Derivation of the Immunosuppressive Macrolide Rapamycin: Chemical, Structural and Biological Aspects

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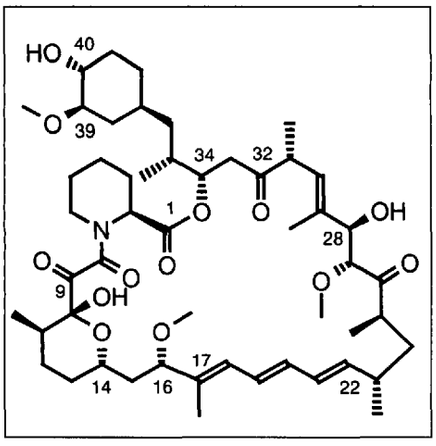
The macrolide rapamycin was discovered in 1975 at *Ayerst* in a screen for novel antifungal agents. It has attracted interest since the beginning of the nineties because of its remarkable immunosuppressive properties and is presently undergoing clinical trials for the prevention of allograft rejection.



Rapamycin exerts its immunosupressive activity by forming, in a first step, a complex with its intracellular receptor FKBP12. This complex formation involves the 'binding domain'. The latter consists mainly of the C(1)–C(14) subunit, but also includes the C(40) hydroxy group, which makes a hydrogen bond with FKBP12. Binding of rapamycin to FKBP12 is necessary, but not sufficient, for immunosupressive activity. Indeed, in a second step, the FKBP12/rapamycin complex binds to a protein termed FRAP, and it is this second interaction which is ultimately responsible for immunosuppression. The interaction of the FKBP12/rapamycin complex with FRAP is mediated by the 'effector domain' which comprises the region between C(14) and C(28) and, thus, includes the conjugated triene subunit.

Through chemical derivation of the macrolide, we could show that the triene moiety is indeed critical: partial or complete hydrogenation and dihydroxylation drastically decreased or even abrogated the immunosuppressive activity. Modification of the allylic C(16) methoxy group, on the other hand, led to potent derivatives. While replacement of this group by larger alkoxy moities resulted in loss of activity, the introduction of propargylic ethers led to derivatives which were up to threefold more potent than rapamycin.

Methylation of the C(28) hydroxy function resulted in a thousandfold decrease in activity, though binding to FKBP12 was not affected. The X-ray crystal structure of 28-*O*-methylrapamycin complexed with FKBP12 showed that the cyclohexyl ring of this derivative has a completely different orientation compared to the one found for bound rapamycin. Structural evidence indicated that the cyclohexyl ring in its new position impedes the second complexation step with the target protein FRAP, apparently mainly for steric reasons. These results prompted us to further investigate the influence of the cyclohexyl moiety on the immunosuppressive activity. We found that cleavage of the C(39),C(40) bond, a modification which does not add steric bulk to the molecule, abrogates the activity. This finding indicated that part of the cyclohexyl subunit, which until now was thought to be only involved in binding to FKBP12, might also be important for interaction with FRAP, and thus might also belong to the effector domain of rapamycin.

Somatic Cell and Gene Therapy: Perspectives in Oncology

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The *ex vivo* expansion of primitive haematopoietic cell harvests is of interest for gene therapy and transplantation applications. The possibility of maintaining, manipulating and expanding harvested human haematopoietic stem cells in *ex vivo* culture could help to provide autologous and allogeneic transplants of improved purity and performance. Such improved cell yields may be of clinical benefit to poor-mobilising patients and those requiring multiple transplantation, and enable access to gene therapy of the haematopoietic system. Efficient gene transfer into haematopoietic stem cells could improve the treatment of inherited diseases, viral infections and cancer, and aid the therapeutic evaluation of tumor-cell purging in autologous stem cell transplantation. Successful gene transfer into haematopoietic repopulating cells has been reported at low levels, but improvements are needed for clinical application.

Serum-free GMP culture conditions have been developed for the expansion, differentiation, purging and gene transfer of haematopoietic stem cells. Such serum-free conditions have theoretical advantages for the biological safety and standardisation of clinical gene transfer into haematopoietic cells and have been shown to allow greater cell-culture expansion than serum-containing conditions. Under these conditions, efficient purging (>3 logs) with recombinant immunotoxins or dendritic cell differentiation can readily be obtained. The same GMP medium (CellGro = AE) also allows serum-free production of retroviral vector-containing medium using the gibbon ape leukaemia virus (GALV) pseudotyped packaging cell line PGT13/LN. The GALV envelope binds to a spe-

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cific receptor expressed at high density on human cells but absent on producer cells, thus increasing biosafety.

In chronic phase CML, Ph-stem cells can be mobilised efficiently into the peripheral blood by intensive chemotherapy. In most CML patients, Ph+ cells have a CD34+/HLA-DR+ phenotype which can be depleted from the harvest by extended long-term marrow culture. In a phase-I/II trial of haematopoietic stem cell autotransplantation for patients with CML, serum-free *ex vivo* gene marking has been performed after *in vivo* and *in vitro* purging of Ph+ cells. Retroviral gene transfer was performed in committed and primitive progenitor cells (long-term culture-initiating cells, LTC-IC) for 7 days in suspension culture with serum-free vector-containing media supplemented by stem-cell factor (SFC), interleukin-3 (IL-3) and flt-3 ligand. Gene-transfer efficiency was determined as the percentage of G418-resistant colonies relative to the total LTC-IC or CFU-C-derived colonies.

After 7 days of transduction culture, the overall number of LTC-IC derived colonies in CD34+ and CD34+/HLA-Drlo cells increased on average 3.9 ± 2.3 and 2.7 ± 2.4-fold, respectively. The transduction efficiency was 49 ± 1.3% and 57 ± 8.4% for CFU-C and LTC-IC, respectively, generated from CD34+ peripheral blood progenitor cells. The marker gene is present in the peripheral blood for 200 days. Preliminary analysis suggests that up to 10% of those cells from the *ex vivo* culture that contribute to long-term haematopoiesis contain the transgene. A new method of viral integration-site analysis demonstrates oligoclonal haematopoiesis with deferent clones of the marked population detectable at varying time points.

While extended static culture (>7 days) leads to telomere shortening and decreased telomerase activity, shorter culture periods maintain telomere length and increase telomerase activity. Telomere loss after 12 days *ex vivo* expansion culture is not significantly greater than following standard-dose chemotherapy. Human peripheral blood-derived haematopoietic stem cells (HPSC) retain all biological *in vitro* and *in vivo* properties for long-term haematopoietic reconstitution.

In conclusion, retroviral gene transfer into primitive haematopoietic progenitor cells can be achieved using long-term *ex vivo* transduction in serum-free culture conditions supplemented by flt-3 ligand and SCF, with or without IL-3, IL-6 or thrombopoietin. Preliminary data indicate that efficient gene transfer into repopulating haematopoietic stem cells is possible.

Gene Transfer for Immune Therapy of Cancer

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The first clinical gene therapy protocol began in 1990 for the correction of adenosine-deaminase (ADA) deficiency. Since then, retroviral, adenoviral, lentiviral, adeno-associated and non-viral gene delivery techniques have been explored for treatment of inborn diseases. An explosive second phase began when the technology was exported to treatment of cancer and AIDS.

In the context of T-cell-depleted allogeneic bone-marrow transplantation (allo-BMT), delayed donor-lymphocytes infusion plays a central therapeutic role in both graft-*versus*-leukemia and immune reconstitution. However, the use of donor lymphocytes is limited by the risk of severe graft-*versus*-host disease (GvHD) which is a frequent and life threatening complication of BMT. Patients who relapsed or developed an *Epstein-Barr*-virus-induced lymphoma after BMT were treated with donor lymphocytes transduced with the thymidine kinase suicide gene of the Herpes Simplex virus (HSV-tk). HSV-tk expression confers to donor lymphocytes a selective *in vivo* sensitivity to the drug ganciclovir allowing a specific treatment of GvHD, thus avoiding immunosuppressive therapies.

The SFCMM-3 vector that encodes the gene for HSV-tk was generated under the control of a viral LTR promoter as well as the gene for a truncated and, therefore, non-functional version of the low-affinity receptor for nerve-growth factor (LNGFR). This LNGFR gene served as a marker for successful transduction and was under the control of the SV40 promoter. The transduced cells survived for over 12 months, in high proportions (up to 13.4% of circulating peripheral blood lymphocytes (PBL)) resulting in antitumor activity in over 50% of patients. Complete remission could be achieved in patients affected by EBV-lymphoma, chronic myelomonocytic leukemia, acute myeloid leukaemia, non-*Hodgkin* lymphoma, and multiple myeloma.

Among three patients who developed a GvHD after donor lymphocytes infusion, two patients showed complete remission after treatment with ganciclovir, whereas one patient with chronic GvHD showed partial remission. Circulating PBLs obtained from the patient before and after ganciclovir treatment showed no difference in ganciclovir sensitivity *in vitro* and high expression levels of the cell-surface marker LNGFR, suggesting that ganciclovir resistance of chronic GvHD is mainly related to the cell-cycle dependent activity of the HSV-tk/ganciclovir system.

Three patients treated with donor T-cells, which were genetically engineered to express HSV-tk, developed a specific immune response against the tk protein, which resulted in immediate elimination of the infused genetically engineered cells. This phenomenon was observed in patients who received the first infusion of genetically modified donor cells late after BMT, documenting the role of the recipient immune constitution. To circumvent the intrinsic limitations of the HSV-tk based strategy, such as immunogenicity, partial ganciclovir resistance and cell-cycle dependence, modified vectors for the new and promising suicide gene-strategy will be developed.