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The Continuous Story of the Miraculous Drug, Rapamycin

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Abstract: *Being of high potency and importance, rapamycin attracts continuous attention for further investigations and studies. Rapamycin exhibits various biological activities especially as a potent immunosuppressant. Also it exerts antitumor, antiviral, anti-inflammatory, antiproliferative, antiangiogenic, antifibrotic, anticancer, lifespan extension and antiaging, neuroprotective and neuroregenerative activities, in addition to its role in organs transplantation. Rapamycin is characterized also by causing no nephrotoxic effect. This review aim to understand the importance of rapamycin, its chemistry, production, biosynthesis, and biosynthetic gene cluster. In addition to highlighting its current status in drug market.*

Keywords: *Rapamycin, Streptomyces hygroscopicus, polyketides, production, biosynthesis, gene cluster.*

1. Introduction

Polyketides are diverse class of secondary metabolites that exert a wide range of biological activities such as antimicrobial, antitumor, antiviral, immune-suppressing, anti-inflammatory, and hypocholesterolemic activity **[1-5]**. Actinomycetes are known as famous natural producers of polyketides, especially the genus *Streptomyces*. Examples of some polyketides produced by this genus are rapamycin, daunorubicin, caprazamycin, oleandomycin, and actinorhodin. [**6-12**].

Rapamycin (Rap), also named (sirolimus), is a macrolidepolyketide that has been discovered in 1975 [**13-15**], as an antifungal compound produced by *Streptomyces hygroscopicus* with strong activity against *Candida spp*. rap shows stronger antifungal action when compared with amphotericin B in murine systemic candidiasis [**16**].

Since its discovery, Rap has exhibited a list of biological activities such as antifungal, antitumor, antiviral [**17**], anti-inflammatory [**18**], antiproliferative activity[**19**], antiangiogenic and antifibrotic properties [**20**], anticancer, lifespan extension and antiaging action [**21**], neuroprotective [**22**] and neuroregenerative activities [**23**]. In addition to be characterized by causing no nephrotoxic properties, rap has been proven as a potent immunosuppressant with activity 150 times greater than that of cyclosporine A in addition to a remarkable lower cytotoxicity. Moreover, there are some trials to use Rap intreatment of acute myeloid leukemia [**24**], retinal and choroidal vascular diseases, preventing early development of diabetic nephropathy in rats [**25**], participate in healing and closure of bladder and abdominal wounds **[26]**, besides its role in organs transplantation [**27**].Two approvals were recorded for rap from the American food and drug administration (FDA). Preventing host rejection in kidney transplantation was the first one (August 1999), while the second one was in 2003 for preventing restenosis of coronary arteries following angioplasty. **Awla et al., [28] reported that** rapamycin has promising agricultural applications as antifungal activity.

Due to the potency and known importance and potential applications of rap, this review aims to discuss the chemistry, production, biosynthesis, and mode of action of rapamycin. In addition, the current status of rapamycin in drug market was highlighted.

2. Chemistry of Rap

The structure of Rap (Fig 1) was determined by combination of X-ray crystallography and 1H and 13C NMR data [29]. It consists of very large (31-membered) lactone ring with characteristic α-

ketonicgroup. The large ring (macrocyclic) is commonly known as macrolide ring. Macrolide ring of Rap is biosynthesized by condensation of acetate and propionate molecules and so-called "polyketide ring'. The backbone of the ring has three conjugated double bonds allowing Rap to be classified as triene compound. The 31-membered ring includes the sole nitrogen atom presented in the antibiotic and so considered a heterocyclic ring. Also, the Rap structure contains six membered hemiketal ring (C-10 to C-14) that is characterizing the isomer B of Rap. Outside the macrolide ring is a trisubstituted cyclohexane ring (C-37 to C-42). [29].

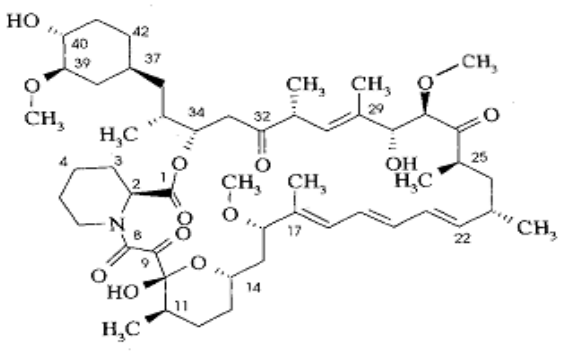

Fig1. *Structure of Rap (the predominant isomer B).*

3. PHYSIOLOGY OF RAP PRODUCTION

While majority of conducted studies focused on investigating the biological activities of Rap, inadequate number of studies concentrated on production of Rap [**30**-**32]**.

Streptomyces is famous for being the major antibiotic-producing genus in the field of microbiology till now **[33].** Members of the genus *Streptomyces* are aerobic, Gram positive soil saprophytes with extensive branching substrate and aerial mycelia [**34**]. **Watve*et al.* [33]**engaged themselves with evaluating the capacity of this genus for producing bioactive compounds and they succeeded to establish a mathematical model that estimated the total number of antimicrobial compounds that this genus is capable of producing to be of the order of a 100,000 – a tiny fraction of which has been discovered so far. **[35]**, basing on detailed literature survey and on studying various databases, approximated the total number of bioactive microbial metabolites

that are already discovered to be 22500 of which 7630 were derived from *Streptomyces* i.e., from only the genus *Streptomyces*, one third of all microbial bioactive compounds are produced. Of these bioactive metabolites produced by *Streptomyces* there were 6550 antibiotics (having antibacterial, antifungal, antiprotozoal, antitumor and antiviral activities) and 1080 specified as bioactive metabolites having no antibiotic activity. Out of all *Streptomyces* species, **Tanaka and Omura [36]** published a survey showing that the *Streptomyceshygroscopicus* (*S. hygroscopicus*) and *Streptomycesgriseus* strains are superior to other actinomycete strains in their ability to produce large numbers and varieties of bioactive metabolites. As such, **Hayakawa et al. [34]** developed a selective isolation technique for targeting actinomycetes belonging to the *Streptomyces violaceusniger* phenotypic cluster which contained *Streptomyceshygroscopicus* with three other species. Antibiotics formed by the *Streptomyces hygroscopicus*group belong to different chemical classes e.g.,aminoglycosides, peptides, polyenic and non-polyenic macrolides, polyethers**[37]**. The strain *Streptomyces hygroscopicus* ATCC 29253 (NRRL 5491; AY B-994; DSM 41530; IMET 43975) was thefirst reported Rap producer. Its morphological characteristics were assigned as an aerial mycelium that is monopodially branched; sporophores are terminated spore chain in the form of short, narrow, compact and closed coils (spirals) of three or more turns; ten or more spore are present in each spiral [**13**]. Two another strains of *S. hygroscopicus* (strain AY B 1206 and C9) were also employed by **Kojima et al.[30]** in Rap production. Rap has been also produced by the strain *S. hygroscopicus* FC904 that was isolated from Fuzhou, China [**38**]. Out of the remarked predominance of *S. hygroscopicus* in Rap production, **Nishida et al. [39]** reported the unique investigation on the isolate *Actinoplanes* sp. N902-109 that was recovered from soil sample in Shizuoka Prefecture, Japan and was recorded as novel Rap producer.

Rap production was usually conducted in shaken flask cultures using *Streptomyces hygroscopicus* as a producer strain in chemically defined medium [**30**], or in media composed of natural components

[**14**]. In the chemically defined media, fructose and mannose were the best combination of two carbon sources whereas acetate and propionate, even being a building unites in Rap biosynthesis, failed to support the growth, rather than Rap biosynthesis, of the tested strain of *Streptomyces hygroscopicus*[**30**]. The study of **Cheng et al. [40]** pointed out to the existence of phosphorus, magnesium and nitrogen-negative regulation mechanisms for Rap biosynthesis. Addition of amino acids to chemically defined medium containing aspartate, arginine and histidine to support good growth was investigated where it was found that amino acids interfered in Rap production by different manners; a 150% increase in productivity was achieved upon addition of L-Lysine, propably due to its conversion to pipecolic acid, while some other amino acids, like methionine and phenylalanine, caused remarkable negative effect on productivity. Rare studies about production physiology in complex media have been previously reported. Of this was the finding that shikimic acid failed to increase Rap titer in complex medium [**41**]. In a recent study, the influence of stressing-out the rap producer strain *Streptomyces hygroscopicus* ATCC 29253 was investigated [**42**]. Promising results were reported when rap production was performed in a medium containing 1% NaCl which resulted in 56.4% increase in the rap production yield. Rap yield increased to 129.7 % when production was conducted using 1.5-fold concentrated production medium. Moreover, production under fluctuated incubation temperatures achieved 132% increase in rap production. On the other hand, investigating the effect of nano-sized soymeal, which is a key nutrient important for rap production, resulted in minor enhancement in production yield especially during using size 89 nm. On the contrary, repeated inoculation of the production medium with the producer strain, co-culturing the yeast *Candida albicans* with the producer strain, and addition of camel milk to the production medium have been proven to be non-effective. [**42**].

4. BIOSYNTHESIS OF RAP

Biosynthesisof Rap has been reviewed by **Reynolds and Demain [43]**. The precursors are acetate, propionate, shikimate, L-methionine, and L-lysine. Rap biosynthesis starts with a cyclohexane moiety (derived from shikimate) to which seven acetate and seven propionate units then participate to build up a polyketide backbone in a head-to-tail fashion. Finally, pipecolate (synthesized from L-lysine) attaches to the polyketide chain, followed by ring closure via lactone formation. Three methyl groups are transferred from methionine via S-adenolsyl methionine to form the three methoxy groups [**44**].

5. BIOSYNTHETIC GENE CLUSTER INVOLVED IN RAP BIOSYNTHESIS

The biosynthetic gene cluster of rapamycin has been published for the first time in 1995 [45], from the producer strain Streptomyces rapamycinicus NRRL 5491, this sequence has been deposited in GeneBank data base under the accession number X86780. Huang et al. [46] had cloned the biosynthetic gene cluster of rap produced by Streptomyces hygroscopicus using a probe designed based upon the polyketide synthase-encoding sequence of erythromycin biosynthetic cluster [46]. The biosynthetic gene cluster of rap produced by Streptomyces hygroscopicus ATCC29253 is illustrated in Fig. (1), and the function of each gene is elucidated in table (1). The polyketide synthase (PKS) genes are rapB, A, and C and NRPS-like gene rapP. The genes rapO, N, M, L, K, J, I, H, and G were found downstream of the PKS genes, and are responsible for precursor synthesis, regulation, and tailoring of macrolactone [46].

Analyzing the gene cluster of rap contributed in understanding the role of each gene in rap biosynthesis and regulation [47-49]. Furthermore, knowing such information has contributed in successful construction of many bioactive analogs of rapamycin using genetic manipulation and precursor-directed biosynthesis [32, 50, 51].

Table1.*Rapamycin biosynthetic genes in Streptomyces hygroscopicus*

Gene	Size (aa)	Putative function
rapA	8563	Polyketide synthase (PKS)
rapB	10223	Polyketide synthase (PKS)
rapC	6020	Polyketide synthase (PKS)
rapP	1541	NRPS
orfD	387	Unknown
orfE	465	Unknown
orfF	453	Transporter
rapJ	386	Modification

rapL	345	Precursor synthesis
rapO	77	Modification
rapQ	317	Modification
rapM	317	Encoding a methyltransferase
rapN	404	Modification
rapH	872	regulation
rapG	330	Regulation
rapI	260	Modification
rapK	334	Starter unit biosynthesis

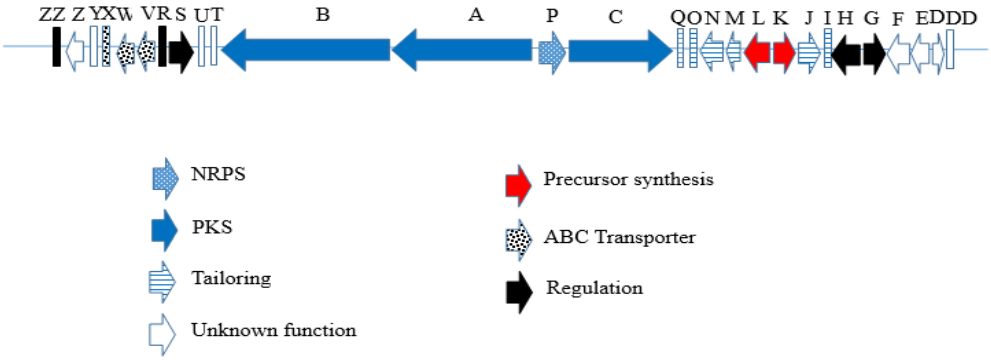

Fig2. *Organization of the rapamycin biosynthetic gene cluster*

6. Rap Action Mechanism

Investigating for the molecular target of rapamycin resulted in the identification of its target mTOR (mammalian Target of Rapamycin) and hence clarification of its action mechanism **[52]**. The mode of action of rapamycin starts by binding to FK506 Binding Protein 12, followed by forming a complex (rapamycin–FKBP complex) which interacts with mTOR and results in blocking of the cell-cycle progression of T-cells, which consequentially suppress proliferation of T-cell induced by cross-linking of T-cell receptors, antigenic peptides, or cytokines such as interleukins [**16**].

7. Current Status of Rapamycin in Drug Market

Rap price is high due to the insufficient microbial production of Rap [42]. The price of oral tablets of sirolimus (concentration of 0.5 mg) is about $226 /30 tablets, which is so expensive compared with other generic drugs [53]. Rapamycin is produced commercially under many trade names such as Rapamune®™ is released in markets in 1999 and come in two different formulations: Oral solution, and coated tablets (Fig. 3 a,b). Sirolimus® 1mg and 2mg (Fig. 3 c), Rapamycine® (Fig. 3 d).

Other derivatives of rapamycin such as everolimus; temsirolimus; and zotarolimus under the trade names Certican®, Afinitor®, Xience V®, Endeavor®, and Torisel®. These drugs were released in markets starting from 2004, and are used as anticancer, immunosuppressant, and stent coating drug [46].

Fig3. *Some Rapamycin products (a).Rapamune 1mgwww.indiamart.com ; (b). Rapamune 2mg www.drugsdepot.com; (c). Sirolimus 1mg and 2mg www.drugstorenews.com ; (d). Rapamycinewww.Longlong life.org*

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