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Review

The Immunosuppressant Tacrolimus (FK506) Facing the 21st Century: Past Findings, Present Applications and Future Trends

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Abstract: The confluence of a large variety of factors, achievements and developments has resulted in the current long-term success in graft transplants. Some of these events are reviewed, paying special attention to immunosuppressant drugs, which are one of the most relevant milestones in the prevention of organ transplant rejection. The discovery, industrial exploitation, mechanisms of action and side effects of several drugs exhibiting immunosuppressive effects (e.g., corticosteroids, nitrogen mustards, mycophenolic acid, rapamycin) are deeply detailed. Furthermore, new trends in immunosuppressant research, improvement and reformulation are also reviewed. Nevertheless, the core of the manuscript is the immunosuppressant tacrolimus, also called FK506, which has been sought after due to the commercial success of cyclosporine and other immunosuppressant compounds, but also because of the side effects of those previous compounds. Thus, in the mid-1980s tacrolimus was described as a more potent immunosuppressive molecule, with less undesirable effects. Currently, tacrolimus is a well-stablished API that is used as a clinical treatment to avoid graft rejection, but also shows interesting properties in terms of decreasing the impact of some autoimmune diseases and acting as an enhancer of nerve regeneration treatments. Thus, in the 40th anniversary of its discovery, this paper describes the current state of the art of this drug and how it is adapting to new social needs and clinical trends.

Keywords: corticosteroids; FK506; graft; immunosuppressant; mycophenolic acid; nitrogen mustards; omics; rapamycin; *Streptomyces*; *Streptomyces tsukubaensis*; synthetic biology; tacrolimus

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1. Introduction

The vegetal grafts observed in nature between compatible trees that grow contiguously result in a natural union of the different trees' branches. This phenomenon likely inspired the idea of combining different organs or parts into a single body, an idea which has existed in humankind for a long time. Such concepts are reflected in legends and miracles of both Eastern and Western cultures (e.g., the Hindu icon Ganesha was the result of the combination of an elephant head onto a child body by Shiva; the Chimaera, the fabulous creature, is an amalgamation of lion, goat, and serpent; or the Christian saints Cosmas and Damian, who miraculously transplanted the black leg of the dead Ethiopian gladiator onto the white body of their verger, Justinian, with a "cancerous" leg) [1,2]. Therefore, the image of Dr. Christiaan N. Barnard's face on the cover of the weekly magazine Time (15 December 1967) because of the first human heart transplant undoubtedly summarized several centuries of longing, research, and findings regarding the idea of graft transplants. Though his first patient, Mr. Louis Washkansky, died after only 18 days, the conceptual seed of transplantation was sown, and different groups worldwide were able to achieve new and successful results in terms of longevity, ranking from a few days to several years (e.g., Barnard's fifth and sixth patients, who lived for almost 13 and 24 years, respectively) [3].

Successful graft transplants are the result of the confluence of a huge variety of factors, such as surgical developments, technological achievements, microbial screenings, drug discovery and approval, ethical and legal aspects, and religious concerns. Table 1 summarizes some of the most relevant milestones, which have conditioned or boosted the current success of organ transplantation and have paved the way for future achievements.

Table 1. Chronological description of different relevant events that have resulted in the existing status of graft transplantation. Clinical, surgical or research group leaders are indicated as representativess of their entire team.

Year	Discovery, Finding or Development	Refs.
c. 1550 BC	First written mention of skin grafting for the treatment of burn is attributed to the Ebers Papyrus.	[2,4]
800–600 BC	First operative description of nose and ear reconstructions based on auto-transplants by the surgeon Sushruta are documented in Sushruta Samhita medical text.	[5–7]
30	The Roman physician and surgeon Cornelius Celsus, through his *De Medicina*, reported important advances in surgery (cautery to make incisions, hemostasis achievement, tumor ablation).	[8]
348	"The Miracle of the Black Leg", included in the Jacobus De Varagine's *Legenda Aurea*, mentions a limb transplantation carried out by the saints Cosmas and Damian.	[2]
1215	Pope Innocent III bans higher ministers from performing any surgical procedure, though most European medical knowledge at that time rested with the Catholic Church.	[4,9]
1537	French barber–surgeon Ambroise Paré defines practical interventions to decrease pain and suffering (e.g., battlefield burn wounds).	[10]
1597	The Italian surgeon Gaspare Tagliacozzi describes the "Italian method" of nose reconstruction from a pedicled arm flap.	[11]
1668	In a posthumous publication, the Dutch surgeon Job Janszoon van Meekeren describes a tale about a bone section transplantation from a dog's skull into a soldier's cranium that was carried out by a Russian surgeon. The soldier was excommunicated and the dog bone was removed to reaccept him again into the church.	[12,13]
1770	The Scottish experimentalist surgeon John Hunter auto-transplants cocks' spurs into their combs.	[14]
1804	The Italian physician Giuseppe Baronio carries out the first scientifically documented reports of effective autologous skin grafts in mammals.	[15]
1846	The American dental surgeon William T. G. Morton performs a successful public demonstration of diethyl ether anesthesia during surgery at the Massachusetts General Hospital.	[4]
1869	The Swiss surgeon Jacques-Louis Reverdin authors the first publication of successful skin grafts for wounds.	[16]
1870	The British surgeon Joseph Lister defines how to prevent infection in wounds during and after surgery.	[17]
1901	The Austrian American immunologist and pathologist Karl Landsteiner discovers AB0 blood groups and their heritability.	[18]
1902	The French surgeon Alexis Carrel reports on the triangulation with fine silk material as a technique of vascular suture.	[19]
1916	A dextrose-based solution allows for human blood preservation and its transfusion.	[20]
1933	The Soviet surgeon Yurii Voronoy achieves the first human-to-human kidney transplant. The patient survived for 2 days. Later, in 1953, the first long-term (8 years) kidney transplant is achieved between twin brothers.	[21,22]
1936	Peter Gorer describes for the first time a histocompatibility system (Major Histocompatibility Complex: MHC) in mice.	[23]
1944	Medical team at the Valley Forge General Hospital (Pennsylvania) successfully grafts skin onto the World War II pilot Charles Woods (±70% of body burned) from a recently deceased soldier.	[24]
1945	The German American pathologist Leo Loeb tackles biological individuality through the genetic disparity of donors and recipients.	[25]

Table 1. *Cont.*

Year	Discovery, Finding or Development	Refs.
1949	Cortisone becomes available. Later, in 1951, a cheap synthesis method from diosgenin is discovered, which allows the production of glucocorticoids (greater effectiveness and lifespan).	[26,27]
1956	The American physician Edward D. Thomas performs the first bone marrow transplant, treating a leukemia patient with radiotherapy followed by healthy marrow from an identical twin.	[28]
1963	The United States surgeon James Hardy achieves the first lung transplant procedure.	[29]
1964	Declaration of Helsinki establishes a code of ethics on human experimentation by the World Medical Association.	[30]
1967	The American physician Thomas Earl Starzl carries out the first liver transplant with long-term survival.	[31]
1967	The South African cardiothoracic surgeon Christiaan N. Barnard achieves the first human-to-human heart transplant.	[2,3]
1975	Rapamycin (sirolimus), produced by the soil bacterium *Streptomyces hygroscopicus* NRRL 5491, is described as a potent antifungal metabolite and, later, as a cytostatic against immune cells.	[32,33]
1976	Cyclosporin A, a fungal lipophilic cyclic peptide discovered in 1970, is presented by Sandoz Co. as an immunosuppressive agent.	[34,35]
1981	The American cardiothoracic surgeon Bruce A. Reitz leads the first combined heart–lung transplantation.	[36]
1981	Uniform Determination of Death Act (UDDA) is approved, which provides the comprehensive bases for determining the legal definition of death in all situations.	[37]
1983	FDA approves Cyclosporin A for the prevention of allograft rejection. In 1987, it is registered for several autoimmune disorders, and in 2003 it is approved for dry eye disease.	[38]
1984	Tacrolimus (FK506) is discovered in the culture broth of *Streptomyces tsukubaensis* by the Fujisawa Pharmaceutical Co. staff.	[39,40]
1986	The American thoracic surgeon Joel Cooper completes the first successful double-lung transplant.	[29]
1987	The surgeon Folkert Belzer and the biochemist James Southard enhance the organ preservation before transplantation after development of the University of Wisconsin cold storage solution (ViaSpan).	[41,42]
1989	The French professor of pediatrics Olivier Goulet achieves the first small intestine transplantation.	[43]
1989	The American physician Thomas E. Starzl tests renal transplantation in unrelated baboons under the FK 506 (tacrolimus) drug.	[44]
1990	The American cardiothoracic surgeon Vaughn A. Starnes performs the first lung lobar transplant from a living donor.	[45]
1991	Pope John Paul II states that medicine "*has found in organ transplantation a new way of serving the human family*". In 2000, he defines voluntary donation as a "*a genuine act of love*".	[46,47]
1991	Mycophenolate mofetil, a prodrug derived from mycophenolic acid, is developed for the prevention of organ transplant rejection.	[48,49]
1992	The American physician Thomas E. Starzl attempts at baboon-to-human liver xenotransplantation.	[50]
1994	Tacrolimus is approved by the FDA for primary immunosuppression in adult and pediatric liver transplantation.	[51]
1997	Sarah Marshall, a 6-month-old baby, becomes the youngest pediatric multi-organ transplant (stomach, pancreas, liver, and bowel).	[52]
1998	The Australian microsurgeon Earl R. Owen and the French medical doctor Jean-Michel Dubernard transplant the right distal forearm and hand from a brain-dead man to a living patient.	[53]
1998	Donor-derived cell-free DNA (ddcf-DNA) is presented as a non-invasive approach to prevent allograft rejection. It is proposed in the late 2010s as a biomarker detection procedure.	[54–56]
2001	The European Union Directive 2001/20/EC implements the good clinical practice in clinical trials on medicinal products for human use.	[57]

Table 1. *Cont.*

Year	Discovery, Finding or Development	Refs.
2002	The Additional Protocol to the Convention on Human Rights and Biomedicine concerning Transplantation of Organs and Tissues of Human Origin is established.	[58]
2005	The maxillofacial surgeon Bernard Devauchelle, assisted by the transplantation specialist Jean-Michel Dubernard, tackles the first partial face transplant of a woman with serious dog bite injuries.	[59]
2010	The European Union Directive 2010/45/EU defines the standards of safety and quality of human organs for transplantation.	[60]
2010	The Spanish plastic surgeon Juan P. Barret achieves the first full face transplant, including all integral aesthetic and functional units.	[61]
2012	The Spanish team headed by Juan F. Martín reports the first genome of a tacrolimus-producer strain, *Streptomyces tsukubaensis* NRRL 18488.	[62]
2018	The FDA approves a medical device (ReCell®) for processing skin samples into a sprayable cell suspension.	[63]
2022	U.S. reaches one million transplants.	[64]
2023	Spain is a global leader in organ transplants for over three decades, showing 48.9 donors per million people and 122.1 transplants per million, in 2023.	[65]
2024	The surgeon Tatsuo Kawai achieves the first transplant of a genetically modified kidney from a pig to a living human.	[66]

2. Immunosuppressive Drugs: History and Evolution

All along the millenary tradition of graft transplant attempts, setbacks or failures have been more common than advances. However, different transplants of organs and tissues from humans (living and dead) and animals are nowadays achieved worldwide. Thus, the development of immunosuppressants has meant an improvement of the lifespan of the organs after transplantation and patient quality of life, even though the side effects are a worrying drawback. Thus, this section describes several compounds used as immunosuppressants through their historical discovery, type of molecule, action mode, side effects, and new trends in their research, development or reformulation.

2.1. Corticosteroids: An Initial Step in Immunosuppression

Immunosuppressants have played an outstanding role as part of the current status of long-term transplant success. Initially, **corticosteroids** were relevant compounds in the preliminary steps of organ transplantation, where Azathioprine was developed in the late 1950s, as the first immunosuppressive agent for human kidney transplant. Glucocorticoids (corticosteroids) have well-known inhibitory effects on a broad range of immune responses and they are used to treat rheumatoid arthritis, inflammatory bowel disease, asthma, allergies and many other conditions. Corticosteroids act on the immune system at two levels: (i) altering the circulating lymphocytes (reticuloendothelial system knap the CD4+ T lymphocytes), and (ii) blocking several lymphokines and cytokines, which inhibit the proliferation and function of lymphocytes [67,68]. Despite the recognized undesirable effects of chronic corticosteroid therapies [e.g., predisposition to infection, weight gain, cataracts, glucose intolerance, sodium retention (edema and hypertension), skeletal defects (osteopenia, aseptic necrosis)], steroid doses were used both for prevention of acute rejection and as a main immunosuppression treatment of maintenance up to the mid-1990s. However, aimed to decrease the side effects, dosage lowering was studied and subsequently described in the 1970s [68].

When Cyclosporin A was approved by the Food and Drug Administration (FDA) (see below) for the prevention of allograft rejection in the 1980s, steroid-free immunosuppression gained attention (see Table 1) [68]. The discussion around the use of steroids in kidney transplants has continued for a long time [69,70] and clinical trials about early corticosteroid cessation in kidney transplant recipients continues to this day [71]. However, several corti-

costeroid drugs (e.g., dexamethasone, betamethasone, prednisone, methylprednisolone, hydrocortisone) are routinely used at present and appear in the pharmacopoeia.

2.2. Nitrogen Mustards: A Collateral World War II Effect

Nitrogen mustards are powerful cytotoxic agents, and show chemotherapeutic and mutagenic effects, which make them useful as therapeutic agents for cancers, sarcomas, and hematologic malignancies [72]. They are alkylating agents that include the 7-nitrogen atom of guanine and other DNA constituents as final targets, being able to interact with nucleotides of opposite strands, which results in covalent linkage adducts (DNA interstrand cross-links) [73]. These skills gained attention in both World Wars, as lymph ablative agents in chemical warfare [27]. When the information about nitrogen mustards was declassified in 1946, these reagents were considered for cancer therapy. Thus, **cyclophosphamide** was observed as the most versatile of the nitrogen mustards suppressing antibodies production, which placed it in routine treatment when autoantibodies play a pathogenetic role, such as autoimmune diseases (e.g., systemic lupus erythematosus) and hematopoietic stem cell transplantation. Cyclophosphamide metabolism generates phosphoramide mustard as a final product, which alkylates DNA, resulting in the suppression of the cell division mainly in B-cells. This occurs because B-cells have a slower turnover than T-cells, which present timing and dosage-dependent effects [27,74]. Its effects on these two types of immune cells influence both humoral and cell-mediated immunity.

Cyclophosphamide presents well-known side effects, such as leukopenia, hemorrhagic cystitis, cardiotoxicity or alopecia, in addition to its mutagenic effect, which increases the risk of cancer [27]. However, the trending development of targeted small molecules and monoclonal antibodies as part of the treatment of malignant and autoimmune diseases, in combination with nitrogen mustards, have recently reached Phase I and II clinical trials [72], which can extend the lifespan of some nitrogen mustards (e.g., cyclophosphamide and melphalan) (Figure 1).

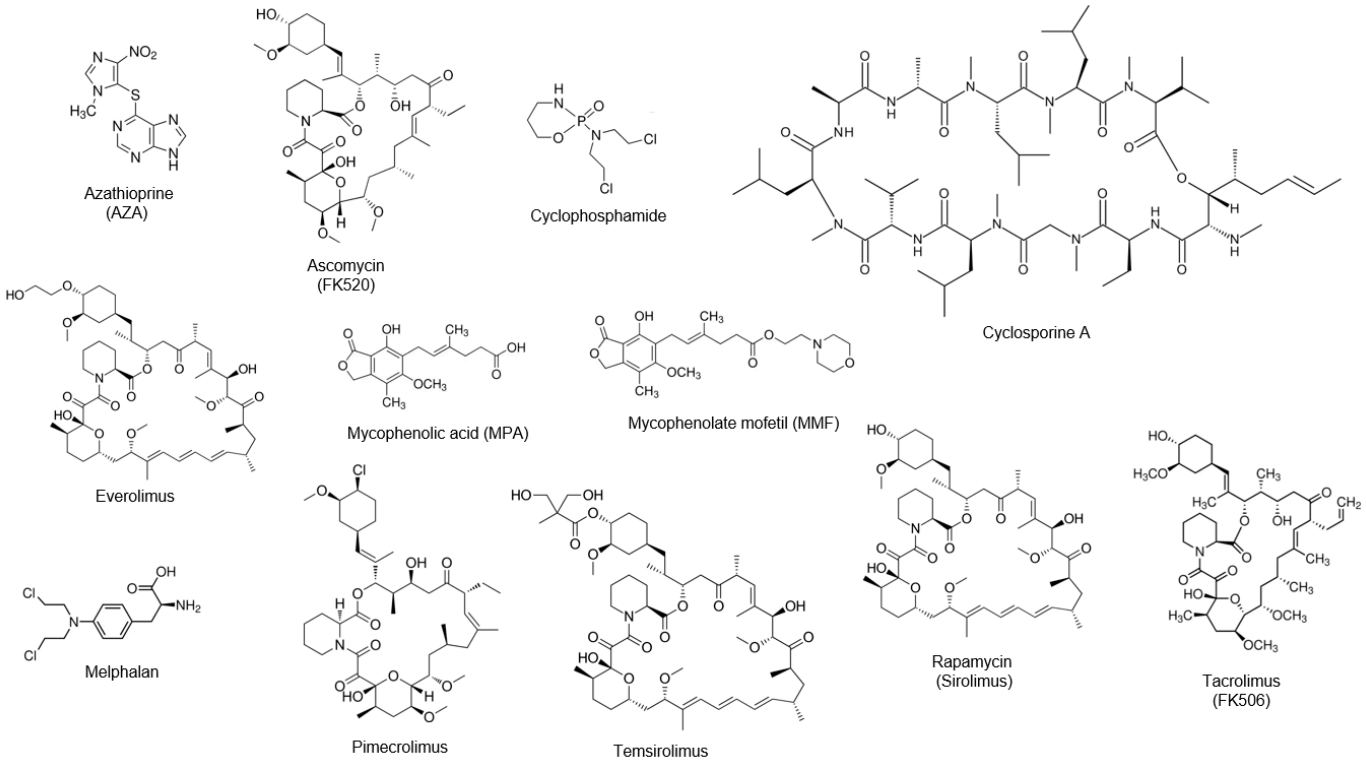

Figure 1. Chemical structure of different immunosuppressant agents described throughout the text.

2.3. Antimetabolites: Controlling Nucleotide Biosynthesis

Antimetabolites, which act as inhibitors of purine metabolism, were also considered for transplant immunosuppression since the early 1960s, where the prodrugs mycophenolate mofetil (MMF) and azathioprine or AZA (6-[(1-Methyl-4-nitro-1H-imidazol-5-yl)sulfanyl]-7H-purine) were the best-known examples [75] (Figure 1). **Azathioprine** was described in 1961 as an analogue of 6-mercaptopurine (6-MP), a previously described compound evaluated in allograft rejection [76]. Thus, in 1963, the first kidney transplant was reported where AZA was used as an immunosuppressant [77]. AZA is also prescribed to treat inflammatory conditions (rheumatoid arthritis, Crohn's disease and ulcerative colitis [78], severe inflammation of the liver, skin diseases [79], arteries and cardiac disorders [80]) (https://www.nhs.uk/medicines/azathioprine/about-azathioprine/, accessed on 6 April 2024).

Azathioprine metabolism is carried out in the liver and excretion is through the kidneys [81]. It is based on its almost full conversion to 6-MP, which is metabolized by three different pathways: (i) the conversion into its active nucleotides, 6-thioinosinic acid and 6-thioguanine acid; (ii) the production of thiouric acid by means of the enzyme xanthine-oxidase (target of the allopurinol drug); and (iii) the transformation into the inactive metabolite 6-methyl-MP through the enzyme thiopurine methyltransferase. 6-thioguanine acid is the most active metabolite and it is in charge of its major effect through inosine-monophosphate (IMP) metabolism [(i) IMP to adenosine-monophosphate (AMP), (ii) adenosinetriphosphate (ATP), (iii) synthesis of RNA and DNA in the salvage pathway], inhibiting purine synthesis [75,81]. Thus, azathioprine was a pillar of chronic immunosuppression protocols until the mid-1990s, when mycophenolate mofetil demonstrated higher effectiveness for the prevention of renal acute rejection by means of double-blind randomized trials [82–84]. As a result of its lack of specificity and mutagenicity, AZA is less effective than mycophenolate mofetil in preventing allograft rejection [27,85]. However, AZA is one of the oldest pharmacologic immunosuppressant agents in use, and research about the compounds has continued throughout the last 60 years. Hence, it has been proposed as a basis for the development of drugs that could induce allograft-specific tolerance [86] and, due to its activity as a promoter of the differentiation of the intestinal epithelial cell into Paneth cells, it can be considered as a drug to decrease the severity of ileal Crohn's disease [87].

2.4. Mycophenolic Acid: A Fungal Product "Rediscovered" Time After Time

Fleming's serendipity allowed the discovery of *Penicillium*, as the penicillin producer fungus [88–90] boosted the screening of new antibiotics. Thus, a strain of *Penicillium brevicompactum* able to produce a compound capable of inhibiting the growth of *Staphylococcus aureus* was presented by Wilkins and Harris in 1943 [91]. The active compound was previously discovered in 1893 by the Italian physician Bartolomeo Gosio as a growth inhibitor of *Bacillus anthracis*, although he was looking for a metabolite causing pellagra [92–95]. In fact, it was the first antibiotic produced by a mold isolated in its pure and crystallized form, as stated Howard Florey, head of the staff involved in the harsh process of penicillin purification [96]. Furthermore, in 1913, a fungal resorcylate isolated by Alsberg and Black from spoiled corn samples was named **mycophenolic acid** (MPA, an acidic phenol from a fungus), which resulted in the same compound crystallized by Gosio some years before [97] (Figure 1). Even though MPA was initially defined as an antimicrobial drug, it was soon described as both an antitumoral and immunosuppressive agent. In the 1970s, it was tested as a treatment for psoriasis [98,99] and, in the early 1990s, it was considered as an immunosuppressant for transplanted patients [49,100]. As a result, in 1994, MMF, a semisynthetic morpholinoethyl ester of MPA developed to improve its bioavailability, was approved by the FDA for the prevention of acute rejection in kidney transplantation [48,75]. Nowadays, MPA is available in two formulations: (i) MMF, which improves its bioavailability, and (ii) enteric-coated mycophenolate sodium (EC-MPS), which reduces the gastrointestinal effects (see below) and delays the compound's release into the small

intestine (https://www.uptodate.com/contents/mycophenolate-overview-of-use-and-adverse-effects-in-the-treatment-of-rheumatic-diseases/print, accessed on 6 April 2024).

MMF is rapidly hydrolyzed after oral administration by plasma and tissue esterases into MPA, which acts over both isoforms (type I and II) of the inosine monophosphate dehydrogenase, which is a crucial enzyme required for lymphocyte proliferation through the de novo synthesis pathway of purines. It is a reversible, selective and non-competitive inhibitor of this enzyme, although type II is 4–5 times more sensitive than type I [48,101,102]. MPA bioavailability is up to 94% after oral administration. However, a drawback is its high protein affinity (97–99%, mostly to albumin) in the pharmacologically available format, which only allows a small fraction of MPA to circulate in its free form in patients with normal renal and liver function (99.9% plasma fraction and 0.01% cellular components) [103,104]. Even though both MMF and EC-MPS are highly selective of lymphocyte growth and the side effects are lower than other immunosuppressive drugs, some adverse effects have been described, such as gastrointestinal disturbances (e.g., nausea, diarrhea, vomiting, abdominal cramps), infections (genitourinary tract, respiratory system, wounds), or blood dyscrasias (leukopenia, thrombocytopenia, anemia) [48].

2.5. Cyclosporine A: The Immunosuppressant That Almost Was Not

Microorganisms are an endless source of metabolites and biotechnological solutions. Thus, in the early 1970s, the immunosuppressant **cyclosporine A** (Figure 1) was characterized from two fungal species isolated from soil samples of different regions [*Tolypocladium inflatum* Gams (Wisconsin, USA) and *Cylindrocarpon lucidum* Booth (Hardanger Vidda region, Norway)] by the staff of Sandoz Ltd. [later Novartis, and in October 2023, again spun off as Sandoz (https://www.sandoz.com/, accessed on 6 April 2024)]. Since only *T. inflatum* was able to grow successfully under fermentative conditions, it was the selected candidate to upgrade the cyclosporine manufacturing process, which initially was a mixture of two metabolites: cyclosporine A and B [35]. However, cyclosporine A demonstrated a higher inhibitory level over the proliferation of lymphocytes in a very selective way but did not inhibit the proliferation of other somatic cells, which presented it as a unique metabolite at that time [34,35,105]. Interestingly, due to the scarce transplants market in the 1970s and the large investment needed to get a new drug approved by the FDA (about USD 250 M), it was on the brink of oblivion unless a new application was proposed to justify the expenses, which was its anti-inflammatory activity in rheumatoid arthritis, even though the most prominent application was as an immunosuppressant [35].

Cyclosporine A is a neutral lipophilic cyclic undecapeptide (Figure 1) that specifically inhibits T-cell activation by two complementary ways (Figure 2). On the one hand is its blocking of the transcription of cytokine genes (e.g., IL-2, IL-4, CD40L), since cyclosporine A produces cyclophilins [ubiquitous cytosolic proteins with peptidyl-proline-cis-trans isomerase activity (PPIase), possibly involved in protein folding], which block calcineurin (PP2B) activity. Calcineurin regulates, by dephosphorylation, the nuclear translocation and subsequent activation of NFAT (nuclear factor of activated T cells) transcription factors, which are directly involved in the transcriptional activation of genes encoding cytokines [106–108]. On the other hand, several transcription factors, such as AP-1, NF-κB, and NFAT, are involved in the transcriptional activation of the IL-2 gene. These factors are affected by the presence of cyclosporine through its effect over two (JNK and p38) mitogen-activated protein kinases (MAPKs), which are relevant to the activation of different transcription factors, such as AP-1 [106,108,109]. Thus, the presence of two pathways as targets of cyclosporine A activity in T cells justifies its high specificity as an immunosuppressant. Furthermore, cyclosporine A decreases the activity of dermal and epidermal lymphocytes, as well as macrophages, and also inhibits the activation of antigen-presenting cells, natural killer cells, and keratinocyte hyperproliferation. The expression of cellular adhesion molecules on dermal capillary endothelium is downregulated too [110].

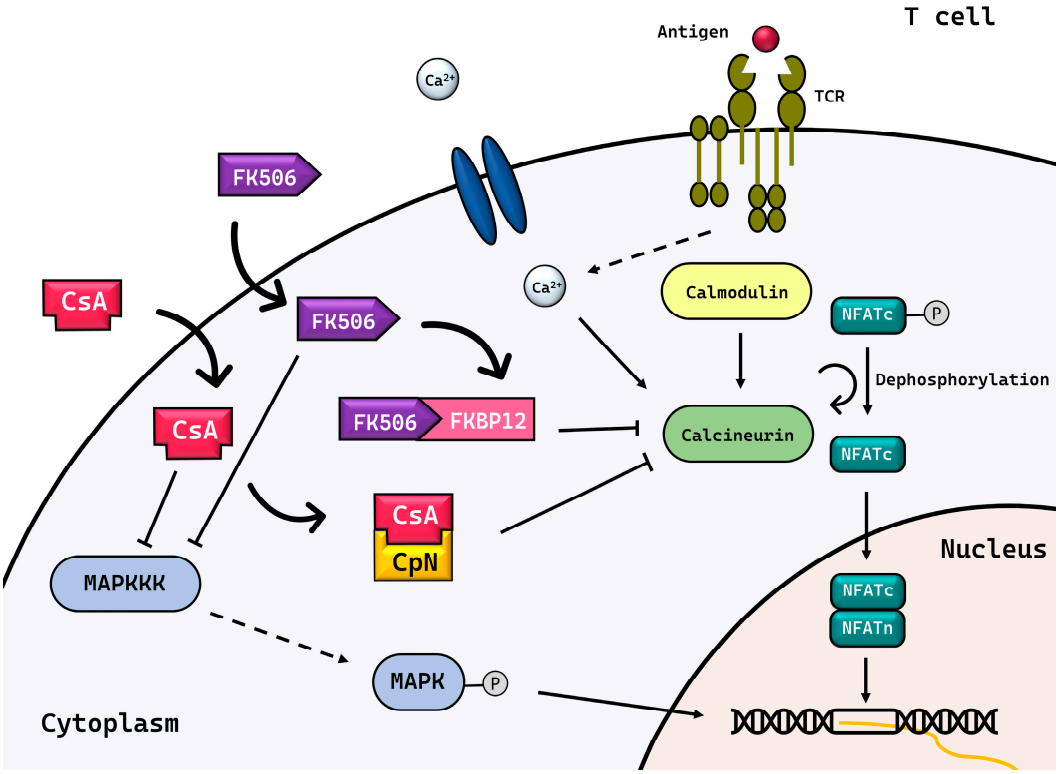

Figure 2. Scheme of the pathways controlled by the calcineurin inhibitors cyclosporine A (CsA) and tacrolimus (FK506). FKBP12 denotes the binding protein for tacrolimus, which inhibits calcineurin activity. CpN represents the cyclophilin molecule, which binds with cyclosporine A to similarly disrupt calcineurin. NFATc refers to nuclear factors of activated T cells, both phosphorylated (with a P) or dephosphorylated. The MAPKKK cascade pathway is also presented, highlighting its role in transmitting signals through a series of phosphorylation events that activate MAPKs, which subsequently regulate key cellular processes. Arrows with flat ends indicate inhibition, whereas arrows with pointed ends represent activation. The diagram provides a visual overview of how these immunosuppressive drugs exert their effects by disrupting both calcineurin activity and MAPK signalling pathways.

The main side effects of cyclosporine A are related to (i) renal dysfunction, which is the most notable due to its nephrotoxicity mainly in prolonged therapy; (ii) cardiovascular disorders, such as hypertension, arrhythmia, thrombotic microangiopathy; (iii) endocrine and metabolic diseases (dyslipidemia, hypomagnesemia, hyperkalemia, hypercholesteremia, gynecomastia, hypertrichosis); (iv) neurologic conditions (encephalopathy, seizures, anxiety, paresthesia, headache, tremors and fever); (v) gastrointestinal ailments (gingival hyperplasia, anorexia, nausea, vomiting, diarrhea, abdominal pain); (vi) musculoskeletal conditions (myalgia, muscle cramps); (vii) bone loss; (viii) cosmetic effects (hirsutism, acne, hypertrichosis) [110–112]. However, even though the collateral effects of cyclosporine are significant, the research around this immunosuppressant drug continues nowadays from different approaches, including (i) delivery procedures to enhance the bioavailability due to its poor water solubility, also aimed at decreasing adverse effects of current formulations in human and animal health [113–115], and (ii) new approaches based on its putative neuro- and cardio-protective activities [38].

2.6. Rapamycin (Sirolimus): A Compound That Refused to Be Forgotten

Rapamycin (Figure 1), also known as sirolimus by its generic name assigned by USAN (United States Adopted Names [116]), is an inhibitor of the regulator named mammalian target of rapamycin (mTOR). This drug is recommended as an immunosuppressant under special circumstances when the circumvention of the calcineurin inhibitor is desirable (such as skin cancer) [117]. Sirolimus discovery began in 1964 by means of the METEI (Medical Expedition to

Easter Island), a Canadian-led medical expedition team of about 40 medical and scientific staff to the Chilean territory of Rapa Nui, commonly known as Easter Island [118,119]. This island had captured the minds of explorers for more than a century due to the monolithic human figures called "moai", but plans for an airport in 1966 threatened to disrupt the biosphere of this unique ecological niche. Because of this, a medical expedition to document the population and biosphere was planned by Dr. Stanley Skoryna (1920–2003, a McGill University surgeon and gastroenterologist) (https://healthenews.mcgill.ca/in-memoriam-stanley-constantine-skoryna-1920-2003/, accessed on 21 July 2024) and Georges Nogrady (1919–2013; resident bacteriologist of the Université de Montreal's Faculty of Medicine) (https://www.legacy.com/us/obituaries/legacyremembers/georges-nogrady-obituary?id=43867343, accessed on 21 July 2024). Aiming to find new antibiotics from natural sources, soil samples from different parts of the island were collected by Nogrady and taken back to Canada, where they were handed over to Suren Neth Sehgal (also spelled as Surendra Nath Sehgal [120]) (1932–2003; Ayerst Laboratory in Montreal, later merged with Wyeth and from 2009 acquired by Pfizer: https://www.pfizer.com/news/press-release/press-release-detail/pfizer_and_wyeth_become_one_working_together_for_a_healthier_world, accessed on 21 July 2024). Thus, an antibiotic-producing strain, classified as *Streptomyces hygroscopicus* NRRL 5491, was isolated in 1972 from these soil samples. It produced an inhibitor of the microbial growth of *Candida albicans*, *Microsporum gypseum* and *Trichophyton granulosum* [32,33]. Furthermore, it also blocked the production of immune cells, which was a drawback for patients fighting off an infection and definitively ended its chance as an antifungal drug. The compound was initially called rapamycin [etymology: rapa- (Rapa Nui = Easter Island), -mycin] [32]. Subsequent studies carried out by the National Cancer Institute (NCI) Developmental Therapeutics Program established the rapamycin-mediated inhibition of tumoral cell growth in tumor cell lines [121,122]. Sehgal stated: "*it was a totally new class of anticancer agents we were looking at*" due to its cytostatic activity, unlike the traditional cytotoxicity of previously developed chemotherapies. However, in 1982, the rapamycin project was temporarily shut down due to (i) the low scientific and clinical relevance of the signal transduction blockage, which was totally unknown at that time, (ii) the closure of the Ayerst facilities at Montreal, and (iii) the difficulty of formulating the compound into an intravenous drug, making clinical trials impossible [119,120]. In 1987, American Home Products (owner of Ayerst) decided to merge Ayerst with Wyeth, and a year later, Sehgal found a chance to revive rapamycin as an immunosuppressant (Sandoz's cyclosporine had been approved in 1983), and NCI resumed the research around its anticancer properties. Finally, rapamycin could be formulated for oral consumption, and it was approved in September 1999 as an immunosuppressant to prevent organ transplant rejection under the commercial name of Rapamune (https://www.accessdata.fda.gov/drugsatfda_docs/nda/99/21083A.cfm, accessed on 21 July 2024) [119,120,123]. Later, in 2015, it was approved as a treatment against a rare progressive lung disease called lymphangioleiomyomatosis (https://www.pfizer.com/news/press-release/press-release-detail/pfizer_s_rapamune_sirolimus_becomes_first_fda_approved_treatment_for_lymphangioleiomyomatosis_lam_a_rare_progressive_lung_disease, accessed on 21 July 2024).

Polyketides are a class of natural products, including macrolides, which demonstrate a characteristic macrocyclic lactone ring. Rapamycin is a macrolide compound (Figure 1) which interacts with a group of intracellular binding proteins named FKBPs (FK binding proteins), in a similar way as the structurally related immunosuppressant tacrolimus does. In contrast, cyclosporine A acts through cyclophilin [116,124]. These receptors, where some immunosuppressant agents bind, are known as immunophilins (intracellular binding proteins), which act as cis/trans PPIases that are inhibited when the drugs bind to them. This is a crucial step in their immunosuppressive actions. However, this fact is not essential for immunosuppression. Thus, the drug–immunophilin interaction modulates other specific intracellular targets, such as mTOR in the case of rapamycin. mTOR is a 289 kD protein that activates S6K1 (p70 ribosomal S6 kinase). The activation of S6 through S6K1-mediated phosphorylation boosts the translation of a specific class of mRNA transcripts characterized by the presence of polypyrimidine tract (5′ TOP) [125], which includes some ribosomal proteins or elongation factors (e.g., eEF-2) for protein synthesis.

Sirolimus effectively blocks the protein synthesis mediated by mTOR, but it also interferes with different regulatory systems, such as (i) the transcription of IL-2 (interleukin-2) in T lymphocytes, since sirolimus affects the CD28-mediated response [126]; (ii) the translation of some specific mRNAs necessary for cell growth and proliferation though the inhibition of the phosphorylation of the eIF-4E binding protein (4E-BP1), which controls cap-dependent translation initiation [127]; (iii) the G1-to-S-phase progression of the cellular cycle, which, as a result of rapamycin's effect on IL-2, allows Cdk (cyclin-dependent kinase), which leads to cell cycle detention in mammals and yeasts [116,128].

The adverse reactions of sirolimus can be considered severe (e.g., thrombosis, thrombotic micro-angiopathy, pulmonary fibrosis), moderate (e.g., hypertension, chest pain) or mild (e.g., fever, pruritus) (https://www.pdr.net/drug-summary/?drugLabelId=2097, https://www.drugs.com/sfx/sirolimus-side-effects.html, accessed on 21 July 2024), and most of them are associated with glucose-related metabolic defects (hyperglycemia, hyperlipidemia, insulin resistance and new-onset type 2 diabetes) [129], and tumors regrow upon treatment cessation [130].

By the mid-2000s, different researchers reported how a decrease in mTOR activity extended the lifespan of diverse species (e.g., yeasts, nematodes, fruit flies), which seems to be relevant in the aging process [119]. Since sirolimus turned down the mTOR receptor similarly to a genetic knockout, research was focused on animal feeding with this immunosuppressant to analyze the effect on their lifespan. Thus, using genetically heterogeneous mice, an increase of 14% in females and 9% in males was described [131]. Hence, marmosets, pet dogs and even elderly people were part of the anti-aging experiments by using rapamycin or its analogues (rapalogs), including everolimus or temsirolimus [119,132] (Figure 1). As a result, different parameters conditioned by aging in the immune, cardiovascular, and integumentary systems were improved in patients with aging-related diseases, and even in healthy individuals. Although no serious effects were reported in healthy people, those showing aging-related diseases presented an increased number of infections as well as increases in total cholesterol, LDL cholesterol, and triglycerides [133]. While more studies are needed, rapamycin seems to be the only drug that has been consistently demonstrated to increase mammalian longevity [134].

3. Tacrolimus (FK506)

Tacrolimus (Tsukuba macrolide immunosuppressant), also known as FK506 or Fujimycin, is a 23-membered macrolide lactone immunosuppressant presenting a characteristic allyl lateral chain (Figure 1). It is structurally like ascomycin (FK520, FR-900520 or immunomycin), which is a 21-carbon molecule carrying an ethyl lateral chain [135] (Figure 1). However, it is highly dissimilar to sirolimus, a polypeptide consisting of 11 aminoamides, which conform a larger macrolide lactone of a 31-membered ring with three double conjugated bounds [32,33] (Figure 1).

The initial discovery of tacrolimus was fuelled by the confluence of different industrial, economic and clinical factors, such as (i) the commercial success of cyclosporine, previously isolated and produced by Sandoz Ltd.; (ii) the side effects described for cyclosporine that needed a solution [136]; and (iii) the screening of more potent drugs (tacrolimus is up to 100-fold compared to cyclosporine [137]) once the financial viability of the immunosuppressants was noticed.

The history of the industrial, economic and scientific achievements around tacrolimus from its initial discovery to the present can be divided into different periods, such as (i) the discovery and industrial exploitation; (ii) the elucidation of its metabolism; (iii) the enhancement of microbial growth conditions and drug production; and (iv) the development of novel derivates. These periods are carefully detailed below.

4. Tacrolimus: Industrial Discovery and Exploitation

Revising a series of industrially relevant compounds such as food and feed additives (e.g., glutamic acid [138]), drugs (e.g., steroids [139]) and antibiotics (e.g., penicillin [90]),

their discovery and industrial manufacturing usually came much sooner than the scientific achievements. This was also the case of tacrolimus, where its isolation in 1984 [39] and the initial characterizations in 1987 [40,140] came almost 25 years before the major developments concerning to its metabolism, biosynthesis or genetic regulation, which were fuelled when the expiry of the patents was approaching (see below).

4.1. Discovery of Tacrolimus

The discovery of cyclosporine by Borel and co-workers in 1976 [105] boosted the screening of new immunosuppressants by different pharmaceutical companies. Thus, Fujisawa Pharmaceutical Co. faced the screening of several thousands of fermentation broths, where strain no. 9993, found in Tsukuba (Japan), produced a potent immunosuppressive drug named FK506, without any inhibition of constitutive cell proliferation [39]. According to Kino and Goto [39], the actinobacterial producer strain showed a "*gray mycelium color*, *rectiflexible spore chains with smooth spore surfaces*, *nonchromogenicity*, *and a limited carbohydrate usage*". It was designated as *Streptomyces tsukubaensis*, referring to the area where the soil sample was collected. The immunosuppressant accumulation in the fermentation broth was detected after 40 h of cultivation, which was the timepoint when the culture achieved the stationary phase. Therefore, this new compound was presented as a secondary metabolite [39,40].

Initial structure determination was carried out by means of extensive chemical degradation and spectroscopic studies supported by X-ray crystal analyses, which established L-pipecolic acid as a component of the newly discovered molecule and presented it as a member of a new class of macrolide lactones (Figure 1) [39,140].

The in vivo and in vitro capacity as an immunosuppressant was subsequently analyzed. Thus, tacrolimus was observed as highly effective in suppressing the proliferative response in murine and human MLR (mixed lymphocyte reaction). However, no effect was observed on constitutive cell proliferation or bone marrow colony formation, which presented FK506 as a selective agent inhibiting T-lymphocyte generation through lymphokine IL-2 [39,140]. Once the cellular activity was validated, its use as an immunosuppressant was tested in vivo in animals by means of renal allografts (38 beagles [141]) and humans (14 liver recipients [142]; 36 kidney transplant recipients [143]). Hence, the team of Thomas E. Starzl, specialized in human graft transplantation, stated in 1989, "*the seeming safety*, *efficacy*, *and relative freedom from side effects of FK 506 encourage further trials in kidney transplantation*" [143]. Although, in 1999, ten years after the introduction of tacrolimus into clinical medicine, more testing was still advisable, the comparative clinical data available for tacrolimus were better than for any of its predecessors, and the risk–benefit balance was in favor of tacrolimus [144].

All of these findings paved the way for FDA's final approval of tacrolimus for primary immunosuppression in adult and pediatric liver transplantation in 1994 (April 8th) under the request of Fujisawa Pharmaceutical Co., with the trade name of Prograft™ (tacrolimus capsules and tacrolimus for intravenous injection) (https://www.accessdata.fda.gov/drugsatfda_docs/nda/pre96/050708_prograf_toc.cfm; accessed on 21 July 2024). Since then, a clear transition from cyclosporine A to tacrolimus took place, as Meier-Kriesche and co-workers described in 2006 [51] and the Annual Data Report of the US Organ Procurement and Transplantation Network (OPTN) and the Scientific Registry of Transplant Recipients (SRTR) have validated over recent years (https://srtr.transplant.hrsa.gov/, accessed on 26 November 2024). Thus, they reported in 2022 that immunosuppression induction was utilized in 92.1% of adult kidney transplants (mainly in combination with corticosteroids, mycophenolate mofetil, or both), where 92.9% of these cases used tacrolimus for post-transplant treatment. Similar trends are observed in pancreas, liver, and lung transplants, where the use of other treatments is below 20% across all cases.

4.2. Clinical Applications of Tacrolimus: Pros and Cons

Once FDA approved tacrolimus as an immunosuppressant drug, new clinical possibilities were evaluated. Thus, in addition to its effect in the prevention of transplantation rejection of solid allogeneic organs such as kidney, liver or heart, and the treatment of allo-

graft rejection resistant to treatment with different immunosuppressive compounds [145], tacrolimus was also tested and subsequently used as a treatment of inflammatory skin diseases in a topical solution (e.g., atopic dermatitis [146]). Other autoimmune diseases like rheumatoid arthritis [147,148] or inflammatory bowel diseases (refractory ulcerative colitis, Crohn's disease) were also addressed by means of tacrolimus [149–152]. Tacrolimus was also considered as a coating substance in drug eluting stents due to the antimitotic, anti-inflammatory, and anticoagulant properties shown by some immunosuppressants in humans [153–155] and in animal models [156]. Furthermore, certain neuroregenerative properties were already reported in 1994 for tacrolimus, since it promoted in vitro sensory neurite outgrowth [157]. Thus, nerve regeneration and protection are activities where tacrolimus was attempted [158], and nowadays, it appears as a promising solution in surgical nerve repair [159–161]. Additionally, the treatment of glomerular diseases, especially refractory glomerular diseases, is frequently addressed by means of tacrolimus used off-label, as well as several dermatologic diseases. However, evidence is still needed to substantiate its off-label use [162,163].

In spite of its beneficial properties, calcineurin inhibitors, such as cyclosporine and tacrolimus, also produce undesirable effects at different levels, including (i) cardiovascular (e.g., angina pectoris, cardiac arrhythmias, hypertension), (ii) central nervous system (e.g., headaches, insomnia, tremors), (iii) gastrointestinal (e.g., abdominal pain, vomiting, diarrhea), (iv) immune system (e.g., post-transplant lymphoproliferative disorders), (v) infection (e.g., urinary tract infection, candidiasis, Epstein–Barr infection, herpes simplex, cytomegalovirus), etc. [164–166]. However, while both drugs share a similar side effect profile, the frequency of certain adverse effects varies. Tacrolimus is more commonly associated with alopecia, tremors, and new-onset diabetes mellitus, whereas cyclosporine is linked to hyperlipidemia, hypertrichosis, and gingival hyperplasia. Some studies suggest that tacrolimus may be less nephrotoxic than cyclosporine, although this conclusion remains controversial. Many investigations into renal injury are conducted in kidney transplant patients, making it challenging to differentiate between drug-induced nephrotoxicity and other factors contributing to renal dysfunction [167]. For instance, baseline and 5-year post-transplant kidney biopsies in pancreatic transplant recipients revealed comparable chronic nephrotoxic effects for both tacrolimus and cyclosporine [168]. Additionally, several concerns about fertility, pregnancy and lactation are also described in the tacrolimus leaflets, although some studies question these claims [169–171], but the precautionary principle must prevail, which recommends that "*it is better to not do something, or to do nothing, than to produce damage*".

Regarding the interaction of immunosuppressants with other drugs, infectious diseases top the scale of immunosuppression complications for solid organ transplantation, and subsequently, the infectious diagnosis is disturbed by the lack of signs and symptoms [172,173]. This fact jeopardizes patients, posing potential adverse drug reactions and prolonged hospitalization. Interactions of immunosuppressants with anti-infective agents can be classified into (i) pharmacokinetic, when drug–drug interactions result in altered concentrations of immunosuppressants, antibiotics or their by-products in stages of absorption, metabolism, distribution, or elimination, and (ii) pharmacodynamic, due to the increased or decreased toxicity and/or efficacy of each other [174,175].

A peculiar concern is the interaction of tacrolimus with plastic compounds. Drug adsorption and absorption (drug sorption) into the plastic tubes used to deliver tacrolimus result in unpredicted drug loss. The highest sorption effect of tacrolimus has been observed with PVC-based (polyvinyl chloride) tubes [176]. Thus, the medication guide of the injectable formulation of Prograft (FDA-Approved Drugs: https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm; accessed on 30 November 2024) shows that "*the diluted infusion solution should not be stored in a polyvinyl chloride (PVC) container due to decreased stability and the potential for extraction of phthalates*".

These side effects of tacrolimus are mainly the result of its own immunosuppressant capability, which decreases the immune response, but other factors also exacerbate this problem. Even when it sounds estrange, human errors in tacrolimus dosage (e.g., mix-ups

between drug strengths, various release formulations, look-alike medication names, etc.) have been described, and they could partially cause the undesirable effects [177].

5. Tacrolimus Metabolism: Mechanisms, Genetics and Biochemistry

In 2014, we highlighted the temporal gap between the initial description of the polyketide synthase (PKS) genes involved in tacrolimus biosynthesis by Motamedi and Shafiee's group, in the late 1990s [137,178–180], and the renewed interest in tacrolimus genetics around 2000–2010, mainly fuelled by the off-patent state at that time [181]. Throughout the following sections, the FK506 action mechanisms, biosynthetic gene cluster, its regulation and the drug biosynthesis are deeply analyzed. Furthermore, the metabolic pathways of immunosuppressant degradation by the human body have been partially revealed in recent years and are also presented in this section.

5.1. Action Mechanisms

Immunosuppressant compounds such as cyclosporine A and tacrolimus generally operate by inhibiting T-lymphocytes (Figure 2). Tacrolimus diffuses through the plasma membrane of T-cells into the cytoplasm, where it binds to the immunophilin FKBP12 (FK506-binding protein-12KDa), whereas cyclosporine A binds to cyclophilins. These binding events inhibit the activity of calcineurin [also known as PP2B (protein phosphatase-2B)], a calcium- and calmodulin-dependent serine/threonine phosphatase, preventing its access to substrates such as NFAT (nuclear factors of activated T-cells) family members (NFAT1, NFAT2, and NFAT4) and their dephosphorylation, which is important for regulating cellular signalling [106–108]. Once the cytoplasmic fraction of NFAT is dephosphorylated, it is translocated to the nucleus, forming an active transcription factor complex that regulates those genes related to B-cell activation, such as interleukin-4 (IL-4) and CD40 ligand, as well as genes involved in T-cell proliferation and differentiation, including interleukins 2 (IL-2), 3 (IL-3), 4 (IL-4), and 5 (IL-5) [182,183]. Hence, cytoplasmatic internalized tacrolimus inhibits calcineurin signalling, resulting in reduced IL-2 production. Additionally, calcineurin facilitates a secondary wave of IL-2 transcription via the transcription factor NF-κB, where calcineurin leads to IκB degradation, allowing NF-κB-mediated transcription of pro-inflammatory genes. Thus, inhibition of calcineurin by tacrolimus increases the fraction of NF-κB bound to IκB, thereby inhibiting the NF-κB-mediated transcription of pro-inflammatory genes [184,185] (Figure 2).

Both tacrolimus and cyclosporine A also exhibit pleiotropic effects on T-cells beyond calcineurin inhibition, influencing signalling pathways of MAPK, TGFβ and Toll-like receptors [184,185]. Some in vitro data suggest that tacrolimus may enhance the expression of TGFβ1 mRNA in T-cells, whereas cyclosporine promotes it in peripheral blood cells, though the underlying mechanisms remain unknown [184]. Similarly, the mechanisms controlling the Toll-like receptor (TLR) signalling pathways are also not fully understood. Even though TLRs, crucial protein molecules in nonspecific immunity associated with systemic autoimmunity, are inhibited by tacrolimus, reducing the inflammatory response and cellular injury in both liver and kidneys, the specific mechanisms remain unknown [185]. On the contrary, the MAPK pathway has been extensively studied. There are three types of MAPK pathways: (i) extracellular signal-regulated kinase (ERK), (ii) Jun N-terminal kinase (JNK1 or MAPK8, inhibited by cyclosporine A), and (iii) p38α (also referred to as MAPK14, inhibited by tacrolimus). These MAPKs are activated through signal cascades, where MAPK kinase kinase (MAPKKK) phosphorylates MAPK kinase (MAPKK), which then activates MAPK. Meanwhile, the JNK and p38 pathways are activated by T-cell responses involving TCR and CD28 co-stimulatory receptor, leading to the translocation of activated MAPKs into the nucleus to phosphorylate transcription factors such as activator protein 1 (AP-1). Activated AP-1, along with NFAT, controls the activation of molecules, such as the IL-2 gene. Both cyclosporine and tacrolimus block upstream of the MAPKKK cascade (e.g., MEKK1/MLK3/TAK1), which inhibits the p38 and JNK pathways without affecting ERK pathway activation [106,108,109,182,184] (Figure 2).

Interestingly, in addition to its immunosuppressive activity, tacrolimus has been identified as a nerve regenerator. Although the mechanism of action of tacrolimus on nerve regeneration is not fully elucidated, its positive effects on the regrowth of regenerating nerve fibers, which primarily target the injured neuron, are distinct from its immunosuppressive actions. One hypothesis suggests that tacrolimus binds to FKBP12, which functions as a TGF-β1 receptor inhibitor, activating the TGF-β1 pathway and stimulating NGF (nerve growth factor) synthesis in glial cells to promote nerve regeneration [182]. Another hypothesis suggests that tacrolimus acts through the FK506-binding protein (FKBP52), which forms heterocomplexes with the 90 kDa heat shock protein (Hsp90) and its co-chaperone p23 within the neural nucleus. FKBP52 plays a crucial role in guiding growth cones of regenerating neurites in response to both attractive and repulsive chemotactic signals. Following neuronal injury, this complex redistributes to the growth cones of regenerating neurites upon exposure to tacrolimus in vitro, promoting accelerated regeneration in vivo [160].

Numerous advances in understanding the mechanisms of action have been made over the past thirty years, and Wang and co-workers have recently compiled these findings in a comprehensive review [185].

5.2. Tacrolimus Gene Cluster and Its Regulation

Whereas tacrolimus was initially described as an immunosuppressant by Kino and co-workers in the late 1980s [39,40], the earliest investigations into the tacrolimus biosynthetic pathway did not begin until the 1990s, led by researchers of Merck [137,178–180]. The tacrolimus biosynthetic gene cluster is a complex set of genes that span approximately 83.5 kb which have been fully uncovered in several *Streptomyces* strains [including but not limited to *Streptomyces* sp. ATCC55098 (MA6858), *Streptomyces kanamyceticus* KCTC 9225, *Streptomyces* sp. KCTC 11604BP, *Streptomyces* sp. VKM Ac-2618D, *S. tsukubaensis* NRRL 18488 and *Streptomyces tacrolimicus* [62,186–190]]. Tacrolimus is synthesized through a hybrid polyketide I synthase–non-ribosomal peptide synthase (PKSI-NRPS) system encoded by the *fkb* cluster, which varies from 19 to 26 genes [189,191]. These clusters exist in two forms: (i) a **shorter version**, consisting of 19 genes, called *fkbQ*, *fkbN*, *fkbM*, *fkbD*, *fkbA*, *fkbP*, *fkbO*, *fkbB*, *fkbC*, *fkbL*, *fkbK*, *fkbJ*, *fkbI*, *fkbH*, *fkbG*, *allD*, *allR*, *allK*, and *allA* (found in *S. tacrolimicus* and *S. kanamyceticus* KCTC 9225), and (ii) an **extended version** found in *S. tsukubaensis* NRRL 18488, *S. tsukubaensis* L19, and *Streptomyces* sp. KCTC 11604BP, which includes five additional genes in the 5′ region of the *fkbG* gene (*allMNPOS*/*tcs12345*) and one or two extra genes (depending on the species) in the 5′ region of the *fkbQ* gene (*tcs6-fkbR*/*tcs67*). However, the deletion of the *allMNPOS* genes in *Streptomyces* sp. KCTC 11604BP does not significantly impact the tacrolimus titer, raising doubts about their involvement in tacrolimus biosynthesis [187,190,192].

The core genes involved in tacrolimus biosynthesis are *fkbA*, *fkbB* and *fkbC*, which encode a polyketide synthase (PKS) comprising 10 modules with a total of 51 domains. Other relevant genes are *fkbD* (C9 hydroxylase), *fkbL* (lysine cyclodeaminase), *fkbM* (31-O-methyltransferase), *fkbO* (chorismatase), *fkbP* (peptidylprolyl cis-trans isomerase) and *fkbQ* (thioesterase) [191,193,194] (see acting mode below) (Figure 3).

Three key regulatory genes have been reported within the *fkb* cluster: *fkbR*, *fkbN* and *allN* (belonging to the LAL, LysR, and AsnC families, respectively). First, the *fkbR* gene is only present in the extended *fkb* cluster and encodes a pathway-specific regulatory protein that enhances the transcription of genes involved in tacrolimus biosynthesis [190,195,196]. Indeed, the inactivation of *fkbR* decreases tacrolimus titer by 20% with reference to the parental strain [197]. On the other hand, *fkbN*, which is present in both the extended and short versions of the cluster, acts as a positive regulator by activating the transcription of most transcription units within the cluster, resulting in a 55% increase in tacrolimus titer when it is overexpressed [190,192]. In fact, the knockout of *fkbN* results in complete cessation of tacrolimus production, showing its essential role in the biosynthesis pathway [197]. Furthermore, the interplay between *fkbR* and *fkbN* is characterized by a complex network of self- and cross-regulation. *fkbR* is transcribed as a leaderless mRNA, suggesting a mechanism of self-regulation, whereas *fkbN* is part of

an operon with *tcs6* and *fkbQ*, and its transcription is controlled by both FkbN-dependent and independent promoters [198]. Interestingly, the *fkbR* gene targets the *fkbN* gene, creating a regulatory loop that coordinates the expression of these biosynthetic genes [192,196]. Furthermore, studies have reported the existence of potential genes outside the *fkb* cluster that may be subject to the regulatory influence of FkbN. These genes include *ppt1*, which encodes a 4′-phosphopantetheinyl transferase, along with genes associated with acyl-CoA dehydrogenase and methoxymalonate biosynthesis pathways [198]. The last regulatory gene present in the *fkb* cluster is *allN*, which is commonly associated with the cluster, but its precise role in tacrolimus biosynthesis regulation remains to be fully elucidated [191,192,195]. Indeed, some studies have not found a correlation between *allN* expression and tacrolimus titer in *S. tsukubaensis* [196,197].

Other regulatory systems influencing the tacrolimus cluster operate beyond its immediate biosynthetic framework. Thus, tacrolimus productivity correlates with a decline in reactive oxygen species (ROS) and an increase in catalase activity, suggesting a role for genes like *katA1* and *katA2* in this process [195,199]. This reduction in oxidative stress is pivotal for tacrolimus pathway activation, particularly in strains with compromised oxidative stress response mechanisms [195,200].

Salehi-Najafabadi and co-workers described in 2014 the *bul* region, which includes several genes involved in the control and biosynthesis of the gamma-butyrolactone autoregulator molecules [201]. The region includes the genes *bulR1* (γ-butyrolactone receptor homologue) and *bulS2* (gamma-butyrolactone synthetase homologue), whose deletion leads to a significant decrease in tacrolimus titer [201,202]. In the same way, BulZ was identified as a *Streptomyces* antibiotic regulatory protein (SARP) family regulator by Ma and co-workers [202], and its deletion resulted in a 47.5% decrease in tacrolimus titer. Furthermore, co-overexpression of *bulZ* and *bulS2* improved tacrolimus biosynthesis yields by 36% compared to the control strain, reaching 324 mg/L [202].

Moreover, fermentation raw materials impact tacrolimus biosynthesis. Thus, the presence of N-acetylglucosamine (GlcNAc) affects both morphological differentiation and secondary metabolism, while carbon catabolite repression can hamper secondary metabolite production in *Streptomyces* [188,191,203,204]. Overarching these environmental and nutritional cues is the global regulator Crp (cAMP receptor protein), which significantly influences the expression of tacrolimus biosynthetic genes. Crp overexpression enhances tacrolimus titer and modulates the expression of regulatory genes like *fkbN* and *allN*, alongside genes involved in primary nitrogen metabolism. This means a role for Crp in coordinating primary and secondary metabolism [191,193,205]. Although the exact mechanisms underlying the regulatory effects of the Crp regulator are not fully elucidated, the conservation of similar regulatory mechanisms, as observed with the Crp-like regulator GlxR in *Corynebacterium glutamicum*, suggests a broad regulatory role for Crp across bacterial species [191,193].

Finally, there is a long history of research into the effect of inorganic phosphate (Pi) on the production of secondary metabolites in the *Streptomyces* genus, a topic that Prof. Juan F. Martín's team (Universidad de León and INBIOTEC) has studied for more than twenty years. Under Pi starvation, a two-component system named PhoR-PhoP is activated, enabling the cellular response to adapt to these dramatic circumstances, trying to obtain phosphate from different sources and by means of diverse mechanisms (e.g., alkaline phosphatases, polyphosphatase, low- and high-affinity transporters, etc.) [181,206,207]. Furthermore, a cross-regulation between phosphate and nitrogen pathways has been also observed though PhoP, which is the central regulator of phosphate response and represses nitrogen metabolism at two levels: (i) controlling the expression of GlnR, the main nitrogen regulator, and (ii) through the expression of genes involved in ammonium assimilation (*glnA*, *glnII*, and *amtB-glnK-glnD* operon) [207,208]. Although the genetic response against phosphate depletion is analogous in different *Streptomyces* species, the fine-tuning is species-specific. Thus, PhoP directly controls the production of secondary metabolites in some species and by an indirect pattern in others [209,210]. Thus, high phosphate concentrations (>30 mM) results in a drastic inhibitory effect on tacrolimus yield in *S. tsukubaensis*, whereas phosphate-limiting conditions (2.5 mM)

improve tacrolimus titer and generate a dark green pigment. Furthermore, a cross-regulation between lysine and phosphate has also been described due to hydrogen bond formation (N^+H – O-P), which arrests phosphate and decreases its cellular availability [211,212]. Nowadays, the regulatory pathway that controls the binding of PhoP to the PHO regulatory boxes in *S. tsukubaensis* has been fully revealed [210,213].

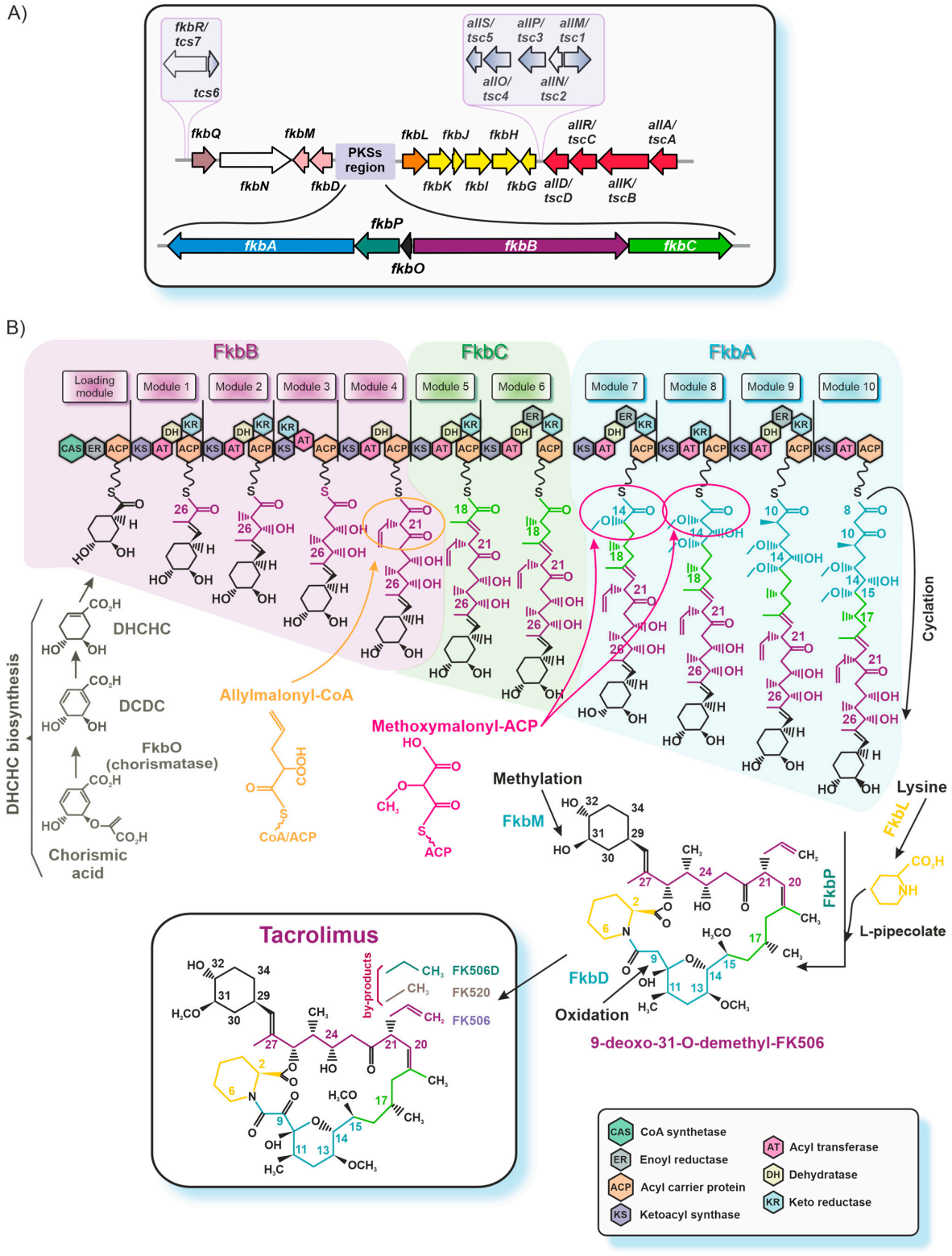

Figure 3. Gene cluster and biosynthetic pathway of tacrolimus (FK506). (**A**) Gene arrangement scheme of the biosynthetic cluster of tacrolimus. Gene colors define the different activities of the genes of the

cluster. The central "PKS region" collects (i) the multifunctional FK506 polyketide synthase genes *fkbA* (cyan), *fkbB* (purple), and *fkbC* (light green); (ii) the gene responsible for the starter unit biosynthesis, *fkbO* (black); and (iii) the NPRS gene *fkbP* (dark green), which forms the macrolactone ring and release tacrolimus from the enzyme complex. Other subcluster genes are labelled as follows: (i) red, *tcsABCD/allAKRD* genes involved in allylmalonyl-CoA biosynthesis; (ii) yellow, *fkbGHIJK* genes involved in methoxymalonyl-ACP biosynthesis; (iii) orange, *fkbL* gene leading the pipecolate biosynthesis; (iv) pink, *fkbDM* genes involved in post-PKS modifications; (v) white, regulation-related genes (*tcs2/allN*, *fkbN* and *tcs7/fkbR*); (vi) dark brown, the thioesterase gene *fkbQ*; (vii) grey, genes with unknown function (*tcs1345/allMPOS* and *tcs6*). Light purple boxes represent a species-dependent feature, highlighting those genes just observed in the largest version of the tacrolimus biosynthesis cluster. (**B**) Tacrolimus biosynthetic pathway. Modules of the PKSs (bottom-right panel) include the following domains: CAS, CoA synthetase; ER, enoyl reductase; ACP, acyl carrier protein; KS, ketoacyl synthase; AT, acyl transferase; DH, dehydratase; KR, keto reductase. Bottom square presents the final structure of FK506 and two by-products of tacrolimus biosynthesis [ascomycin (KF520) or 37,38-dihydro-FK506 (FK506D)] (based on Huang and co-workers [214], Barreiro and Martínez-Castro [181], Ban and co-workers [215]).

5.3. Tacrolimus Biosynthetic Pathway

Tacrolimus biosynthesis in *S. tsukubaensis* is a complex process involving a series of reactions catalyzed by the enzymes encoded within the tacrolimus gene cluster (*fkb*) that guide the assembly of the final macrolide molecule from various precursors (Figure 3) [191,196,214].

The biosynthesis begins with the formation of the starter unit, (4R, 5R)-4,5-dihydroxycyclohex-1-enecarboxylic acid (DHCHC). Previously, it was suggested that DHCHC was derived from the shikimate pathway, although more recent studies showed that the chorismatase encoded by the gene *fkbO* (as well as its homolog *rapK* from the rapamycin gene cluster) is involved in the biosynthesis of DHCHC through the hydrolysis of chorismic acid [215–217] (Figure 3).

Then, the PKSs FkbA–FkbB–FkbC initiate a series of chain elongation cycles on DHCHC, facilitated by its ten extender units: two malonyl-CoAs, five methylmalonyl-CoAs, two methoxymalonyl-ACPs, and one allylmalonyl-CoA/ACP. This demonstrates that the use of allylmalonyl-CoA/ACP distinguishes tacrolimus from ascomycin, which uses ethylmalonyl-CoA instead [186,192]. Notably, the modular PKS structure consists of three multi-enzymes, with each module typically containing essential domains: β-ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP). Specifically, FkbB incorporates a loading module and four extension modules (with the fourth being in charge of transferring an allylmalonyl unit to the ACP domain), while FkbC contains the subsequent two modules responsible for continued chain elongation. The final four modules in FkbA are then used to complete the biosynthesis of the linear polyketide chain [215]. The introduction of the latter two extender units leads to the formation of the methoxy group at C13 and C15, and the allyl radical at C21. Furthermore, the *fkbGHIJK* sub-cluster plays a pivotal role in tacrolimus synthesis, as it encodes relevant enzymes for the biosynthesis of methoxymalonyl-ACP from 1,3-biphosphoglycerate [192] (Figure 3).

After the assembly of the polyketide chain, the NRPS enzyme encoded by *fkbP* incorporates L-pipecolate (a L-lysine derivative generated by FkbL) into the molecule to form the macrolide ring structure of tacrolimus [187,218,219]. The L-pipecolate molecule is detached from the PKS by the thioesterase FkbQ [178]. Following the PKS process, a series of post-modifications (including hydroxylations, methylations, and oxidations) are mandatory for the biological activity of tacrolimus [181,215,218,220]. One of the key enzymes in charge of this process is a cytochrome, P450 hydroxylase, encoded by *fkbD*, which catalyzes C9 oxidation. Another essential modification for tacrolimus activity is the methylation of the 31-OH group, which is carried out by S-adenosylmethionine (SAM)-dependent methyltransferase, encoded by *fkbM*. The existence of both pathways has presented it as two parallel post-PKS

events, leading to FK506 production [198]. Thus, the deactivation of *fkbD* and *fkbM* genes results in the accumulation of the biosynthetic intermediates 9-deoxo-31-O-demethyl-FK506 and 31-O-demethyl-FK506, respectively [137,220,221] (Figure 3).

5.4. Metabolism of Tacrolimus in the Human Body: The Complexity of Dosing

Tacrolimus, when used as an immunosuppressant drug, is mainly administered in oral formulation (capsules, tablets, oral suspension) (Table 2). Its intestinal absorption is limited, with most of the drug being eliminated via feces. Additionally, approximately 99% of the absorbed tacrolimus is bound to erythrocytes, with the remaining 1% entering the lymphatic system, where it exerts therapeutic effects [184]. Furthermore, tacrolimus is a highly lipophilic compound, and it is very susceptible to hydrolysis [222]. Thus, new application forms are being developed, aimed at achieving a more homogeneous distribution of tacrolimus in formulations, ensuring a physical stability to the compound, such as nanostructured lipid carriers (NLCs). This development of nanoparticles represents a significant methodological breakthrough [223].

Table 2. Summary of current tacrolimus formulations and providers obtained from the list of FDA-approved drugs (https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm; accessed on 30 November 2024). NDA (New Drug Application) code indicates those compounds approved by FDA as new pharmaceuticals for sale and marketing in the US. ANDA (Abbreviated New Drug Application) code presents those compounds submitted to FDA for review and potential approval as a generic drug product. The drug name of these generic compounds is "Tacrolimus".

Administration	Company	Drug Name	NDA/ANDA Code	Strength
Extended Release (Oral Capsule)	Astellas Pharma Inc. (Tokyo, Japan)	Astagraf XL	204096	EQ 0.5/1.0/5.0 mg base
	Chengdu Suncadia Medicine Co., Ltd. (Sichuan, China)	Tacrolimus	215012	EQ 0.5/1.0/5.0 mg base
Extended Release (Oral Tablet)	Veloxis Pharmaceuticals, Inc. (Cary, NC, USA)	Envarsus XR	206406	EQ 0.75/1.0/4.0 mg base
Oral Capsule	Astellas Pharma Inc. (Tokyo, Japan)	Prograf	050708	EQ 0.5/1.0/5.0 mg base
	Sandoz GmbH (Basel, Switzerland)	Tacrolimus	065461	EQ 0.5/1.0/5.0 mg base
	Dr Reddys Labs Ltd. (Hyderabad, India)	Tacrolimus	090509	EQ 0.5/1.0/5.0 mg base
	Mylan (part of Viatris, Canonsburg, PA, USA)	Tacrolimus	090596	EQ 0.5/1.0/5.0 mg base
	Strides Pharma (Bangalore, India)	Tacrolimus	090687	EQ 0.5/1.0/5.0 mg base
	Panacea Biotec Limited (New Delhi, India)	Tacrolimus	090802	EQ 0.5/1.0/5.0 mg base
	Accord Healthcare (Middlesex, UK)	Tacrolimus	091195	EQ 0.5/1.0/5.0 mg base
	Alkem Labs Ltd. (Mumbai, India)	Tacrolimus	203740	EQ 0.5/1.0/5.0 mg base
	Belcher Pharmaceuticals, LLC (Largo, FL, USA)	Tacrolimus	206651	EQ 0.5/1.0/5.0 mg base

Table 2. *Cont.*

Administration	Company	Drug Name	NDA/ANDA Code	Strength
Oral Capsule	Glenmark Pharms Ltd. (Mumbai, India)	Tacrolimus	206662	EQ 0.5/1.0/5.0 mg base
	Hangzhou Zhongmei (Hangzhou, China)	Tacrolimus	210929	EQ 0.5/1.0/5.0 mg base
	Biocon Pharma (Bangalore, Karnataka, India)	Tacrolimus	212297	EQ 0.5/1.0/5.0 mg base
	Concord Biotech Ltd. (Ahmedabad, India)	Tacrolimus	213112	EQ 0.5/1.0/5.0 mg base
Injectable (Injection)	Astellas Pharma Inc. (Tokyo, Japan)	Prograf	050709	EQ 5.0 mg base/mL
Oral Suspension	Astellas Pharma Inc. (Tokyo, Japan)	Prograf	210115	EQ 0.2/1.0 mg base/Packet
Topical (Ointment)	Leo Pharma As (Ballerup, Denmark)	Protopic	050777	0.03%/0.1%
	Fougera Pharms Inc. (Melville, NY, USA)	Tacrolimus	200744	0.03%/0.1%
	Glenmark Pharms Ltd. (Mumbai, India)	Tacrolimus	210393	0.03%/0.1%
	Accord Healthcare (Middlesex, UK)	Tacrolimus	211688	0.03%/0.1%
	Encube (Mumbai, India)	Tacrolimus	212387	0.1%

Once tacrolimus is in the human body, extensive metabolism takes place in the intestinal mucosa and liver cells, primarily through O-demethylation, hydroxylation, and/or oxidative reactions. Initially, O-demethylation destabilizes the macrolide ring of tacrolimus, leading to the formation of secondary and tertiary metabolites [224]. This results in an increase in the half-life from 12 h to 15 h, with less than 0.5% of tacrolimus being excreted unchanged in urine or feces. Hence, around 95% of tacrolimus metabolites are eliminated through bile, while 2.4% are excreted via the urinary system [184,224].

Human metabolism of tacrolimus is mainly dependent on two cellular processes. First, the **CYP3A** enzyme system, which includes CYP3A5, CYP3A4 (also referred to as cytochrome P450 3A4), CYP3A7 and CYP3A43, plays a crucial role. This enzymatic complex is mainly present in the small intestine, liver, and kidneys. However, compared to CYP3A5, the catalytic efficiency of CYP3A4 is relatively low, whereas CYP3A7 has little influence on the metabolism of tacrolimus and the role of CYP3A43 remains unclear. The second metabolic process involves the activity of **P-glycoprotein**, a protein pump that transports tacrolimus out of cells. P-glycoprotein is found in the liver, as well as in barriers such as the blood–brain barrier, placenta, and intestinal epithelium [184]. For instance, some tacrolimus metabolites formed in mucosa may return to the intestinal lumen via P-glycoprotein transport. In the kidney, P-glycoprotein may contribute to renal elimination, whereas on the canalicular surface of hepatocytes, it controls excretion into bile [225].

However, tacrolimus metabolism and dosing are conditioned by, in some way, the patients' genetic polymorphisms, which results in great inter-individual variability. As an example, some polymorphisms in CYP3A4 are responsible for the clearance of tacrolimus and, consequently, the dose of tacrolimus needed for effective treatment [184]. In the same way, polymorphisms in the CYP3A5 gene may explain up to 50% of variability in tacrolimus dose requirement [226]. Thus, CYP3A5*1 is the most important functional variant of the CYP3A5 gene, and is highly dependent on ethnicity, being present in only a

minority of Caucasians (5–15%), Asians (15–35%) and Mexicans (25%), but it is present in the majority of people of African descent (45–73%) [226]. Furthermore, the genetic variant of the cytochrome P450 CYP3A5*3 means that almost 80% of Caucasians are weak tacrolimus metabolizers and need lower doses when compared to extensive metabolizers [227]. In addition, but to a lesser extent, the genetic variants of the P-glycoprotein pump (MDR-1), which modulates its bioavailability, and a second cytochrome P450 (named Cyp3A4), related to tacrolimus metabolism, seem to play a role in the complexity of tacrolimus dosing as well [228–231]. This genetic variability allows patients to be categorized into groups of fast, intermediate and slow metabolizers, for whom tacrolimus doses range from 20% to 60% [230].

Additionally, the metabolism of tacrolimus in the human body itself may be responsible for its well-known side effects. Regarding nephrotoxicity, ABCB1, the gene encoding P-glycoprotein in renal tubules, may limit the local accumulation of tacrolimus and its metabolites in kidney by facilitating their excretion into urine. Thus, lower ABCB1 expression in the kidney may be associated with increased risk of chronic kidney damage caused by tacrolimus, although the reported results remain inconsistent. Furthermore, cytochrome P450 CYP3A5 may have different interplay with ABCB1 in vascular and tubule-interstitial compartments of the kidney [225]. On the other hand, neurotoxic effects of FK506 include tremor, headache, insomnia, and peripheral neuropathy. Although the exact pathophysiology of tacrolimus-induced neurotoxicity is unclear, it has been suggested that the CYP3A5*1 allele could increase the risk of neurotoxicity, probably due to tacrolimus secondary metabolites [226].

The deviations among patients of immunosuppressive therapies due to their drug resistance is a serious clinical concern as a result of the divergent cellular pharmacodynamics of each individual. Hence, the use of peripheral lymphocytes derived from each patient to simultaneously test a battery of different immunosuppressive drugs in a quantitative assay (immunobiograms) is a key approach to achieve individualized and more precise therapies [232,233]. Furthermore, fast monitoring methods for whole blood determination of tacrolimus and cyclosporine A have been developed recently by using HPLC-MS/MS [234].

6. Tacrolimus Production and Yield Improvement

The yield enhancement of a clinically valuable API (Active Pharmaceutical Ingredient) is imperative for the development of a competitive manufacturing process, and tacrolimus is no exception. Thus, FK506 is a peculiar compound from the industrial point of view, and several reasons, which act as bottlenecks in the scale-up process, support this statement. First of all, the selection of the **workhorse strain** is a tricky matter, since the *Streptomyces* strains described as "potential" producers total more than eighteen [181]. This fact presents the *fkb* cluster as one of the most promiscuous in the *Streptomyces* genus. Thus, the horizontal transfer [235] of this cluster has been proposed as a reasonable explanation [236,237]. However, there may be different reasons to question this statement, such as (i) a poor patent description of the strains; (ii) unreliable taxonomical analyses, since just a few strains are properly classified (e.g., the poor tacrolimus producer *S. tacrolimicus* [189] or *Streptomyces durmitorensis* [238]); (iii) the obstacles among culture collections to deposit a type strain in at least two publicly recognized ones (e.g., *S. tsukubaensis* [181,212,237]).

Second are the traditionally **low tacrolimus titers**, with the best producers reaching 972 mg/L by a random mutagenized strain (*Streptomyces* sp. TST10) or up to 1500 mg/L by means of a Plackett–Burman Design analysis (see below) [239,240].

Finally is the **downstream process** (DSP), due to (i) the tacrolimus chemical characteristics (e.g., hydrophobicity) that stick the compound to the external surface of *Streptomyces* biomass, and (ii) the natural production process, which yields similar compounds in activity and structure, but just differing in some chemical groups, such as ascomycin (KF520) or 37,38-dihydro-FK506 (FK506D) [181,192]. In this regard, ascomycin production can account for 8% of tacrolimus production in *Streptomyces clavuligerus* KCTC 10561BP [241] and even reach 20% in other *Streptomyces* strains [242]. The presence of these by-products

in the culture broths, and tacrolimus chemical structure, complicates subsequent extraction and purification. Consequently, several DSP approaches have been developed, typically involving sequential steps of extraction with organic solvents, resins, and chromatographic separation steps, thereby increasing production costs [181,241]. Some of these DSP strategies are discussed in the following paragraphs.

6.1. Nutritional Improvements and Classical Mutagenesis: Establishing an Enhancement Foundation

The first work on tacrolimus production improvement was focused on optimizing fermentation conditions. This involved the formulation of fermentation media and feeding or removal of specific raw materials (e.g., carbon or nitrogen sources, precursors, stressing compounds, among others) to determine their stimulating or inhibitory effects on both the growth of the microorganism and tacrolimus productivity.

The first medium for the growth of *Streptomyces* sp. ATCC 55098 was formulated by Yoon and Choi in 1997 [211]. Years later, Martínez-Castro and co-workers [212] reported two additional media (e.g., MGm-2.5 and ISPz) for *S. tsukubaensis* fermentation. Then, the exploration of different carbon and nitrogen sources played a crucial role in enhancing the tacrolimus titer of several strains. On the one hand, while some studies reported the negative impact of easily digestible carbon sources such as glucose and glycerol [243], the role of glucose in the regulation of tacrolimus biosynthesis remains controversial, and feeding glucose, starch, or corn dextrin increased tacrolimus titer in *S. tsukubaensis* NRRL 18488 [212]. Nonetheless, the differences in media composition, as well as the specific growth phase during glucose supplementation, might account for such discrepancies in the results. In the same way, the use of soya oil in combination with soybean meal and L-lysine increased tacrolimus titer by about 1.73-fold in an isolate of *Streptomyces* sp. [205]. On the other hand, changes in the nitrogen source also resulted in improved tacrolimus productivity. For example, the addition of several amino acids, including lysine, cysteine, leucine, glutamic acid or ornithine, enhanced tacrolimus productivity of *Streptomyces* sp. MA6858 [211,244], while the addition of ammonium sulphate at optimal concentrations (2 g/L) improved titer in *S. tsukubaensis* NRRL 18488 [212].

Indeed, many of these compounds act as precursors in the synthesis of tacrolimus. As an example, L-lysine is the precursor of L-pipecolic acid, which closes the macrolide ring of tacrolimus [181]. Furthermore, certain amino acids, such as proline, leucine, isoleucine, threonine or valine, induce a significant increase in tacrolimus precursors (e.g., acetyl-CoA and methylmalonyl-CoA), ultimately leading to the stimulation of tacrolimus productivity [245,246]. Therefore, feeding tacrolimus precursors has been tackled as a realistic option for enhancing yields. The enrichment of the fermentation medium with precursors such as picolinic acid (pyridine-2-carboxylic acid), pipecolic acid (piperidine-2-carboxilic acid) and, to a lesser extent, methyl oleate, has shown a notable increase in tacrolimus titer in *S. tsukubaensis*, ranging from 3- to 7-fold [193,219]. Similarly, feeding methyl oleate to the culture medium enhanced tacrolimus biosynthesis 2.5-fold in *S. clavuligerus* CKD1119 [247]. Furthermore, three-carbon compounds such as propylene glycol, propanol or propionic acid may act as precursors of the macrolide structure and promote the growth of *S. tsukubaensis*, increasing tacrolimus titer by 1.8–5.5-fold [248]. However, it is interesting to note that (i) the effect of a precursor depends on its concentration, (ii) the combination of two compounds showing a positive effect does not always result in a synergistic effect, and (iii) a positive effect can be exerted through both growth promotion and/or productivity stimulation [192].

Similarly, growth stimulators like nicotinic acid (pyridine-3-carboxilic acid) and nicotinamide (pyridine-3-carboxilic acid amide) may stimulate NAD/NADP biosynthesis, resulting in a modest increase in tacrolimus titer [181,219]. Furthermore, stressing agents like DMSO or sodium thiosulfate have been demonstrated to slightly stimulate polyketide productivity in different bacteria, as well as tacrolimus in *Streptomyces* strains [192,240,249]. Furthermore, the addition of Tween 80, as a surfactant agent, promotes the homogeneity of raw materials, allowing for the uniform distribution of oxygen and other components,

leading to a significant increase in tacrolimus titer [250]. As much as 3% of Tween 80 was needed to maximize tacrolimus productivity in *S. tsukubaensis* [240]. Finally, the use of adsorption resins (e.g., Diaion HP-20, Amberlite XAD-4, Amberlite XAD-7H, or Amberlite XAD-16) in the fermentation medium also increases tacrolimus titer. These resins adsorb tacrolimus and its derivatives, reducing cell hydrophobic substances (such as cell membrane or wall components) and leading to better solubilization and secretion [186].

The Plackett–Burman Design allows for the swift and efficient determination of the most significant parameters in a metabolite titer among numerous nutrients, additives or conditions. Recently, 19 different raw materials, including starch, glucose, peptone, peanut oil, glycine, or L-lysine, among others, were analysed. Three of them (ammonium sulphate, yeast extract, and 1,2-propylene glycol) exerted the most significant effect on tacrolimus titer in *S. tsukubaensis* NBRC 108819. This optimization process resulted in a 3-fold increase in tacrolimus productivity, reaching more than 600 mg/L at both flask and fermenter scales [251]. Similar findings were reported by Yan and co-workers [240], who analyzed a set of nine raw materials, highlighting the importance of soluble starch, peptone, and Tween 80 in improving tacrolimus productivity in the mutant strain *S. tsukubaensis* FIM-16-06, whose titer was approximately 3.7 times higher than that using the standard medium, improving from around 400 mg/L to over 1500 mg/L at the fermenter scale.

Nevertheless, the use of additives is not always an efficient strategy from an economic standpoint, since some of these compounds are expensive (e.g., shikimate, chorismate or pipecolate) [192]. Thus, alternative strategies have emerged, primarily focusing on mutagenesis and screening of tacrolimus-producing strains. Traditionally, mutations were induced through random methods [e.g., UV irradiation or N-methyl-N-nitro-N′-nitrosoguanidine (NTG)]. For example, UV irradiation has paved the way for the development of strains able to use specific raw materials to achieve a cost-effective production of tacrolimus. Such is the case of the strain *Streptomyces* sp. P5C3 FERM BP 0927, which uses soybean oil as the only carbon source [252]. Furthermore, both processes are not mutually exclusive, and most of the strains selected through mutagenesis have been analyzed under different nutritional conditions to find those ensuring the highest production yield. For instance, optimization of the fermentation parameters of *S. clavuligerus* CKD 1119 lipase-overproducing mutants generated by UV irradiation led to a 100-fold increase in tacrolimus titer compared to the wild-type strain, demonstrating a close relationship between lipase biosynthesis and tacrolimus production [253]. Furthermore, the combination of chemical and physical mutagens (dual mutation) may further enhance tacrolimus titer even higher than a single mutagenesis treatment. Thus, Singh and co-workers reported a significant improvement of tacrolimus titer (from 10.5 mg/L in the control to 82.5 mg/L) in *S. tacrolimicus* ATCC55098 when mutagenesis was developed with UV, NTG and ethyl methanesulfonate (EMS) in a two-step process [254].

An interesting strategy involves the development of precursors- and product-tolerant mutant strains. The sequential adaptation of a producer strain to increased concentrations of tacrolimus, or its precursors, allows the selection of mutants which can tolerate higher concentrations of these biomolecules, potentially increasing their tacrolimus titer. An example is the strain *Streptomyces* sp. TST10 (a strain developed from *Streptomyces* sp. TST8), which is able to produce up to 972 mg/L of tacrolimus after 7 days of fermentation [239]. Interestingly, also *S. tsukubaensis* TJ-01, modified by different concentrations of disodium methylmalonate or disodium malonate, and then subjected to UV and NTG mutagenesis, led to the development of the strain TJ-P325, which exhibits genetic stability and the ability to get a final titer of more than 500 mg/L at fermentation scale [255].

6.2. Genetic Engeneering Tools: Settling Down the Basis for Improvement

Microbial wild-type strains are traditionally recalcitrant to genetic transformation and, subsequently, to genome manipulation, mainly due to their poor physiologic and genetic characterization and their active DNA repair systems. Prior to the genome sequencing era and CRISPR (clustered regularly interspaced short palindromic repeats)

technology, the main advance in gene replacement in *Streptomyces* was REDIRECT technology (RED-based PCR targeting) [256], which needed long DNA fragments (>10 kb) flanking the target region to allow a fruitful double recombination. Cosmids were a suitable solution to achieve stable extrachromosomal genetic material (±40–45 kb), allowing conjugation and later gene replacement as a genetic tool. Thus, an initial attempt to ease the REDIRECT approach emerged with the development of a pyramidally ordered cosmid library of different tacrolimus producer strains as a tool to enable a dual and efficient screening approach by means of PCR and/or in situ colony hybridization [257]. This process eased detection and mutation of the γ-butyrolactone receptor genes in the tacrolimus producer strains (i) *S. tacrolimicus* (formerly *Streptomyces* sp. ATCC 55098 [189]) (gene *gbr*) [258] and (ii) *S. tsukubaensis* (genes *bulR1* and *bulR2*) [201], aimed at increasing immunosuppressant productivity.

The first genome sequence of *S. tsukubaensis* was published by Barreiro and co-workers in 2012 [62]. Nowadays, there are nine entries, corresponding to eight genomes sequenced and validly deposited in the NCBI database (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=83656, accessed on 17 October 2024) [(i) NRRL 18488 (*twice*: INBIOTEC plus Universidad de León (Spain) [62] and KAIST); (ii) AT3 (Shandong First Medical University); (iii) L20 (Zhejiang university); (iv) VKM Ac-2618 (FRC Pushchino Center for Biological Research of the Russian Academy of Sciences) [259]; (v) F601 (Shandong Academy of Medical Sciences) [260]; (vi) NPDC093257 (The Herbert Wertheim UF Scripps Institute for Biomedical Innovation and Technology); (vii) NPDC002422 (The Herbert Wertheim UF Scripps Institute for Biomedical Innovation and Technology); (viii) NPDC008303 The Herbert Wertheim UF Scripps Institute for Biomedical Innovation and Technology)]. Once the first genome was released, the genetic engineering approaches were improved in relation to FK506 production. Undoubtedly, the RNA-guided DNA editing technology CRISPR/Cas9 [261–263] has been the most significant development in genome editing, including for the *Streptomyces* genus. It allows double-stranded breaks into genomes, which eases the subsequent site-specific replacement of genetic material insertions/deletions. As a result, the expended time in one round of genome modification is decreased by one-third or one-half of the traditional methods, with high efficiencies (45–54%) [264]. Thus, different applications have been carried out through the CRISPR/Cas9 procedures in *Streptomyces*, such as discovery and characterization of biosynthetic gene clusters [265] or generation of FK506 analogues by the modification of different modules of the polyketide synthases of the *fkb* cluster, as occurred in *S. tsukubaensis* T857 (derived from *S. tsukubaensis* NRRL 18488) [266]. However, the CRISPR method does not always work properly in industrial strains of *Streptomyces* (e.g., *Streptomyces chattanoogensis* L10, *S. tsukubaensis* YN06, *Streptomyces albus* ZD11). Then, parallel deletion systems independent of episomal vectors have been recently obtained for the deletion of large DNA fragments (10 kb to 200 kb), which decrease by 25% the time needed to achieve the mutation when compared to the traditional procedures [267].

In addition to efficient genome editing systems, fast-screening procedures of the resultant mutants are needed to ease the analyses. Thus, the finding of different *Saccharomyces cerevisiae* mutant strains (e.g., TB23) sensitive to cyclosporin and hypersensitive to FK506 and FK520 allowed a friendly mutant-based screening process [268]. This fact connects the antimicrobial activity against yeast and pathogenic fungi (e.g., *Cryptococcus neoformans*) of some immunosuppressants (e.g., tacrolimus, rapamycin) with the affected genes (cyclophilin A and FKBPl2 genes) of the *Saccharomyces* strains used as reporters, since the immunosuppressants could be considered as part of the arrays of compounds generated by bacteria to inhibit the growth of competing yeast and fungi [269]. More recently, a yeast cell-based strategy (based on *S. cerevisiae* BY4741 and its calcineurin mutant strain *cnb1*Δ) has been devised again for the swift screening of *S. tsukubaensis* producer strains. These bioassay methods decrease the time-consuming fermentation process, HPLC measurements, and extensive incubation space, while significantly increasing screening throughput [270].

6.3. Synthetic Biology and Omics: Redirecting the Metabolism

To deepen the understanding of tacrolimus production, researchers have sequenced, partially or fully, the FK506 biosynthetic gene cluster from several *Streptomyces* strains, including *Streptomyces* sp. MA6548 (ATCC 53770) [178], *Streptomyces* sp. KCTC 11604BP, or *S. kanamyceticus* KCTC 9225 [190], among others. Traditionally, one of the most effective strategies for advancing the study of individualized natural products involved heterologous expression of the biosynthetic genes in a tractable host organism or microbial chassis. Thus, aiming to enable the expression of the tacrolimus biosynthetic cluster in a microbial platform, Jones and co-workers [271] devised the BAC vector pAC20N, carrying the complete tacrolimus biosynthetic cluster, along with additional flanking regions of about 8.5 kb and 17.5 kb, respectively. As a result, this heterologous expression of the tacrolimus biosynthetic cluster in model strains of *Streptomyces coelicolor* shed light on the significance of *fkbN* and *fkbR* as regulators of tacrolimus [271].

In the same way, sequencing and comparative analysis of the biosynthetic clusters from different *Streptomyces* strains that produce either tacrolimus or ascomycin revealed specific genes responsible for supplying the critical extender units needed for both compounds. For instance, the inactivation of the *allR* genes (e.g., homologous to crotonyl-CoA carboxylase/reductase encoded in the ascomycin biosynthetic cluster in *S. tsukubaensis*) inactivates the production of both tacrolimus and ascomycin. However, tacrolimus production can be re-established without by-product synthesis when allylmalonyl-S-N-acetylcysteamine precursor is added to the fermentation broth [241]. At the heart of this process lies the *allR/tscC* gene, a pivotal player that bridges both the allylmalonyl-CoA and ethylmalonyl-CoA biosynthetic pathways [181].

The application of omics technologies has simplified the systematic design of nutrient supply strategies, thereby contributing to the enhanced yields of tacrolimus achieved nowadays. As a secondary metabolite, tacrolimus biosynthesis and its regulation happen through a complex process, so every comprehensive understanding of the biological mechanisms governing tacrolimus overproduction is always welcomed. Hence, the release of the first draft genome sequence of *S. tsukubaensis* (NRRL 18488) in 2012 [62] (see above) opened the door to further genomic studies. As an imaginative example, Wu and co-workers identified three PKS/PKS-NRPS gene clusters in the genome of *S. tsukubaensis* L19, able to compete for common acyl precursors, such as malonyl-CoA. By deleting the genes encoding core PKS in these three clusters, they increased FK506 production from 140.3 mg/mL to 170.3 mg/mL at 168 h, which means a 21.4% improvement [246].

Transcriptomic [243] and **proteomic** [195] approaches have also shed light on how environmental factors and nutrient availability affect tacrolimus biosynthesis. One standout example is the addition of N-acetylglucosamine, which stimulates the transcription of genes responsible for tacrolimus and other polyketides in *S. tsukubaensis* NRRL 18488 [203]. Another interesting example is how stress adaptation consequences play a pivotal role in orchestrating the metabolic shift from primary to secondary metabolism in *Streptomyces* strains. In the case of tacrolimus biosynthesis, the redox-based signalling network enhances the availability of tacrolimus precursors, thereby increasing the overall product yields [195].

On the other hand, **metabolomics** has played a crucial role in unravelling the metabolic pathways that drive tacrolimus production, identifying key precursors along the way. Hence, metabolomic analyses revealed several pathways in *S. tsukubaensis* closely linked to tacrolimus biosynthesis, including TCA cycle, pentose phosphate pathway, shikimate, and amino acid metabolism, all of which contribute essential intermediates for production [218,245]. In fact, although metabolites such as pyruvate, lactate, or valine play crucial regulatory roles in tacrolimus biosynthesis, two critical metabolites (methylmalonyl-CoA and shikimate) were pinpointed as the major limiting factors due to their roles as extender and initiator molecules, respectively [245]. These results highlight the relevance of some nutrients indirectly tied to tacrolimus biosynthesis, making them potential targets for industrial optimization. In the same way, Wang and co-workers [272], with metabolomic techniques, explored how well-known additives such as DMSO and sodium butyrate

influence tacrolimus yields. Their work identified thirteen distinct metabolic modules and sixteen hub metabolites associated with these stimulatory effects. The analyses spanned central carbon, amino acid, and fatty acid metabolism, offering valuable insights for further boosting tacrolimus titers. Moreover, a deeper understanding of these metabolic pathways could minimize the production of unwanted by-products, like ascomycin and 37,38-dihydro-FK506, simplifying downstream processes [245].

Therefore, this relentless progress in the omics field has opened a wealth of opportunities, highlighting key metabolic pathways for advancements in the **genetic engineering** of tacrolimus production [186,246,273,274]. In fact, several studies have successfully combined genetic engineering with nutritional improvements, leading to significant increases in tacrolimus yields. First, the increase in the copy number of tacrolimus biosynthetic genes, as well as the overexpression (*accA2*, *aroH*, *pntAB*, *zwf2*) or deletion (*ghdA*, *ppc*) of primary metabolism-involved genes, combined with an appropriate supplementation (pipecolate, lactate, succinate, shikimate, etc.), have boosted from 10 to 70% the tacrolimus titer of *S. tsukubaensis* D852 compared to the wild type [275]. Similarly, the shikimate pathway is nowadays a well-known metabolic pathway involved in tacrolimus production. The combined overexpression of shikimate kinase and dehydroquinic acid synthetase encoding genes led to a 33.1% enhancement of tacrolimus titer in *S. tsukubaensis* NRRL 18488, whereas the knockout of the D-lactate dehydrogenase gene, combined with the overexpression of tryptophan synthase and aspartate 1-decarboxylase genes, led to a 29.8% increase [276]. These genetic modifications aim to enhance the precursor molecules of tacrolimus [246], such as shikimate [276], methylmalonyl-CoA [247] chorismate or lysine [218], in parallel with the decrease in unnecessary secondary metabolites that consume these precursors (such as lactate) [276].

6.4. Downstream Process to Pure Tacrolimus: A Real Headache

From the very beginning of tacrolimus discovery and production, several structurally related compounds have been isolated from *S. tsukubaensis* fermentation broths, such as methyl (FR900425), ethyl (FR900520), and proline (FR900525) analogues [39,277]. The co-production of tacrolimus and structural analogues shows an impurity profile quite similar in different fermentation broths, with ascomycin (FK520) and 37,38-dihydro-FK506 (FK506D) being the main by-products [244,278]. In fact, the initial manuscript by Kino and co-workers in 1987 describing the fermentation parameters, chemical characteristics and isolation of FK506 [40] already illustrated the complex process needed to the obtain the final "pure prisms of white powder". However, according to the US Pharmacopeia, tacrolimus content should be not less than 98%, with unidentified impurities limited to no more than 0.1%. As for the concentrations of tacrolimus analogues, they have been set at no more than 0.5% for ascomycin and 0.15% for tacrolimus 8-propyl analogue (also referred to as 37,38-dihydro-FK506 or FK506D) [279]. This stringent regulatory framework elucidates the rationale behind the attempt at tacrolimus chemical synthesis in the 1990s, a pursuit that was promptly abandoned due to its diminished effectiveness and expensive costs [280,281]. Thus, (i) the development of genetically stable high-titer tacrolimus strains accumulating a low proportion of tacrolimus analogues and (ii) fermentation titer improvement, including the modification of culture parameters and raw materials, have been the main strategies in the industry to address the analogues' drawbacks.

Thus, the tacrolimus DSPs developed involve the extraction of the fermentation broth with organic solvents and the use of resins and chromatographic separation steps to achieve the desired purity. Depending on the quantity and type of tacrolimus by-products, the DSP must be adapted to achieve a reasonable yield and keep the production costs under control at the industrial scale. Due to the structural similarity of tacrolimus and its analogues, its purification process by conventional crystallization methods is not an option, and more expensive methods based on adsorption to resin bound to silver ion and purification by preparative HPLC were developed [282,283]. Alternative solutions, based

on crystallization and extraction under reduced pressure or using different adsorption resins, were also developed [284,285].

Considering that most of the tacrolimus is accumulated intracellularly and bound to the biomass, some industrial-scale processes include (i) filtration of the fermentation broth, (ii) extraction of the biomass with an organic solvent (e.g., acetone or methanol), (iii) binding tacrolimus and by-products to non-ionic adsorbent resins (e.g., HP-20), (iv) evaporation of the purified solution to an oil, (v) purification by silica gel normal phase chromatography, (vi) purification by C18 reverse phase chromatography containing silver ions, (vii) tacrolimus crystallization by solvent exchange, and finally, (viii) filtration and drying.

7. Future Trends: Analogues

Similar to tacrolimus, ascomycin is a potent calcium-dependent serine/threonine protein phosphatase inhibitor. Pharmacological investigations of ascomycin have delineated its immunosuppressant properties, therapeutic efficacy in inflammatory diseases, and anticonvulsant activity [286]. Ascomycin, rapamycin, and tacrolimus share a similar structure that includes a tricyclic skeleton (crucial for the FKBP binding domain) conferring their biological activities [186,271]. However, these biological activities may vary among them, demonstrating how subtle biochemical changes in the composition of a biomolecule can influence its functional effect. As an illustrative case, a tacrolimus analogue produced by *Bacillus amyloliquefaciens* HSSN09 demonstrated antifungal activity against *Fusarium oxysporum* f. sp. *niveum*, the causative agent of watermelon *Fusarium* wilt [287]. Thus, the exploration of new analogues of tacrolimus with improved activity, new biological actions, reduced toxicity, or increased production yields remains a focal point of numerous investigations [215]. Initially, significant chemical modifications were made to various functional groups of FK506, leading to the creation of numerous tacrolimus analogues and the identification of functional moieties that could be altered without compromising its immunosuppressive activity [288].

However, specific chemical modification of these molecules is often impractical due to their structural complexity. Thus, a breakthrough came with the development of analogues containing altered side chains or non-natural starter units through mutasynthesis. In this process, the gene in charge of producing a natural building block is disrupted, allowing for the effective incorporation of modified precursors, which results in the substitution of building blocks from the original molecule [215]. Precursor selectivity for starter and extender units can be predicted from modular acyltransferase domains, which perform gatekeeping functions. Substrate tolerance of the acyltransferase domain, as well as promiscuity of downstream enzymes, furthermore, permit certain flexibility in the biosynthesis route [289]. Therefore, several analogues with modified tricyclic skeletons and polyketide backbones have been synthesized, such as 36,37-dihydro-37-methyl-FK506, 36-methyl-FK506, and 36-fluoro-FK520, which were obtained by feeding the diverse non-natural extender units trans-2-hexenoic acid, 4-methylpentanoic acid and 4-fluorocrotonic acid, respectively, to the cultures of a mutant strain of the tacrolimus producer *Streptomyces* sp. KCTC 11604BP, where the *tcsB* gene was inactivated [190]. The inactivation of the *tcsB* gene, responsible for the biosynthesis of a novel allylmalonyl PKS extender unit, resulted in the more efficient incorporation of non-natural extender units in the absence of competition from the natural extender unit [190]. Interestingly, among these products, 36-methyl-FK506 displayed an improved neurite outgrowth activity in comparison with tacrolimus molecule [190,215].

In the same way, the addition of non-native starter units (specifically 3-cyclohexene1-carboxylic acid) to an *fkbO*-deleted mutant of *Streptomyces* sp. KCTC 11604BP resulted in the biosynthesis of an FK506 analogue. In this case, the inactivation of *fkbO* (the gene in charge of the biosynthesis of the natural starter unit, DHCHC) allowed more efficient incorporation of non-natural starter units, as there was no competition by the natural starter unit. As a result, a new compound called 32-dehydroxy-FK506 was generated, exhibiting similar in vitro neurite outgrowth activity to FK506, while in vitro immunosuppressive activity decreased [215]. In a related study, the strain *Streptomyces* sp. GT110507,

derived from the parental strain *Streptomyces* sp. GT11005 through *fkbO* deletion, when fed with trans-4-hydroxycyclohexanecarboxylic acid and 3-hydroxybenzoic acid, resulted in the biosynthesis of two compounds, 31-desmethoxy-FK506 and TC225, both of which demonstrated enhanced antifungal activity [186].

Among the numerous molecules developed, a few have stood out significantly, demonstrating their clinical utility. Notable examples include (i) temsirolimus (CCI-779, FDA-approved as Torisel®; Wyeth Pharmaceuticals, Inc., Madison, NJ, USA) (https://www.accessdata.fda.gov/drugsatfda_docs/nda/2007/022088s000TOC.cfm; accessed on 3 August 2024), (ii) everolimus (https://www.accessdata.fda.gov/drugsatfda_docs/nda/2009/022334s000TOC.cfm; accessed on 3 August 2024) and (iii) pimecrolimus (Elidel™, SDZ-ASM 981, approved by FDA for the treatment of mild to moderate atopic dermatitis) (https://www.accessdata.fda.gov/drugsatfda_docs/nda/2001/21-302_elidel.cfm; accessed on 3 August 2024), which have shown promising activities as immunosuppressants, anticancer agents, and neuroregenerative compounds (Figure 1) [186]. **Temsirolimus** (Figure 1) is a prodrug of sirolimus, marketed as Rapamune®® (Wyeth Pharmaceuticals, Inc., Madison, NJ, USA) for the prophylaxis of organ rejection after renal transplant. Approved by FDA in October 2006 [290], temsirolimus, like sirolimus, is an inhibitor of the mammalian target of rapamycin (mTOR), an enzyme that regulates cell growth and proliferation, preventing progression from the G1 to S phase of the cell cycle against a variety of human tumor types, such as advanced renal cell carcinoma. Similarly, **everolimus** (Figure 1), also an mTOR inhibitor, has been the subject of extensive clinical investigation since 1996. Initially developed to prevent organ transplant rejection, it has proven to be both effective and safe in treating various cancers as well as treating tuberous sclerosis complex in both adults and children. The extensive scientific evidence gathered from in vitro and in vivo studies laid the foundation for a comprehensive clinical development program, which has led to multiple FDA-approved indications since 2009, including renal cell carcinoma (approved in 2009), progressive neuroendocrine tumors of pancreatic origin (approved in 2010), and certain types of breast cancer (approved in 2012), among others [291]. **Pimecrolimus**, approved by FDA in 2001 as a non-steroidal alternative for treating mild to moderate atopic dermatitis, is a calcineurin inhibitor applied topically as a cream. It functions by blocking the production of inflammatory cytokines, particularly by inhibiting calcineurin, thus preventing T-cell activation. Pimecrolimus has shown particular efficacy in reducing the symptoms of atopic dermatitis, with a lower risk of systemic immunosuppression compared to oral treatments [292].

Sometimes, structural modifications can impact the immunosuppressive capacity of the biomolecule, while enhancing other biologically relevant activities. Hence, numerous studies have explored the synthesis of different analogues demonstrating antifungal activity against a broad spectrum of fungi (e.g., *Cryptococcus neoformans*, *Candida albicans*, and *Aspergillus fumigatus*), albeit in most cases at the expense of immunosuppressive activity [293–295]. Recent breakthroughs, including CRISPR editing, have empowered the creation of analogues tailored for a particular purpose. In the realm of tacrolimus treatment, it has come to light that the immunosuppressive activity through calcineurin binding might pose a risk for patients. Exploring analogues devoid of methoxy groups at positions C15 [266] and C21 [296] has led to analogues lacking calcineurin binding activity while retaining BMP (bone morphogenetic proteins) potentiation. In the same way, separating tacrolimus immunosuppressive activity from its neurotrophic activity is imperative to develop analogues for the treatment of neuronal diseases, as reducing immunosuppression can avoid unwanted side effects. Jung and co-workers [297] reported that the proline substitution in 9-deoxo-36,37-dihydroFK506 and 9-deoxo-31-O-demethyl-36,37-dihydroFK506 significantly reduced immunosuppressive activity by over 120-fold, while preserving nearly identical neurite outgrowth activity and enhancing synaptic transmission strength.

8. Tacrolimus Market

According to the United Network for Organ Sharing (UNOS; https://unos.org/, accessed on 13 June 2024), the U.S. recorded a record-breaking 42,887 organ transplant

procedures in 2022, with over 14,000 deceased organ donors contributing to more than 36,400 transplants. These figures underline the global improvements in organ donation and transplantation, showing a promising trajectory for the tacrolimus market. The increasing incidence of autoimmune disorders, coupled with R&D activities to develop more effective drugs, is expected to become a key contributor pushing the growth path of the market. Thus, autoimmune diseases affect up to 8% of the U.S. population, making them among the most prevalent disorders in the country, and showing a rising demand for advanced solutions in immunosuppression. Furthermore, new immunosuppressive agents have been developed, including the JAK inhibitor tofacitinib and the mTOR inhibitor everolimus, showing promising therapeutic effects in clinical use compared to existing drugs. These new compounds are designed to be more selective and have fewer side effects. This growing prevalence of autoimmune diseases underscores the necessity for such developments, leading to an expanding market in this field.

The recent COVID-19 pandemic had a significant negative impact on the growth of the tacrolimus market, since most of the surgeries and transplantations were either postponed or delayed, reducing the overall adoption rate of tacrolimus medications worldwide. However, the global market sales of tacrolimus increased by 4% in 2023 (USD 2796 M) compared to 2022 (USD 2692 M), showing the following distribution by region in 2023: (i) EU (USD 859 M), (ii) US (USD 516 M), (iii) Latin America (USD 30 M) and (iv) the rest of the world (USD 1391 M).

The consumption of tacrolimus increased by 7% in 2023 (2063 Kg) compared to 2022 (1927 Kg), being shared in 2023 among (i) the EU (619 Kg), (ii) US (500 Kg), (iii) Latin America (21 Kg) and (iv) the rest of the world (922 Kg) (Cortellis Generics Intelligence https://clarivate.com/products/biopharma/generics-and-manufacturing/generics-intelligence-analytics/, accessed on 13 June 2024).

Regarding global tacrolimus market trends, different data providers [GMI (Global Market Insights: https://www.gminsights.com/toc/detail/tacrolimus-market, accessed on 3 December 2024); GVR (Grand View Research: https://www.grandviewresearch.com/industry-analysis/tacrolimus-market-report, accessed on 3 December 2024); Coherent Market Insights (https://www.coherentmarketinsights.com/market-insight/tacrolimus-market-2298, accessed on 17 October 2024)] project a CAGR of 5.2% from 2024 to 2029, estimating Asia–Pacific to grow at the highest CAGR over this period, and with North America accounting for the largest market share in 2024. Another prediction anticipates a market growth from USD 6801.35 million in 2024 to USD 9683.26 million by 2032, exhibiting a CAGR of 4.5% during the forecast period. This market is segmented into dermatitis, immunosuppression, and other applications. The immunosuppression segment is poised to cross USD 6.4 billion by 2032. The product types that can be found in the market include injections, tablets, capsules, ointments and granules.

Bearing in mind that tacrolimus is considered a generic API (Active Pharmaceutical Ingredients) due to its curent off-patent state, the main manufacturers are located in China (9), India (3), the Czech Republic (1), Hungary (1), Italy (1) and all other countries (3). The tacrolimus market is fragmented in nature due to the presence of several companies operating globally as well as regionally. Some of the prominent players within the market include Astellas Pharma Inc., Dr. Reddy's Laboratories Limited, GlaxoSmithKline plc, Glenmark Pharmaceuticals Inc., Leo Pharma A/S, Lupin Pharmaceuticals Inc., Novartis AG, Panacea Biotec, and Pfizer Inc., among others (https://www.mordorintelligence.com/industry-reports/tacrolimus-market, accessed on 13 June 2024).

Finally, the regulatory filings could provide hints about the future market trends. This mainly includes (i) Drug Master File (DMF), which consist of a submission to FDA providing confidential information about facilities, processes, or equipment for tacrolimus manufacturing, and (ii) Certification of Suitability (COS/CEP), which certifies compliance of the pharmaceutical ingredients with that of the rules laid down in the monograph of the European Pharmacopoeia (EP); the manufacturer provides evidence that the quality of the substance is controlled by the monographs of the EP and is granted by the Certification

Secretariat of the European Directorate for the Quality of Medicines (EDQM). Nowadays, in the case of tacrolimus, twenty-three US active DMFs, nine EU COS/CEP, ten Korean registered DMFs, eight Japanese registered DMFs, and twelve Chinese active DMFs can be found, which indicate the current interest in this API.

9. Concluding Remarks

Immunosuppressants have paved the way for the current long-term success in the graft transplants in addition to other clinical, methodological and legal achievements. Despite the uncomfortable side effects of these drugs, their absence would be fatal for many patients. So, the continuous search for new compounds or variants of the existing ones is still a challenge. Thus, the sequential discovery of new immunosuppressant compounds from the 1950s has allowed the study of their action modes, development of novel derivatives (analogues), or fine-tuning of the proper dosing. However, there is room for more improvements. Hence, focusing specifically on tacrolimus, several aspects will be the focus of research in the coming years to enhance its clinical use and to expand its existing application portfolio, which can be summarized as follows: (i) traditional improvement of the manufacturing processes (e.g., strain selection and modification, fermentation and DSP improvement, etc.) to yield more than 2.0–2.5 g/L; (ii) enhancement of tacrolimus absorption by means of new formulations (e.g., encapsulation) to decrease the required dose of patients, lowering the environmental release; (iii) better understanding of its metabolism connected to the patients' genetic profiles (pharmacogenomics) to ease the application of personalized medicine in the prevention of organ transplant rejection; (iv) deeper analysis of the new clinical applications, which are currently being defined (e.g., neuroregeneration) or are being carried out off-label; and (v) generation of new analogues with reduced side effects and boosting new uses, which can transform this current generic API into a repurposed drug.

Thus, tacrolimus/FK506, on the 40th anniversary of its discovery, has a long history of developments from industrial to clinical points of view, and presents promising applications in novel therapies due to its recently discovered effects or as a base for analogues with newly revealed applications.

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