ACTIVITY OF RAPAMYCIN (AY-22,989) AGAINST TRANSPLANTED TUMORS

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(Received for publication June 18, 1984)

Rapamycin exhibits activity against several ascites and solid transplantable tumors; it is slightly active to inactive against leukemias. On a weight basis, rapamycin was less active than 5-fluorouracil, cyclophosphamide and adriamycin, but rapamycin's maximal activity against Colon 38 tumor was similar to that of 5-fluorouracil and cyclophosphamide. Its activity was such that it significantly inhibited tumor growth at any stage of development. In the active dose range, rapamycin appeared less toxic than the other drugs. In the Colon 38 tumor model, rapamycin at a given dose exhibited the same activity when administered ip, iv, im and sc; upon oral administration, its activity was reduced but not abolished. Rapamycin was compatible with 5-fluorouracil and cyclophosphamide. The sequential treatment 5-fluorouracil-rapamycin-cyclophosphamide was superior to the sequence 5-fluorouracil-adriamycin-cyclophosphamide in protecting Colon 38 tumor-bearing mice.

29-Demethoxyrapamycin exerted only marginal activity against P388 lymphocytic leukemia; it was inactive against B16 melanocarcinoma and Colon 38 solid tumor.

Rapamycin is a triene antibiotic produced by *Streptomyces hygroscopicus*[1,2]. Structure elucidation revealed the presence of a pipecolic acid residue in the macrolide[3,4]. Several yeasts, as well as yeast-like and filamentous fungi, are sensitive to rapamycin; however, the main feature of the antibiotic is its high activity against *Candida albicans* (MIC 0.02~0.20 μg/ml)[5]. Nucleic acids synthesis inhibition and degradation in *C. albicans* are the primary modes of action[6]. The LD_{50} ip of rapamycin in the mouse is 587 mg/kg. The antibiotic also has immuno-suppressant activity[7]: it is half as potent as cyclophosphamide in inhibiting experimental allergic encephalomyelitis and is as potent as this standard reference drug in preventing adjuvant-induced arthritis. The mode of action and the pharmacological effects warranted the evaluation of rapamycin in experimental tumor models. The National Cancer Institute (NCI, Division of Cancer Treatment) conducted the initial studies and reported modest activity against P388 lymphocytic leukemia (Increased life span (ILS)=30~40% at 1.25 mg/kg) and no activity against L1210 lymphoid leukemia and Lewis lung carcinoma[8]. Activity was reported against B16 melanocarcinoma (ILS 80% at 100 mg/kg), Colon 26 tumor (ILS 105% at 6.25 mg/kg) and EM ependymoblastoma (ILS 85% at 50 mg/kg and ILS 100% at 200 mg/kg). Rapamycin was also active against the solid tumors, CD8F1 mammary tumor (80% tumor weight inhibition at 25 mg/kg) and Colon 38 tumor (85% tumor weight inhibition at 25 mg/kg). Subrenal capsule CX-1 colon adenocarcinoma xenograft and spontaneous colon adenocarcinoma 11/A were sensitive to rapamycin.

We report here a more detailed evaluation of the efficacy of rapamycin in transplantable tumor models. The effects of dosage, regimen and route of administration were studied. Rapamycin was compared to other antitumor agents; the antineoplastic effects of rapamycin in combination were also evaluated.

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Materials and Methods

Drugs

Pure crystalline rapamycin and 29-demethoxyrapamycin (AY-24,668) were prepared as described previously[2,6]. 5-Fluorouracil (5-FU, 99% pure) was obtained from Aldrich Chemical Co., Milwaukee, WI, and cyclophosphamide (CYP, 99% pure) from Polyscience Inc., Warrington, PA. Adriamycin (ADR) was purchased from a local drug store; each vial contained 10 mg doxorubicin·HCl and 50 mg lactose. For injection, rapamycin was dissolved in absolute ethanol containing 1.0 mg butylated hydroxyanisole/ml, and one volume of this solution was mixed with nine volumes of 10% Cremophor EL (BASF, Aktiengesellschaft, West Germany) in water. Sterile physiological saline was used as a vehicle for the three reference drugs. All drug solutions were prepared just before use.

Tumor Models and Animals

P338 lymphocytic leukemia, B16 melanocarcinoma and Colon 38 tumor lines were obtained from the Mason Institute, Worchester, MASS., a tumor bank for the NCI. The tumors were serially transplanted in appropriate strains of inbred mice: P388 in DBA/2, B16 in C57/B1 and Colon 38 in BDF_1 mice. Male BDF_1 mice (18~20 g) were used in the antitumor tests. All mice were purchased from Jackson Laboratories, Bar Harbor, ME., and were of specific-pathogen-free (SPF) grade.

Antitumor Tests

The experimental procedures were those recommended by the Developmental Therapeutic Program, Drug Evaluation Branch, of the National Cancer Institute[10,11]. They are summarized as follows:

P388 Lymphocytic Leukemia: Ascites tumor cells were aseptically withdrawn from a tumor-bearing mouse. After one washing with saline, the tumor cells were enumerated with a hemocytometer and suspended in saline at a concentration of 5.0×10^6 cells/ml. On Day 0, each test mouse received ip 1×10^6 viable tumor cells suspended in 0.2 ml of saline. Drug treatment was given ip once daily from Day 1 to Day 9. Six mice were used in each test group.

B16 Melanocarcinoma: Tumor nodules from tumor-bearing mice were excised, gently homogenized in a hand-operated tissue grinder and suspended in sterile saline which was then diluted 1: 10 (w/w). On Day 0, each mouse received ip 0.5 ml of the tumor suspension. Treatment was given ip once daily from Day 1 to Day 9. Ten mice were used in each test group.

Colon 38: On Day 0, tumor nodules from tumor-bearing mice were excised and cut into 2~3 mm^3 fragments. One tumor fragment was placed sc in the back of each test mouse through a trocar. Unless otherwise specified, treatment was given ip once on Days 2 and 9 in the 2× treatment schedule, or once on Days 2, 5 and 9 in the 3× treatment schedule. On Day 20, the tumor nodules were excised and weighed individually.

Evaluation

The effects of the drugs in the P388 and B16 test systems were evaluated on the basis of the median survival time (MST, in days). The results are expressed as T/C (%).

$$T/C\% = \frac{\text{MST (treatment group)}}{\text{MST (control group)}} \times 100$$

The effects of the drugs in the Colon 38 tumor model were assessed on the basis of median tumor weight (MTW, in mg) on Day 20, unless otherwise specified. The results are expressed as T/C%,

$$T/C\% = \frac{\text{MTW(treatment group)}}{\text{MTW (control group)}} \times 100$$

or percent tumor inhibition (100−T/C%).

For the evaluation of the effects of rapamycin on established and advanced Colon 38 tumors (Table 3), the width (a) and the length (b) of individual tumors were measured (in mm) at various time intervals. The tumor weight was calculated by the formula:

$$\text{Tumor weight (mg)} = 1/2\ ab^2\ (\text{mm})$$

Results

Activity against P388 Lymphocytic Leukemia, B16 Melanocarcinoma and Colon 38 Tumor

As shown in Table 1, rapamycin was slightly active against P388 leukemia: in two separate experiments, T/C values of 160 and 137% were obtained after the ip administration of 100 mg/kg once daily for 9 days. By comparison, a T/C value of 191% was obtained when 5-FU was given ip at 10 mg/kg/day for the same period. The antibiotic was active against B16 melanocarcinoma; T/C values of 141 and 179% were attained with 100 mg/kg/ip injection in the 9× treatment schedule. This effect is comparable to that observed with 20 mg/kg/ip injection of 5-FU (T/C 143%). Rapamycin also showed antitumor activity against the Colon 38 tumor. Relative to the tumor growth observed in the untreated controls, 400 mg/kg/ip injection (2× treatment schedule) of rapamycin (T/C 7.0 and 3.7%) inhibited tumor weight by 93 and 96.3%. Thus, rapamycin was more active than 5-FU (T/C 23.8%) administered ip at a dose of 70 mg/kg on Days 2 and 9. When given according to the treatment schedules described in Table 1, rapamycin did not cause any early deaths, indicating that it was devoid of acute toxicity at the doses used.

In these experiments, demethoxyrapamycin was compared to rapamycin and found completely inactive against B16 melanocarcinoma and Colon 38 solid tumor; it exhibited slight activity (T/C% 135) against P388 lymphocytic leukemia. Therefore, the absence of the methoxy group in position 29 almost completely abolishes the antitumor activity of rapamycin[6].

Anti-Colon 38 Activity of Rapamycin Administered by Various Routes

In the present experiment five routes of administration were compared with respect to their effects on the antitumor activity of rapamycin (400 mg/kg, 2× treatment schedules). As shown in Table 2, the im and sc (adjacent to the tumor) routes were as effective as the ip route. These three routes afforded over 90% tumor inhibition. When the sc injection was given on the other side of the back, distant from the tumor, the antitumor activity (84.8% tumor inhibition) decreased slightly. Oral administration produced significantly less antitumor activity (64.8% tumor inhibition) than the other routes.

Table 1. Effects of rapamycin and 5-FU against P388 lymphocytic leukemia, B16 melanocarcinoma and Colon 38 tumor (expressed as T/C%)[a].

Dose (mg/kg/ip injection)		P388 lymphocytic leukemia*		B16 melanocarcinoma*		Colon 38 tumor**	
		Expt I	Expt II	Expt I	Expt II	Expt I	Expt II
Rapamycin	400	—	—	—	—	7.0	3.7
	200	—	—	—	—	13.4	7.4
	100	160	137	141	179	38.9	24.2
	50	145	141	134	171	133.4	39.3
	25	145	141	124	150	—	—
	12.5	145	141	129	131	—	—
5-FU[b]	10		191				
	20				143		
	70						23.8

Treatment schedule * 9×(Days 1~9), ** 2×(Days 2 and 9)

[a] Drug considered active when T/C% ≥130 (P388), ≥125 (B16) and ≤42 (Colon 38).

[b] Positive control.

Table 2. Anti-Colon 38 effect of rapamycin administered by various routes (treatment on Days 2 and 9).

Route of administration	Median tumor weight (mg)		T/C%[a]	Tumor inhibition (%)
	Vehicle (0.4 ml ip)	Rapamycin (400 mg/kg)		
ip	1,096	70	6.4	93.6
im	1,061	70	6.6	93.4
sc				
a) Adjacent to tumor	567	52	9.1	90.9
b) Distant to tumor	885	135	15.2	84.8
po	838	262	35.2	64.8

[a] Drug considered active when T/C% $\leq$42.

Effects of Rapamycin on Established and Advanced Colon 38 Tumors

In the standard test, tumor inoculation is conducted on Day 0 and treatment given on Days 2 and 9. In this experiment, rapamycin treatment was delayed until a) Days 6 and 13; b) Days 13 and 20; and c) Days 20 and 27. Tumors were excised and weighed on Day 29 or 30. All treated groups received rapamycin ip at 400 mg/kg/injection, and the corresponding control groups were given the vehicle ip. As shown in Table 3, the tumor weights in the treated and control groups were similar up to the first day of treatment. However, there was a great reduction of tumor growth in all the rapamycin-treated groups in comparison to the vehicle-treated controls. When the tumors were measured on Day 29 or 30, 81.1, 76.1 and 54.8% inhibition occurred in groups (a), (b) and (c), respectively. Thus, there was a direct correlation between the time of treatment and the maximum tumor inhibition attained.

Table 3. Activity of rapamycin against established and advanced Colon 38 tumors.

	Days after tumor inoculation	Average tumor weight (mg)		Tumor inhibition (%)[a]
		Vehicle (0.4 ml, ip)	Rapamycin, ip (400 mg/kg)	
a) Treatment on Days 6 and 13	6	39	38	2.6
	9	66	43	34.9
	13	213	50	76.6
	16	306	70	77.2
	20	564	103	81.8
	23	908	140	84.6
	26	896	194	78.4
	30	1,121	212	81.1
b) Treatment on Days 13 and 20	13	97	128	−31.9
	16	139	220	−58.2
	19	244	139	40.4
	23	415	194	53.3
	26	646	190	70.6
	29	844	202	76.1
c) Treatment on Days 20 and 27	20	594	602	−1.3
	23	1,159	792	31.7
	27	1,975	845	57.3
	29	2,112	955	54.8

[a] Drug considered active when tumor inhibition $\geq$58% (T/C% $\leq$42).

Table 4. Comparative anti-Colon 38 activity of 5-FU, CYP, ADR and rapamycin (treatment on Days 2 and 9).

	Dose ip (mg/kg/injection)	Early deaths	T/C%[a]	Tumor inhibition (%)
5-FU	200	4/10	Toxic	Toxic
	100	0/10	5.8	94.2
	50	0/10	54.4	45.6
CYP	200	0/10	0.8	99.2
	100	0/10	7.9	92.1
	50	0/10	59.0	41.0
ADR	20	8/10	Toxic	Toxic
	10	3/10	26.8	73.2
	5	0/10	89.7	10.3
Rapamycin	400	0/10	6.8	93.2
	200	0/10	9.6	90.4
	100	0/10	12.3	87.7

[a] Drug considered active when T/C% $\leq$42.

Comparison of the Anti-Colon 38 Activity of Rapamycin and Other Antitumor Drugs

In this study, Colon 38 tumor-bearing mice were treated ip on Days 2 and 9 with 5-FU, CYP, ADR or rapamycin. Three dose levels of each drug were tested. As shown in Table 4, the intermediate doses of the reference drugs had very high anti-Colon 38 activity; 5-FU (100 mg/kg), CYP (100 mg/kg) and ADR (10 mg/kg) inhibited tumor growth by 94.2, 92.1 and 73.2%, respectively. The highest doses of 5-FU and ADR were toxic to the tumor-bearing mice, and the lowest doses of 5-FU, CYP and ADR did not exert significant anti-Colon 38 activity. Rapamycin exhibited significant anti-Colon 38 activity at all three doses; tumor growth was inhibited by 93.2, 90.4 and 87.7% at 400, 200 and 100 mg/kg/injection, respectively. No early deaths occurred at any of three doses, an indication of low acute toxicity. Therefore, rapamycin exerted the same maximal activity as 5-FU and CYP against Colon 38, and the activity of rapamycin was observed for a wider range of concentrations.

Combination of Rapamycin, 5-FU and CYP for the Treatment of Colon 38 Tumor-bearing Mice

The purpose of this study was to compare the anti-Colon 38 activity of two regimens: FRC (5-FU, rapamycin and CYP given in sequence) and FAC (5-FU, ADR and CYP given in sequence). Single drug treatments were also included for comparison. Treatment was administered on Days 2, 5 and 9. As shown in Table 5, 5-FU was toxic at 50 and 100 mg/kg/injection and ADR at 5 and 10 mg/kg/injection; CYP and rapamycin exhibited no toxicity at the doses tested. In the FAC regimen, the optimal dose was 50 mg/kg of 5-FU on Day 2, 5 mg/kg of ADR on Day 5, and 50 mg/kg of CYP on Day 9; no early deaths occurred and tumor inhibition was 87.4%.

The FAC regimen was more effective than either 5-FU or ADR given alone, but only the intermediate dose level exhibited high tumor inhibition. In the FRC regimen, activity was obtained at all three dose levels. These results indicate that rapamycin can be combined with 5-FU and CYP to provide a wide range of doses effective against Colon 38 tumor.

Table 5. Anti-Colon 38 activity of 5-FU, CYP, ADR, rapamycin and combinations FAC and FRC (treatment on Days 2, 5 and 9).

	Dose (mg/kg)	Number of injection	Early deaths	T/C%	Tumor inhibition (%)
5-FU	100	3×	9/10	Toxic	Toxic
	50	3×	6/10	Toxic	Toxic
	25	3×	0/10	68.5	31.5
CYP	100	3×	0/10	6.2	93.8
	50	3×	0/10	14.7	85.3
	25	3×	0/10	109.8	−9.8
ADR	10	3×	8/10	Toxic	Toxic
	5	3×	6/10	Toxic	Toxic
	2.5	3×	0/10	22.5	77.5
RAPA*	400	3×	0/10	4.5	95.5
	200	3×	0/10	13.5	86.5
	100	3×	0/10	11.6	88.4
FAC	100(F)+10(A)+100(C)		9/10	Toxic	Toxic
	50(F)+5(A)+50(C)		0/10	12.6	87.4
	25(F)+2.5(A)+25(C)		0/10	70.6	29.4
FRC	100(F)+400(R)+50(C)		2/10	0.7	99.3
	50(F)+200(R)+50(C)		0/10	2.7	97.3
	25(F)+100(R)+25(C)		0/10	10.8	89.2

* Rapamycin.

Discussion

The screening strategy of the National Cancer Institute consists of testing natural products such as antibiotics against the murine transplantable tumor, P388 lymphocytic leukemia[11]; compounds with ILS $\geq$30 are then evaluated in a tumor panel which includes ascites as well as solid tumors. Rapamycin shows only slight activity against P388 (maximal ILS ranges from 30 to 40), and its activity could easily be missed if an activity of ILS $\geq$30 were mandatory for further evaluation.

Rapamycin exhibited little or no activity in the leukemia models. However, it was active against B16 melanocarcinoma, EM ependymoblastoma, CD8F1 mammary and Colon 38 tumors. The resistant tumor P388 and the sensitive B16 and Colon 38 tumors were used in the present study. 5-FU served as the control drug and exhibited the expected activity in all of the experiments reported. Rapamycin was less active than 5-FU on a weight basis, but the antibiotic's maximal activity at $\geq$25 mg/kg against B16 and Colon 38 tumors was higher than that obtained with 5-FU (Table 1). In the Colon 38 tumor model, CYP surpassed rapamycin on the basis of weight and maximal activity attainable; ADR was less active (Table 4).

Rapamycin exhibited the same activity whether administered ip, iv (not shown in Table 2), im or sc; upon oral administration, its activity was reduced but not abolished. The dose-response observed with rapamycin was not as sharp as with the other drugs tested (Tables 4 and 5). In the active dose range, rapamycin appeared less toxic than the other drugs. For example, tumor-bearing mice treated with a single ip injection of 400 mg/kg on Day 1 had an average weight loss of less than 5% by Day 5 (data not shown). In the same experiment, 5-FU, CYP and ADR administered at optimal doses caused a 10 to 15% weight loss. Also, rapamycin was capable of inhibiting the growth of Colon 38 tumors at any stage of development (Table 3). If this effect can be reproduced in humans, rapamycin may prove useful as a pre-operative treatment.

Another feature of rapamycin is its compatibility with 5-FU and CYP in the treatment of Colon 38 tumor-bearing mice (Table 5). This sequential combination afforded better protection than any of the

drugs given alone. It was also superior to the sequence 5-FU, ADR and CYP, a combination that has found applications in cancer therapy[12,13].

The results of these studies demonstrate that the rapamycin treatments afford latitude with respect to the dose, the route of administration and the treatment schedule which can be used. This latitude is useful to the investigator who can therefore select effective, minimal toxic doses for specific clinical situations.

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