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Advances in Brief

Rapamycin Selectively Inhibits the Growth of Childhood Rhabdomyosarcoma Cells through Inhibition of Signaling via the Type I Insulin-like Growth Factor Receptor[1]

Michael B. Dilling, Peter Dias, David N. Shapiro, Glen S. Germain, Randall K. Johnson, and Peter J. Houghton[2]

Departments of Molecular Pharmacology [M. B. D., P. D., G. S. G., P. J. H.] and Experimental Oncology [D. N. S.], St. Jude Children's Research Hospital, Memphis, Tennessee 38105; Departments of Pharmacology [P. J. H.] and Pediatrics [D. N. S.], University of Tennessee College of Medicine, Memphis, Tennessee 38163; and Department of Biomolecular Discovery, SmithKline Beecham, King of Prussia, Pennsylvania 19406-0939 [R. K. J.]

Abstract

We show that cell lines derived from childhood alveolar rhabdomyosarcoma (RMS) are very sensitive to the growth-inhibitory effects of the immunosuppressive agent rapamycin (RAP), compared to other human cell lines (50% inhibitory concentration range of 0.1–8 ng/ml, compared to 1280 to >10,000 ng/ml). Our data suggest that the sensitivity of RMS lines is due to RAP inhibition of insulin-like growth factor 1 receptor-mediated signaling, which is essential for continued proliferation of RMS cells. The embryonal RMS line Rh1, which was resistant to RAP in serum-containing medium (50% inhibitory concentration, 4180 ng/ml), was highly sensitive under autocrine conditions of growth, indicating that resistance was due to paracrine signaling pathways insensitive to RAP action. FK506 reversed RAP action in all cell lines, indicating a dependence on complexing with the cytosolic FK506-binding protein for activity.

Introduction

RAP[3] and its structural analogue FK506 (Fig. 1) are potent T-cell-immunosuppressive agents that act through different cell signaling pathways (for reviews, see Refs. 1 and 2). Both agents suppress T-cell activation through binding to a ubiquitous family of soluble cytoplasmic receptor proteins, termed FKBP. A M_r 12,000 member of this family has been implicated in mediating inhibitory effects on T-cells (3, 4). Initially, inhibition of the peptidyl-prolyl isomerase activity of FKBP by FK506 binding was hypothesized to be a mechanism for FK506-mediated immunosuppression. However, experiments with an FK506 analogue demonstrated that binding to FKBP and inhibition of rotamase activity could occur without producing immunosuppression, indicating that rotamase activity inhibition was insufficient as an explanation for the mechanism of immunosuppression by the FK506-FKBP complex (5).

The FK506-FKBP12 complex blocks T-cell activation by inhibiting signaling from the T-cell receptor prior to the synthesis of IL-2, whereas the RAP-FKBP12 complex blocks signaling from the IL-2 receptor after IL-2 synthesis, thereby interrupting the autocrine loop needed to drive proliferation. Inhibition of the Ca^{2+}-dependent serine/threonine phosphatase activity of calcineurin (protein phosphatase 2B) has been implicated as a mechanism for FK506-FKBP12 inhibition of T-cell receptor signaling (6). Arrest of this enzymatic activity is hypothesized to be the mechanism for preventing nuclear translocation of the transcription factor nuclear factor of activated T-cells in FK506-treated cells (7). Contrasting the actions of FK506, RAP has no effect on calcineurin activity or nuclear translocation of the nuclear factor of activated T-cells. Nor does RAP-FKBP12 interfere with IL-2-dependent synthesis of c-*myc* or c-*jun*, despite accumulation of cells in G_1 phase (8). Evidence that RAP-FKBP12 blocked T-cells late in G_1 led to the hypothesis that the drug-immunophilin complex might inhibit the increase in $p34^{cdc2}$ kinase activity normally seen in late G_1. The histone H1 kinase activity of $p34^{cdc2}$ was shown to be strongly suppressed in RAP-treated cells. RAP has also been indicated to inhibit the M_r 70,000 kinase that phosphorylates ribosomal protein S6 (9). Interestingly, in the human IL-2-dependent T-cell line Kit225, RAP inhibition of p70 S6 kinase was demonstrated but this inhibition did not prevent the cells from entering S phase once they had passed a certain point in early G_1 (10). Furthermore, RAP addition to an IL-2 stimulated exponentially proliferating Kit225 culture had no effect on cell number, indicating that RAP does not have an immediate effect on blocking cell cycle progression once cells are in a cycling status, although it does prevent the entry of resting cells into the cell cycle. Although the enzymatic activities of $p34^{cdc2}$ and p70 S6 kinase are inhibited by the RAP-FKBP complex, it is quite possible that these events are downstream manifestations of the primary locus of RAP-FKBP action.

The possibility of using RAP as an anticancer agent has been examined. Preliminary studies *in vivo* showed that RAP activity was limited to a single murine ependymoblastoma. Recent testing of RAP against the cell line panel used by the National Cancer Institute as a screen demonstrated growth-inhibitory activity with a mean IC_{50} of 3160 ng/ml.[4] This contrasts with the IC_{50} needed for T-cell inhibition, which was 0.1–1 ng/ml. Thus, the potency of the drug as a cytotoxic agent was several thousand-fold less than its potency as an immunosuppressive agent. Additionally, RAP demonstrated no cell-type specificity.

A parallel between T-cell activation and RMS proliferation exists in the IGF-1R pathway. IGF-1R expression is induced in activated T-cells and is required for the proliferation of hemopoietic cells (11). Expression of the IGF-1R was demonstrated to be necessary for progression of mitogen-stimulated T-cells into S phase and for expression of proliferating cell nuclear antigen. An examination of RMS tumors and cell lines revealed that they express high levels of IGF-II mRNA and IGF-1R mRNA indicating the existence of an autocrine signaling pathway mediated by IGF-II binding to the IGF-IR (12). The possibility that RAP might be inhibiting T-cell proliferation in an IGF-1R-dependent fashion caused us to propose that RAP may be an effective agent for inhibiting proliferation of RMS cells. Here, we demonstrate that a number of RMS cell lines are exquisitely sensitive

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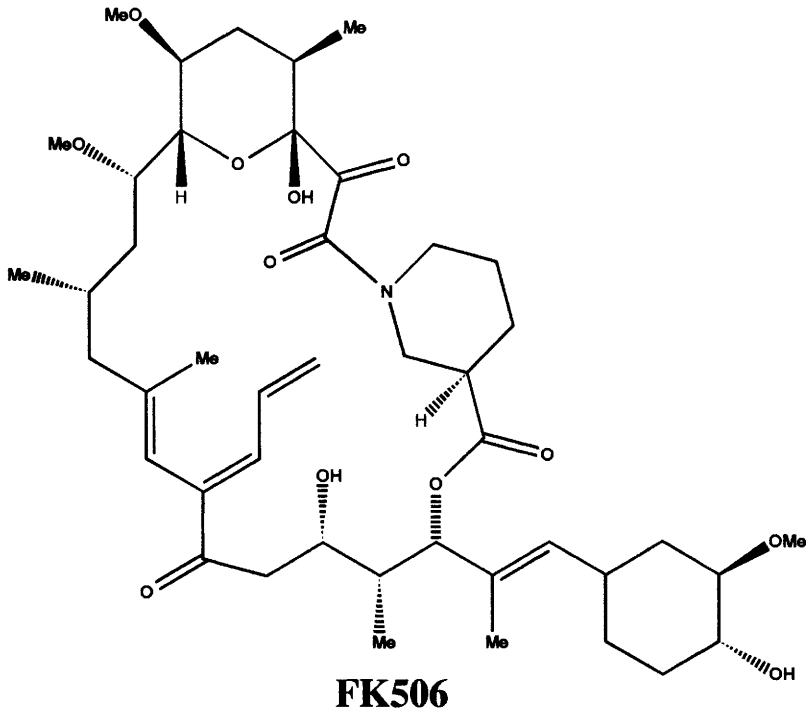
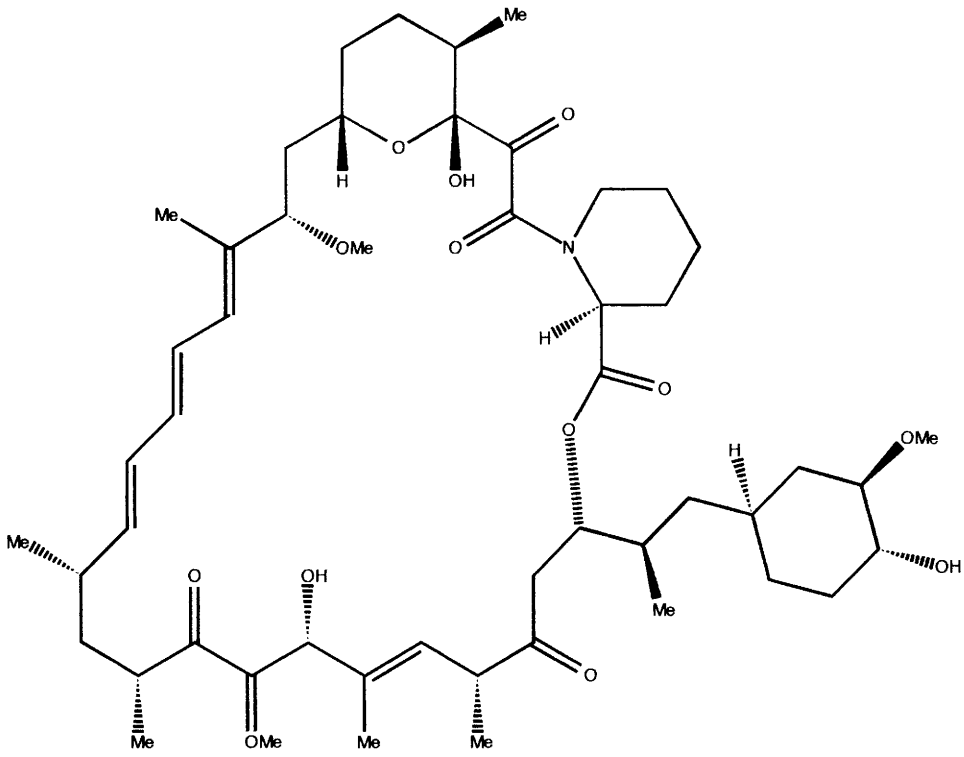
[2] To whom requests for reprints should be addressed, at Department of Molecular Pharmacology, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38105.

[3] The abbreviations used are: RAP, rapamycin; IGF-1R, insulin-like growth factor 1 receptor; RMS, rhabdomyosarcoma; FKBP, FK-binding protein(s); IL-2, interleukin 2; IC_{50}, 50% inhibitory concentration; BSA, bovine serum albumin; Pgp, P-glycoprotein; PI-3-kinase, phosphatidylinositol-3-kinase; IGF-II, insulin-like growth factor II.

[4] A. Monks, personal communication.



FK506



Rapamycin

Fig. 1. Chemical structures of rapamycin and its structural analogue FK506.

to the growth-inhibitory effects of RAP and that this sensitivity correlates with cellular dependence on the IGF-1R pathway for proliferation.

Materials and Methods

Cell Culture. Childhood rhabdomyosarcoma and colon carcinoma cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. For experiments involving serum-free conditions, Rh30 and Rh1 cells were cultured in N2E medium. N2E consists of a basal medium of Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture (50/50) supplemented with 1 μg/ml transferrin, 100 μM putrescine, 20 nM progesterone, and 30 nM sodium selenite (13). Culture in N2E was accomplished using fibronectin-treated (1 $\mu g/cm^2$) cultureware. For experiments involving the addition of BSA to N2E, the protein was added at a concentration of 2.6 g/liter, equivalent to the BSA concentration of medium containing 10% fetal bovine serum.

Drug-mediated Inhibition of Growth and Clonogenicity. For assays designed to measure growth inhibition, cells were plated on 6-well culture plates in triplicate (10^5 cells/well). The following day, serial drug dilutions were

added to the plates and they were incubated for 5–7 days. The cells were lysed under hypotonic conditions, as described previously, and nuclei were counted using a Coulter counter (14). For clonogenic assays, cells were plated in triplicate on 6-well plates (3000 cells/well). Drugs were added the following day and the plates were incubated for 7 days. Cell colonies were stained with crystal violet and quantitated with an Artek counter.

Inhibition of Cell Growth by α-IR-3. Rh30 and Rh1 cells were plated onto fibronectin-treated 6-well plates (10^5 cells/well) in triplicate in N2E medium. The following day, indicated concentrations of α-IR-3 or control IgG (MOPC-21) were added. The plates were incubated for 5 days prior to lysis and nuclei counting.

Results

To determine whether rhabdomyosarcoma cells that demonstrate IGF-1R-dependent growth were more sensitive to RAP than other cancer lines, we compared the sensitivity of Rh30 (an alveolar RMS cell line) to that of the colon carcinoma line GC_3/c1. Rh30 cells were exquisitely sensitive to RAP, whereas GC_3/c1 was resistant (Fig. 2). The IC_{50} values for this experiment were 0.044 ng/ml for Rh30 and 3971 ng/ml for GC_3/c1 (a 95,000-fold difference). We were then interested in determining whether the difference observed between RMS and colon carcinoma cells was of a broad nature; therefore, we tested a number of childhood RMS and colon carcinoma lines for sensitivity to RAP. Three of four RMS lines tested displayed a high degree of sensitivity to RAP, whereas all colon carcinoma lines were relatively resistant (Table 1). The mean sensitivity of the colon carcinoma cells (5260 ng/ml) was similar to that of the National Cancer Institute battery of 60 cell lines (3160 ng/ml).

We were concerned that the differential RAP sensitivity observed between the colon carcinoma cell lines and the RMS lines might be due to high levels of P-glycoprotein expression in the former. To evaluate this possibility, we tested the sensitivities of KB3-1 (which does not express Pgp) and KB8-5 (which overexpresses Pgp) to RAP using clonogenic assays (15). The sensitivities of the two lines were similar, with IC_{50} values of >10,000 ng/ml.

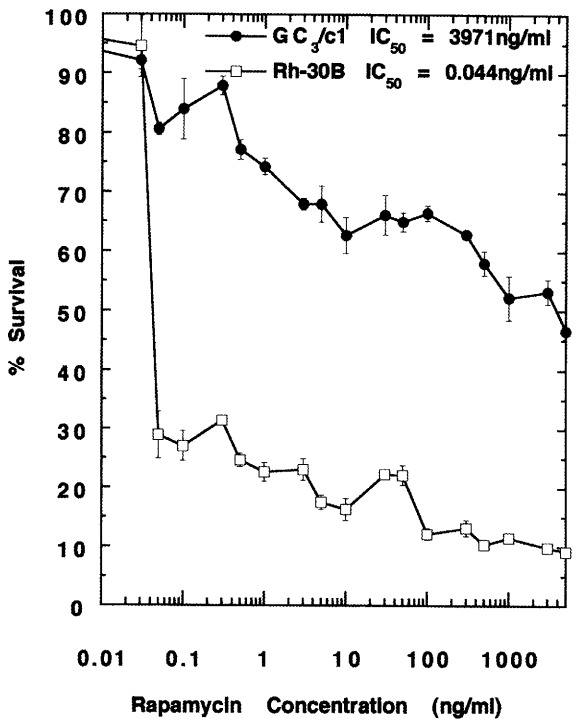


Fig. 2. Differential sensitivity of a human alveolar rhabdomyosarcoma cell line (Rh30) and a colon carcinoma cell line (GC_3/c1) to RAP. Cells were exposed to RAP for 7 days and clonogenic survival was determined. Each point is the mean ± SD for three determinations. IC_{50} values were 0.044 and 3971 ng/ml for Rh30 and GC_3/c1, respectively.

Table 1 *Sensitivities of human childhood rhabdomyosarcoma cell lines and human colon carcinoma cell lines to RAP and geldanamycin*

	RAP IC_{50} (ng/ml)	Geldanamycin IC_{50} (nM)
RMS cell lines		
Rh1	4,680	5.9
Rh18	0.1	14.3
Rh28	8.0	17.9
Rh30	0.37	1.9
Colon carcinoma cell lines		
GC_3/c1	9,800	3.6
VRC_5/c1	1,280	1.4
CaCo	1,570	3.4
HCT8	8,400	ND[a]
HCT29	>10,000	2.6
HCT116	>10,000	ND
National Cancer Institute screen (60 cell lines)	3,160	

[a] ND, not determined.

Whereas three of the four RMS cell lines tested were sensitive to RAP, one cell line, Rh1 (an embryonal RMS), was resistant. We therefore wanted to determine whether this cell line was dependent on the IGF-1R for mitogenic stimulation. A 5-day incubation under serum-free conditions with a monoclonal antibody (α-IR-3) that blocks signaling via the IGF-1R demonstrated that Rh1 cells were not dependent on this receptor pathway for growth (Fig. 3) (16, 17). In contrast, Rh30 cells were highly sensitive to α-IR-3 under conditions that require autocrine growth, indicating a dependence on the IGF-1R.

To further examine the possibility that the RAP-sensitive locus in Rh30 involves the IGF-1R signaling pathway, RAP sensitivity was measured in a derivative of Rh30 that expresses an antisense IGF-1R construct (Rh30AS23). Scatchard analysis revealed that these cells express 60% fewer IGF-1R on their surface, compared with the parental line (18). Rh30AS23 cells were 10-fold more sensitive to RAP than was the parental line (Table 2). Also, the sensitivity of the parental Rh30 line was evaluated under serum-free conditions that require autocrine growth. The RAP IC_{50} of these cells under autocrine conditions did not change from that observed in serum-containing medium (Table 2).

Additional evidence that the growth inhibition observed in RAP-treated RMS cells was a consequence of a specific RAP-sensitive locus present in these cells was provided by testing a panel of RMS cell lines and colon carcinoma cell lines for their sensitivity to the benzoquinonoid ansamycin geldanamycin, a tyrosine kinase inhibitor (19). Both cell types displayed similar sensitivities to this agent (Table 1), with an approximately 10-fold variation in sensitivity being observed among the RMS lines. These data directly contrast with the RAP sensitivity data, in which a large difference in sensitivity was observed between RMS and colon carcinoma cell lines.

To determine whether the sensitivity of RMS cells to RAP was dependent on the drug forming a complex with the cytosolic receptor FKBP, we tested the ability of FK506 to antagonize the effects of RAP in Rh30 cells. Table 2 shows that a nontoxic concentration of FK506 inhibited RAP activity, causing an 18,000-fold shift in the IC_{50}.

Although Rh1 cells were highly resistant to RAP in serum-containing medium, we became interested in determining whether their sensitivity would change under serum-free/growth factor-free conditions that require autocrine growth. In serum-free N2E medium Rh1 cells became highly sensitive to RAP, with a 1600-fold shift in the IC_{50} (Table 2). The addition of BSA at the concentration normally found in serum did not cause a shift in the sensitivity of the cells to RAP. The RAP antagonistic ability of FK506 under these serum-free conditions was evaluated and an IC_{50} shift similar to that seen with Rh30 was observed (Table 2).

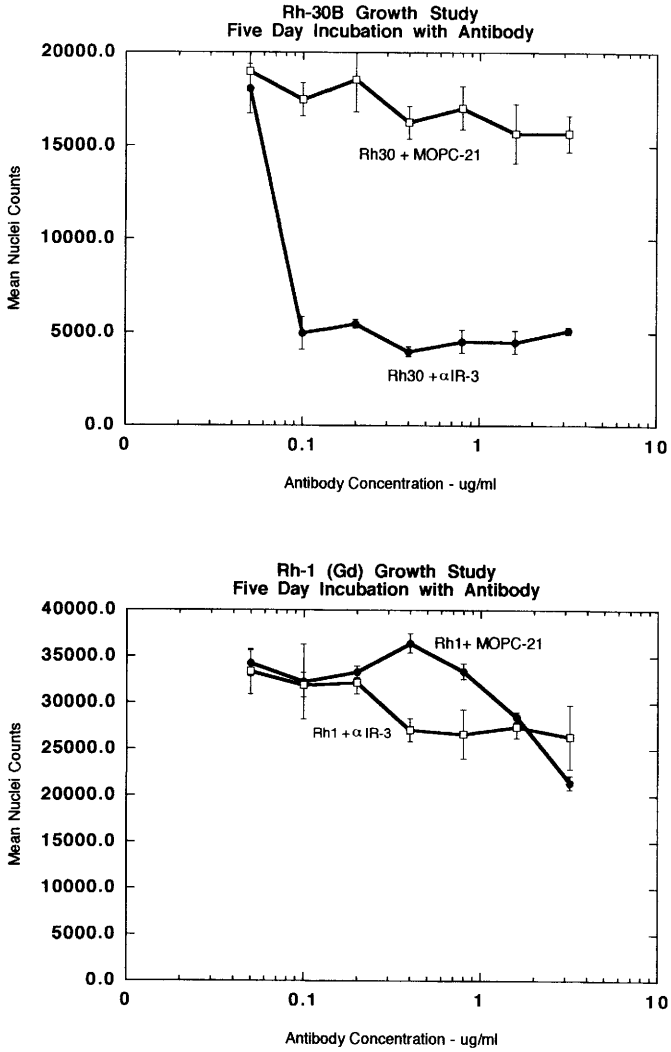


Fig. 3. Inhibition of RMS cell proliferation by an anti-IGF-1R monoclonal antibody (α-IR-3). Cells were cultured under conditions requiring autocrine growth and were exposed to the indicated antibody (α-IR-3 or MOPC-21) concentrations for 5 days. Proliferation was assessed by lysing the cells and counting nuclei. Each point is the mean ±SD for three determinations.

Discussion

In this study we demonstrate that cell lines derived from alveolar RMS are very sensitive to RAP. In contrast, a battery of colon carcinoma cell lines tested for RAP sensitivity were approximately 1000-fold less sensitive than alveolar RMS. Because both RAP and FK506 have been shown to reverse Pgp-mediated multidrug resistance, it was possible that the differential sensitivity between cell lines was related to the level of Pgp expression (20). These concerns were addressed by showing that a well characterized Pgp-negative clone (KB 3-1) and a Pgp-overexpressing clone (KB 8-5) exhibited similar sensitivities to RAP (15). These results indicate that RAP does indeed exhibit cell-type specificity. The basis for the sensitivity of alveolar RMS is likely to be due to the dependence of the cell lines on the IGF-1R for mitogenic signaling. Previous evidence has shown that childhood RMS cell lines as well as adult RMS tumors express IGF-II and the IGF-IR, implicating an autocrine loop involving this ligand and receptor in the proliferation of RMS cells (12). Only one of the RMS cell lines examined, the embryonal RMS line Rh1, was not highly sensitive to RAP.

To further examine the action of RAP on RMS, we focused on Rh30 and Rh1 because of their vast difference in sensitivity to RAP. Rh-1 is not dependent on signaling via the IGF-1R for mitogenesis. This lack of IGF-1R dependence was demonstrated by incubating Rh1 cells under autocrine conditions in the presence of α-IR-3, an antibody to the IGF-1R that blocks mitogenic signaling via this receptor. The level of growth inhibition observed was not significantly greater than that seen with the isotype-matched control, MOPC-21. Conversely, the alveolar RMS cell line Rh30 was acutely sensitive to α-IR-3, demonstrating the dependence of these cells on the IGF-1R. Additionally, the sensitivity of Rh30AS23, a derivative that expresses an antisense IGF-1R transcript and displays 60% fewer IGF-1Rs on its surface, was 10-fold more sensitive to RAP than was the parental line, implicating a component of the IGF-1R signaling pathway as the locus of RAP activity. The sensitivity of Rh30 cells to RAP did not change when the cells were tested under conditions requiring autocrine growth. This finding, coupled with the sensitivity of Rh30 cells to α-IR-3 and the increased sensitivity of the AS23 derivative to RAP, demonstrates that mitogenesis in these cells is maintained almost exclusively via the IGF-1R and that this pathway is RAP sensitive.

The specificity of RAP action against RMS cells was further verified by testing RMS and colon carcinoma cell lines against geldanamycin, a tyrosine kinase inhibitor. It has been postulated that the mechanism of action of this benzoquinonoid ansamycin compound involves an interruption of *src* homology domain interactions that are critical for the function of growth-regulatory signal transduction pathways (19). The finding that RMS and colon carcinoma cells exhibit similar sensitivities to this agent, while demonstrating vastly different sensitivities to RAP, provides additional evidence for the specificity of RAP activity against RMS cells.

The resistance of Rh1 to RAP in serum-containing medium led us to question whether the mechanisms involved were autocrine or paracrine. When Rh1 cells were cultured under serum-free/growth factor-free conditions, they became highly sensitive to RAP, demonstrating a 1600-fold shift in the IC_{50}. We verified that the IC_{50} difference seen with Rh1 in serum-containing *versus* serum-free medium was not likely to be due to serum protein components binding RAP and abrogating its potency. The RAP IC_{50} for Rh1 in N2E medium plus albumin was not different from that in N2E medium alone. The difference in sensitivity to RAP observed when Rh1 cells were tested under autocrine growth conditions *versus* serum-containing conditions indicates that a paracrine pathway is responsible for the resistance of Rh1 cells to RAP in serum. The nature of this putative paracrine pathway is under investigation. As mentioned above, under autocrine conditions Rh1 cells were insensitive to the antibody that blocks mitogenic signaling via the IGF-1R (α-IR-3), demonstrating that the RAP-sensitive locus in these cells is not dependent on sig-

Table 2 *Sensitivities of childhood RMS cells to RAP and FK506*

Cell line	Medium	Drug	IC_{50} (ng/ml)
Rh30	10% FBS[a]	FK506	9,450
		RAP	0.37
		RAP + FK506[b]	6,600
	N2E + BSA[c]	RAP	0.50
Rh30AS23	10% FBS	RAP	0.035
Rh1	10% FBS	RAP	5,800
	N2E	RAP	3.6
	N2E + BSA	RAP	2.1
	N2E	FK506	760
	N2E	RAP + FK506[d]	1,300

[a] Complete medium formulation was RPMI 1640 medium plus 10% fetal bovine serum (FBS) plus 2 mM L-glutamine.

[b] Cells were exposed to FK506 at 1,000 ng/ml and the RAP IC_{50} was determined.

[c] N2E is a serum-free formulation of Dulbecco's modified Eagle's medium/Ham's F-12 (50/50) plus 1 mg/ml transferrin, 100 mM putrescine, 20 nM progesterone, and 30 nM sodium selenite (6). For experiments involving BSA addition, the protein was added at a concentration of 2.6 g/liter, equivalent to the BSA concentration of medium containing 10% fetal bovine serum.

[d] Cells were exposed to FK506 at 300 ng/ml and the RAP IC_{50} was determined.

naling via the IGF-1R. This finding suggests that RAP may have activity against several signaling pathways, or at a locus common to several pathways.

The results obtained with Rh1 could indicate that, while RAP does not inhibit cell growth in serum-containing medium, it does in fact inhibit its sensitive pathway (as suggested by Rh1 sensitivity to RAP under autocrine conditions). Possibly, many other cell lines (*i.e.*, those of the 60-cell line panel of the National Cancer Institute) would be sensitive to RAP under serum-free conditions. Thus, while RAP as a single agent may not inhibit cell growth, it may still inhibit specific signal transduction pathways and could prove to be a valuable component in a strategy of combining specific inhibitors of different signal transduction pathways.

To determine whether the inhibitory effects of RAP on RMS cells were dependent on binding to the cytosolic receptor FKBP, we tested the ability of the RAP structural analogue FK506 to antagonize the effects of RAP. Binding to FKBP has been shown to be necessary for the RAP-mediated suppression of T-cell activation (21). Both Rh30 and Rh1 were relatively insensitive to the effects of FK506 alone. This finding underscores previous evidence that, despite the finding that both drugs bind the same immunophilin ligand, they act through different signaling pathways. In both Rh1 and Rh30 cells, FK506 strongly antagonized the inhibitory effects of RAP on cell proliferation. This finding is consistent with the requirement for RAP binding to the cytosolic FKBP receptor to mediate growth inhibition.

We originally became interested in evaluating the RAP sensitivity of RMS cells because of evidence that implicated the IGF-1R pathway as being a necessary component for resting T-cells to enter S phase (11). If the RAP-mediated block of T-cell activation is linked to the IGF-1R pathway, then this agent might also serve to inhibit the IGF-1R-dependent proliferation of RMS cells. The data presented here indicate that this hypothesis appears to be true. However, an important difference between RAP action on T-cells and RMS cells must be noted. In the T-cell model, RAP does not block proliferation once the cells have entered the cell cycle, despite the inactivation of p70 S6 kinase (10). In contrast, our data obtained with childhood RMS cells demonstrate that RAP inhibits actively proliferating cells.

Data from the closely related insulin receptor indicates that signaling via the IGF-1R proceeds through at least two distinct pathways. Recent data have provided a direct link between this receptor tyrosine kinase and the membrane-bound GTPase p21 Ras (22, 23). PI-3-kinase also associates with the activated insulin receptor complex via an interaction of the *src* homology 2 domains of the p85 regulatory subunit with specific phosphotyrosine residues on insulin receptor substrate 1 (24). The p21 Ras and PI-3-kinase pathways are attractive targets for investigating the molecular mechanism of RAP-mediated growth inhibition in RMS cells. Indeed, recent work in yeast has demonstrated that RAP-resistant mutants map to a PI-3-kinase-like locus whose expression is required for cell cycle progression (25). It will be of great interest to determine whether the mammalian homologue to this enzyme plays a role in RAP activity against RMS.

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