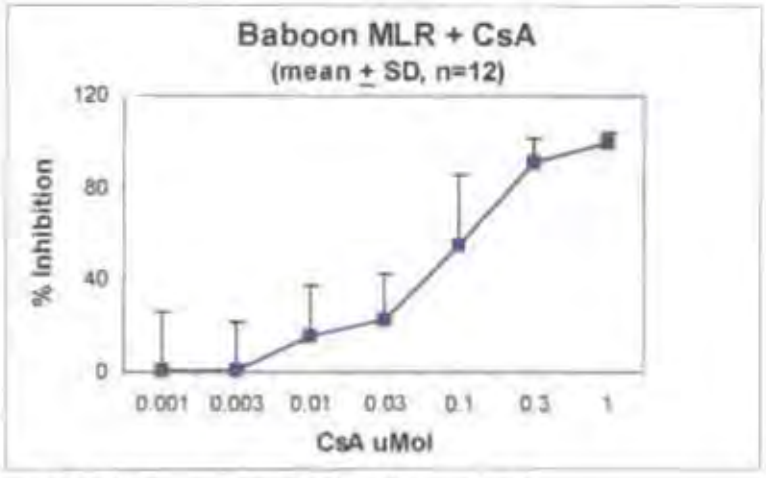


Figure 17A: Inhibitory effect of CsA on Baboon MLR

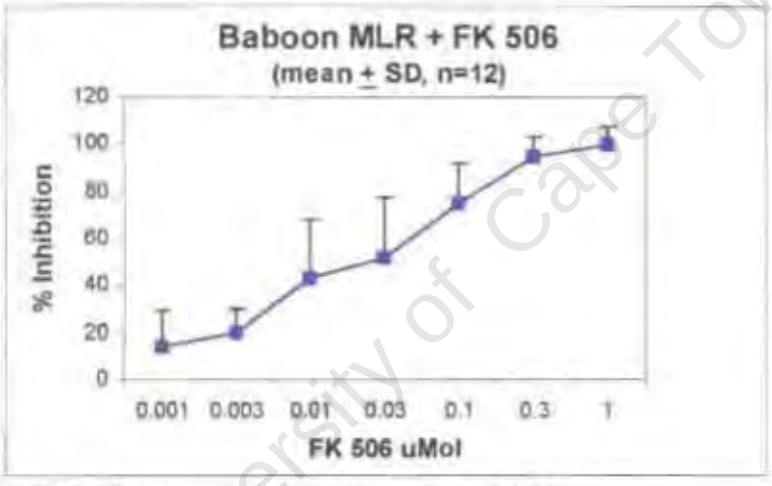


Figure 17B: Inhibitory effect of FK 506 on Baboon MLR

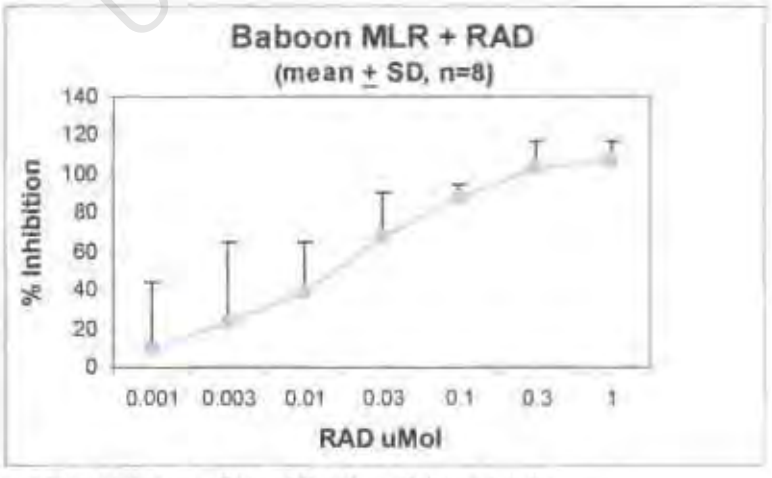


Figure 17C: Inhibitory effect of RAD on Baboon MLR

Table 9: IC50 concentrations of immunosuppressive drugs in baboon MLR

Drug	IC50
CsA	100.8nM
FK505	34.4nM
RAD	21.2nM
FTY720	>10000nM

FTY720 has been reported as being much more effective in vivo than in vitro. Figure 17D shows the effect of FTY720 on baboon MLC in vitro. 10μM FTY720 showed an inhibition of 36.3% and the highest dose of 30μM was not sufficient to completely inhibit the MLR but did cause a 77.7% inhibition. Concentrations of FTY720 below 10μMol showed a minimal response to the drug. The IC50 of FTY720 was 11.17μM, a dose which is likely to cause apoptosis and thus could be a non-specific effect (e.g. non-specific for inhibition of the allo-response).

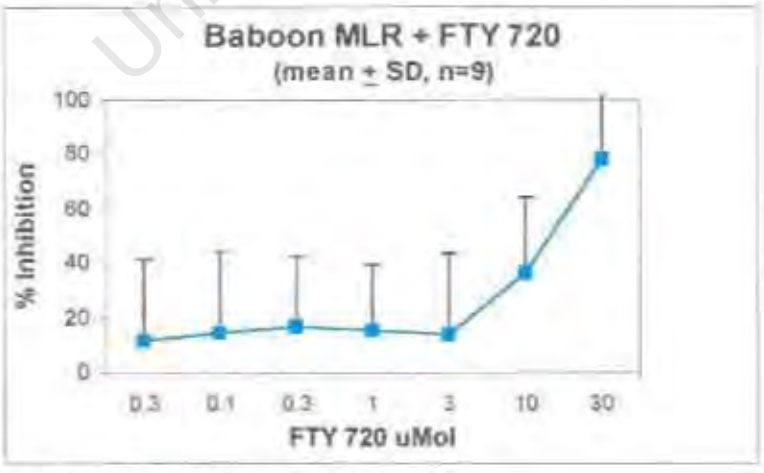


Figure 17D: Effect of FTY720 on baboon MLR

5.9 Effect of Immunosuppressive drugs on Baboon WB

Baboon whole blood, stimulated with 4µl/ml Con A, was treated with increasing concentrations of immunosuppressive drugs. Figures 18A, B and C show the effect of CsA, FK506 and SDZ RAD respectively on stimulated baboon whole blood. Table 10 demonstrates the mean maximum stimulation of baboon WB by Con A, mean background counts and mean SI for each set of experiments using the immunosuppressive drugs (100% stimulation indicates no drug added). The obvious difference in the background counts between the MLC and WB could be attributed to the foetal calf serum, which is omitted in the whole blood assays.

FTY720 was not inhibitory at all even at high doses of 30µMol and appeared to have an unexplained stimulatory effect on the whole blood.

Table 10: Effect of Immunosuppressive drugs on Baboon WB in response to Con A.

Drug	100% Stimulation (cpm)	Background (cpm)	SI
CsA	32650	685	99.52
FK506	19323	601	87.1
RAD	29603	626	97.5
FTY720	33084	713	76.2

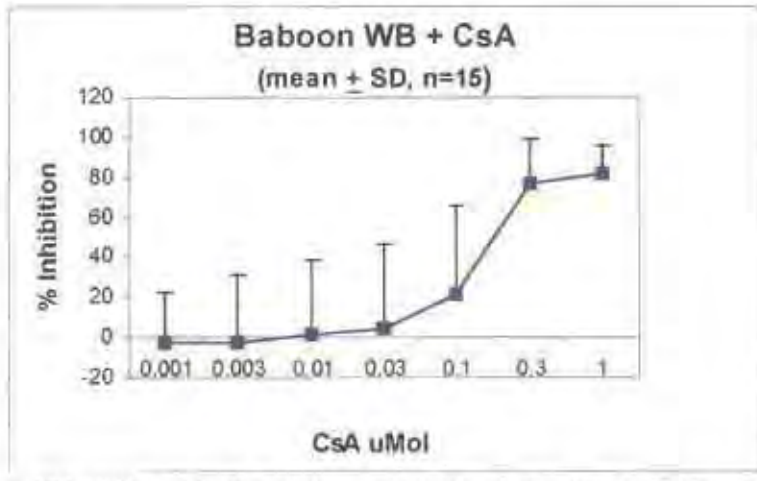


Figure 18A: Effect of CsA on Baboon whole blood stimulated with Con A

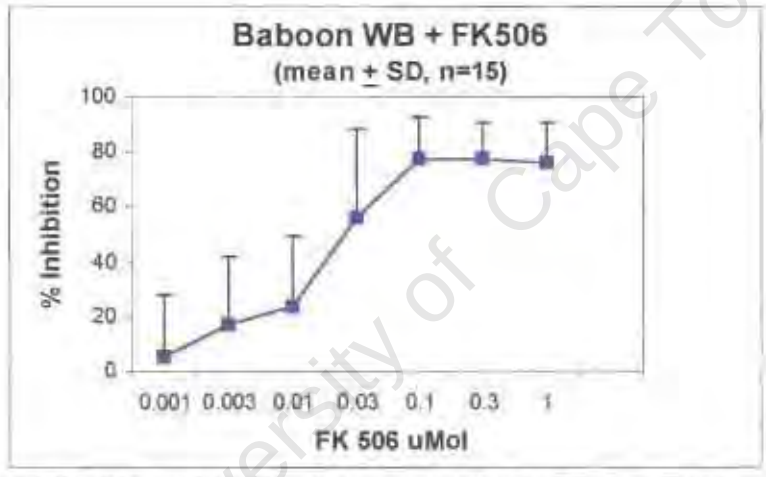


Figure 18B: Effect of FK 506 on Baboon whole blood stimulated with Con A

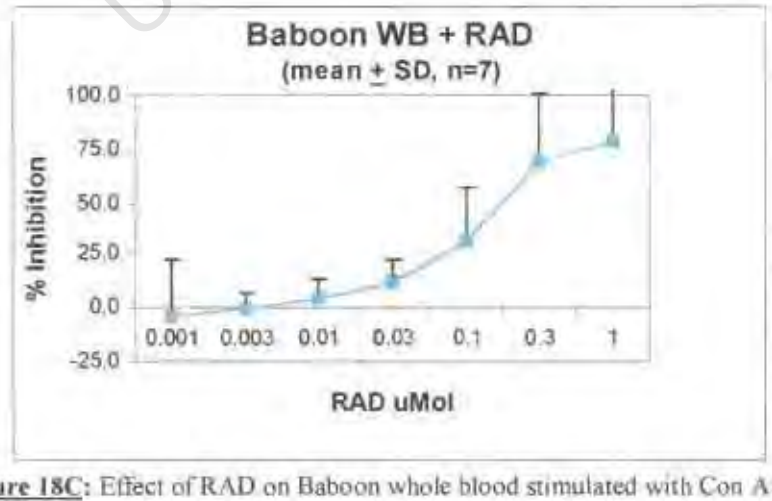


Figure 18C: Effect of RAD on Baboon whole blood stimulated with Con A

Table 11: IC50 concentrations of immunosuppressive drugs in baboon WB

Drug	IC50
CsA	136.8nM
FK505	19.8nM
RAD	125.7nM
FTY720	N/A

The IC50 of CsA and FK 506 in WB were similar to those using PBMC (Table 11). However, the IC50 of SDZ RAD in whole blood was six-fold more than in the MLC; the MLC is an allostimulation rather than a mitogen-induced response. This result may be affected by the "partitioning" of the drug from the plasma and red blood cells when preparing PBMC as doses of approximately 1.5-fold CsA but 36-fold more Rapamycin, a derivative of SDZ RAD, is needed for the same response (data not shown).

6. DISCUSSION

Pharmacokinetic and pharmacodynamic studies of new immunosuppressants are essential to establish dose response and to design new drug regimens for transplantation experiments, especially in non-human primates. Depending on the drug under study, haematological parameters or immunophenotyping of the different lymphocyte populations by flow cytometry can be used as a "read-out' of the drug effect. Animal experiments have demonstrated the powerful immunosuppressive properties of FTY720 in rodent and canine transplantation models. The immunosuppressive effect of FTY720, a synthetic analog of the drug Myriocin, is related to a rapid and profound decrease of peripheral lymphocytes in the blood circulation. Cyclosporine, FK506 and RAD do not affect the number of lymphocytes in circulation, but rather their responsiveness to activation stimuli. Therefore, *ex vivo* lymphoproliferative assays may be useful to assess the pharmacodynamics of these immunosuppressive drugs.

Whole blood assays are preferred for pharmacodynamic evaluations since they avoid drug re-distribution during lymphocyte separation, and require small quantities of blood, thus allowing for repeated sampling for kinetic studies. In this study, pharmacodynamics of FTY720 in baboons were performed using whole blood to assess lymphocyte phenotyping and *ex vivo* lymphocyte proliferation, as an introductory study for the use of FTY720 in baboon allo- or xenotransplantation.

Three doses of FTY720 were tested viz. 0.03, 0.1 and 0.3 mg/kg. All baboons treated with FTY720 were exposed to the drug. There was a good correlation between the dose applied and the FTY720 24-hour trough levels as seen after one or two daily doses of FTY720 administration at 0.03, 0.01 and 0.3 mg/kg/day. After the third dose of 0.3 mg/kg FTY720, the mean levels of drug in the bloodstream was ± 0.9 ng/ml on day seven, whereas that of 0.1 mg/kg FTY720 was ± 0.6 ng/ml in the same time period. The ten-day protocol of 0.03 mg/kg FTY720 reached a maximum of ± 0.7 ng/ml after seven days, then reached a plateau at this level, in two out of three baboons. Thus, there was a clear drug accumulation in the baboons treated with a low dose over a longer period. There was a relatively low inter-animal variability in the concentration profiles.

All but one baboon (0.03mg/kg) showed a response to FTY720 in terms of a decrease of lymphocyte populations from the peripheral blood. The quickest onset of lymphodepletion was clearly seen at the highest dose. The pharmacokinetic study revealed that a decrease in circulating lymphocytes was already observed between 1-2 hours after administration of 0.3 mg/kg FTY720 reaching 50% after four hours and 77% after twenty four hours (n=3). This correlated well with the mean lymphocyte depletion twenty four hours after the 0.3 mg/kg/day dosage, indicating that a sustained anaesthesia did not interfere with the effect of the drug. The inter-individual variability of the response appeared to increase with a lower dosage of the drug. At a higher dose there was little inter-baboon variability in the FTY720-induced lymphopenia in regards to T-cells, B-cells, and T-cell subsets including CD4+ and CD8+ cells, whereas a strong variation was seen in the recovery phase. The duration

of the effect was clearly longer after the higher doses of 0.1 and 0.3 mg/kg/day. However, the maximal effect in terms of peripheral blood lymphocyte depletion was similar in all three treatments. Therefore, there was no clear dose-response effect within the three individual treatments. The data suggests however, that high induction doses e.g. 0.3 mg/kg/day should be used to optimise immediate response and that a reduced dose regimen e.g. 0.03 mg/kg/day should be sufficient for drug maintenance.

The prevention of graft rejection involves the inhibition of active T-cells and B-cells, as delayed xenograft rejection is mediated by cellular and humoral mechanism. FTY720 caused a depletion of both T and B-cells in the peripheral blood circulation, an effect that is unique to this immunosuppressant agent. Although the effect of the drug was more rapid and pronounced on T-cells than B-cells, the effect was more persistent on B-cells which took longer to recover to their original values. After two days of 0.3 mg/kg FTY720, T-cells where reduced by $\pm$ 75% and B-cells by $\pm$51% whereas in the 0.1 mg/kg/day the effect on T and B-cells was essentially equivalent. The continuous dose of 0.03 mg/kg for ten days revealed similar values (on day seven) to the 0.3 mg/kg dose. The kinetic study compared favourably with the data of the 0.3 mg/kg/day FTY720 treatment. Thus, FTY720 is effective in decreasing both T and B-cells in the peripheral blood of baboons. This is not consistent with previous reports in other species which suggest that the predominant effect of FTY720 is on T-cells (Yanagawa *et al.*, 1998; Troncosco and Kahan, 1998; Enosawa *et al.*, 1996; Adachi *et al.*, 1995).

There was little correlation between the 24-hour FTY720 trough blood levels and the lymphocyte depletion. The correlation was slightly better at very low FTY720 blood levels of 0-0.7 ng/ml, whilst values above a concentration of 1 ng/ml resulted in the lymphocyte depletion being in the maximum range. There appeared to be a threshold of FTY720 blood levels above which cells were affected and consequently left the blood circulation. Clear discrepancies between FTY720 blood levels and lymphocyte blood counts were also found in the single dose 48-hour pharmacokinetic study, where the circulating blood levels of FTY720 decreased by 30% between 24 and 48 hours after treatment, whereas the lymphocyte counts remained stable. These results suggest that FTY720 accumulates in the tissues and can be detected for a long duration before being cleared. Therefore the data obtained in this baboon study raises questions about the value of FTY720 blood levels as a prediction for drug effectiveness.

The effect that FTY720 had on the T-cell subsets i.e. CD4+ and CD8+ cells was similar in all three doses of FTY720. There was a slightly more pronounced effect on CD4+ cells than CD8+ cells although CD8+ T-cells recovered quicker than CD4+ T-cells. These results were in agreement with those of Enosawa *et al.*, 1996, who reported that CD4+ T-cells were the most sensitive to the effect of FTY720. Since the rejection of allografts has been associated with Th-1 associated cytokines, which are produced by CD4+ T-cells and are involved in the cytotoxic differentiation of CD8+ T-cells, the effect of FTY720 on dramatically reducing the number of CD4+ T-cells holds promise in contributing to the preventing of allograft rejection.

There were some CD4/CD8 double positive T-cells in the peripheral blood of the baboons that essentially doubled within seven days after FTY720 treatment in nine out of twelve baboons. This was also reported by Enosawa *et al.*, (1996) who documented an increase in CD4/CD8 double positive T-cells after a single dose of FTY720 and proposed that FTY720 caused apoptosis of lymphocytes and, that the CD4/CD8 double positive cells were macrophage cells responsible for scavenging the dead cells. Shimizu *et al.*, (1998) reported that the percentage of CD4/CD8 double positive thymocytes decreased after FTY720 treatment in mice suggesting that FTY720 inhibited negative selection of immature thymocytes. It is possible therefore, that the effect FTY720 has on lymphocyte circulation may also affect the trafficking of thymocytes to the periphery.

The increase in CD4+/CD8+ T-cells after FTY720 treatment in baboons could also have been a sign of accelerated T-cell production as a result of a massive disappearance of T-cells due to FTY720-induced apoptosis. However, no apoptotic cells were detected in the peripheral blood of the baboons tested in the pharmacodynamic study. FTY720 induced apoptosis at high molecular concentrations *in vitro* and apoptotic cells were detected in the thymus, spleen and lymph nodes in mice after FTY720 treatment (Suzuki *et al.*, 1996). However, Shimizu *et al.*, (1998) reported that no apoptotic thymocytes or splenocytes were found in mice after a single dose of 1mg/kg FTY720 although 40% of peripheral blood lymphocytes showed signs of apoptosis. In this study, no apoptotic PBMC were detected after FTY720 treatment using a read-out that detects *in vitro* FTY720-induced apoptosis. However, no attempt was made to analyse the lymphoid organs in

terms of cell populations and immunohistochemistry during the FTY720 treatment, since the protocol included follow-up of the post-treatment recovery phase. Therefore, it would be presumptuous to assume that FTY720-induced lymphodepletion in baboons is not caused by apoptosis.

FTY720 induced a rapid and marked decrease in *ex vivo* whole blood proliferation in response to the mitogen Con A. Carry-over of FTY720 in this assay does not explain these results since the maximal FTY720 concentrations seen in blood after treatment were in the range of 0.015μM (5 ng/ml), whereas FTY720 did not inhibit lymphocyte proliferation in baboon mixed lymphocyte cultures at sub-micromolar concentrations. The *in vitro* data shows that the mean IC50 for FTY720 in the baboon MLC was >10000 nMol i.e. 10 μMol, whereas CsA and FK506 and RAD were inhibitory at nanomolar concentrations; the IC50 was 101 nMol for CsA, 34 nMol for FK506 and 21 nMol for RAD. The *in vitro* response of mitogen-stimulated baboon whole blood to FTY720 showed that the drug was not inhibitory at concentrations up to 30 μMol whereas the inhibitory effect of CsA and FK506 was similar to that seen in the MLC. The decrease in whole blood proliferation in the baboons after FTY720 treatment is likely to be due to a numerical deficit rather than a functional deficit in the T-lymphocyte population as the same volume of blood was used regardless of the lymphocyte count. However, the nadir for *ex vivo* proliferation after FTY720 treatment in the 0.03mg/kg dose was seen on day 4 which was two days after the last FTY720 dose when the lymphocyte counts were already recovering. Thus, it is difficult to interpret the decreased *ex vivo* response in terms of lymphocyte unresponsiveness since there was no linearity between the cell concentrations and

proliferative response to Con A in the whole blood proliferation assay. However, the whole blood *ex vivo* proliferation in response to Con A was reproducible, used little material and appears suitable for efficacy studies of immunosuppressive drugs.

The infusion of αGal polymers into baboons resulted in a rapid reduction of natural anti-αGal IgM and IgG. However, within three weeks the titres of these antibodies were recovered. The simultaneous treatment with FTY720 did not prevent the recovery of anti-αGal IgM, IgG and haemolytic titres; within four weeks the anti-αGal titres had essentially recovered although anti-αGal IgG continued to increase slightly up to the termination of the experiment on day 52.

FTY720 induced a marked lymphocyte reduction in LT-α-/-mice and L-selectin-/-mice after a single administration at 1mg/ml. LT-α deficient mice exhibit a phenotype dominated by defects in secondary lymphoid organ development whereby they lack lymph nodes and Peyer's Patches (Banks *et al.*, 1995). The mice were phenotyped in the Department of Immunology at the University of Cape Town and found to be true LT-α-/- mice with no lymph nodes or Peyer's Patches. L-selectin, a cell adhesion molecule, mediates the attachment of lymphocytes to high endothelial venules of peripheral lymph nodes and Peyer's Patches (Arbones *et al.*, 1994). The immunosuppressive effect of FTY720 has been reported to be due to circulating lymphocytes homing to lymph nodes and Peyer's Patches and attaching to high enothelial venules in these tissues via lymph homing receptors. Thus, the profound lymphopenia induced in these mice by FTY720 supports a cytotoxic rather than a lymphocyte "homing" effect of FTY720. However, no apoptotic studies were

conducted on the mice rendering the mode of action of FTY720 inconclusive in this study. The wild-type control C57Bl/6+SV29 mice were similarly affected whilst the lymphocyte counts in the vehicle control mice were essentially unaffected.

No effect of FTY720 was seen on the other haematological parameters including neutrophil, monocyte, platelet and red cell parameters. The chemistry (data not shown) showed normal kidney and liver functioning. No adverse side effects were experienced by the baboons although some of the animals had a slight reduction in weight. The drug was well tolerated even when administered over a long period as seen in the 5-week α-Gal trial where the baboons received 0.1mg/kg/day for three weeks and the dose was increased to 0.3mg/kg/day for the final two weeks.

In conclusion, this study shows that baboons are susceptible to FTY720, and both peripheral T-cells and B-cells are profoundly affected by FTY720 treatment. The higher dose of 0.3mg/kg is the most effective in terms of a rapid and marked decrease in peripheral lymphocytes. The effect is more pronounced and persistent on CD4+ than CD8+ T-cells and this translates into reduced *ex vivo* T-cell proliferation. The mechanism of action of FTY720-induced lymphopenia , which was originally reported as preferentially exerted on T-cells, is still controversial. No apoptotic cells were found in baboon PBMC isolated after treatment with FTY720 in the pharmacodynamic study, although control studies using human PBMC showed that apoptosis was induced after four hours in the presence of 3 μMol FTY720. Although FTY720-induced lymphodepletion was observed in LT-α-/- and L-selectin-/- mice suggesting that lymphocyte homing to other lymphoid organs might not be a mode of

action for the drug, no apoptotic assays were performed to confirm that FTY720 is cytotoxic to lymphocytes. Thus the actual immunosuppressive activity of FTY720 is still under debate.

As a follow-up study, it would be interesting to assess, especially in the context of delayed chronic rejection and xenotransplantation, whether the FTY720-induced reduction of peripheral B-cells also translates in some B-cell immunosuppression in "T-cell independent" B-cell models.

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8.0 APPENDIX A

Phosphate Buffered Saline (PBS, 10x)

14,4g Na_2HPO_4: 2 H_2O

2.4g KH_2PO_4

2g KCl

80g NaCl

Dissolve all the chemicals in 1 litre of double distilled water and pH to 7.3.

FACs Buffer

To 1L of PBS add:

1% Bovine serum albumin

0.1% NaN_3

APPENDIX B

Hepes Buffer for Annexin-V-Fluos

10mM Hepes pH 7.4

140mM NaCl

5mM $CaCl_2$

Mix all the chemicals together. The buffer is stable for three months at 4°C.

Propidium Iodide (PI) solution

Make up 50µg/ml of PI in the Hepes buffer

Staining solution for Annexin-V-Fluos

1000µl Hepes buffer

20µl of Annexin-V-Fluos labelling solution from the kit

20µl PI solution

Prepare the above and use immediately