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Anti-tumor and immunomodulatory activity of resveratrol *in vitro* and its potential for combining with cancer immunotherapy

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Abstract

We evaluated the anti-tumor effect of Resveratrol (RV) on M21 and NXS2 tumor cell lines and its immunosuppressive activity on human and murine immune cells to determine the potential for combining RV and immunotherapy. *In vitro*, concentrations of RV $\geq 25\text{mcM}$, inhibited cell proliferation, blocked DNA synthesis and induced G1 phase arrest in tumor and immune cells. RV at 12–50mcM inhibited antibody dependent cell mediated cytotoxicity (ADCC) of tumor cells facilitated by the hu14.18-IL2 immunocytokine (IC). The *in vivo* anti-tumor and immunomodulating activity of RV given systemically were assessed in mice. Results showed that this RV regimen inhibited the growth of NXS2 tumors *in vivo* but did not appear to interfere with blood cell count, splenocyte or macrophage function. Thus, RV may be a candidate for combining with immunotherapy.

Keywords

Resveratrol; Immunocytokine; anti-GD2 antibody; Neuroblastoma; hu14.18-IL2

1. Introduction

Resveratrol (RV) is a plant-derived polyphenol most commonly found in grapes [1, 2]. It is produced by plants in response to stress [1]. RV has shown *in vitro* anti-tumor activity on tumor cells and *in vivo* inhibition of tumors in experimental animal models [3, 4, 5, 6, 7, 8, 9,

10, 11, 12, 13]. It is able to inhibit cancer growth in a time- and dose-dependent manner [11]. RV causes cell cycle arrest accompanied by a time- and concentration-dependent induction of cell death through apoptosis [9, 12, 14, 15, 16, 17, 18].

RV has relatively little toxicity to non-transformed tissue *in vivo* [12]. However the potential *in vivo* immunomodulatory activity of RV has not been completely elucidated. RV was found to be immunosuppressive *in vitro* but not *in vivo* in certain mouse models [6, 19, 20, 21]. One major discrepancy in these studies was that the concentrations exerting anti-tumor and immunosuppressive activity *in vitro* are 25 to 50 fold higher than the peak plasma levels of RV achieved in mice (~1mM) after oral administration with doses showing anti-tumor activity [12]. Systemic regimens of RV show inhibition of tumor growth but normally do not induce regression [4, 12], suggesting that there may be potential therapeutic benefit from combining RV with other therapies. Thus, RV was found to synergize *in vitro* and *in vivo* with agents such as paclitaxel and 5-FU [22, 23], to produce a better anti-tumor effect. RV combined with immunotherapy may have therapeutic potential, but it has not been experimentally explored.

Hu14.18-IL2 immunocytokine (IC) is a novel form of immunotherapy composed of the humanized IgG 14.18 monoclonal antibody linked to interleukin-2 (IL-2) [24, 25]. This IC targets GD2, a disialoganglioside expressed with relatively little heterogeneity and at high density on tumors of neuroectodermal origin [26, 27]. The IL-2 component attracts and activates effector cells carrying IL-2 receptors. IC enables ADCC and anti-tumor effects executed mainly by Natural Killer (NK) cells, *in vitro* and *in vivo* [25, 28, 29]. The current studies were performed to determine whether the actions of RV suggest that it should be analyzed pre-clinically in combination with IC.

Here we report on: 1) the anti-tumor activity of RV seen *in vitro* on tumor cells using concentrations detected in mice after oral administration; 2) the effect of RV on proliferation of lymphocytes stimulated with mitogen; and 3) the ability of RV to influence tumor-cell cytotoxicity in NK and ADCC assays *in vitro* using IC as the source of antibody and immune activation. We also report on the anti-tumor and immunomodulatory activity of RV in pre-clinical *in vivo* models.

2. Materials and methods

2.1 Cells, media and reagents

M21 human melanoma and NXS2 murine neuroblastoma cell lines both express GD2 and were provided by Dr. R. Reisfeld (Co-author). SH-SY5Y is a GD2⁺ human neuroblastoma cell line, provided by Dr. A. Polans (Co-author). YAC-1, a murine lymphoma cell line was used as a NK target [25]. Cells were cultured in RPMI or high glucose DMEM (NXS2 only) media supplemented with 10% fetal calf serum (FCS), 100 units/ml of penicillin, 100mcg/ml streptomycin and 2mM L-glutamine (Fisher Scientific, Pittsburgh, PA) and maintained at 37°C/5% CO₂. RV was obtained from Cayman laboratories, Dallas, TX. The hu14.18-IL2 immunocytokine was obtained from EMD-Lexigen-Research Center (Billerica, MA).

2.2 Animals

Female 4–5 week old athymic (*nu/nu*) mice were purchased from Harlan Sprague Dawley laboratories (Madison, WI). Female 6–8 week old A/J mice were purchased from Taconic laboratories (Hudson, NY). Animals were housed in the university-approved facilities and handled according to the NIH and UW-Madison Research Animal Resource Center guidelines.

2.3 Human peripheral blood mononuclear cells (hPBMC) isolation

Human PBMC from healthy volunteer donors (approved by the UW Health-Sciences Human Subjects Committee) were isolated from heparinized blood by centrifugation over a Ficoll-Hypaque density gradient.

2.4 Splenocyte isolation

Splenocytes were isolated from non tumor bearing A/J mice that had been injected daily with 10^5 units of IL-2 in 0.5ml of PBS intra-peritoneally (i.p.) for 3 days and from SH-SY5Y tumor-bearing athymic mice that had been treated daily with 50mg/kg RV via intragastric gavage (i.g.) [12]. Briefly, mice were euthanized by CO₂ asphyxiation. Spleens were harvested and disaggregated by chopping and mashing them between sterile glass slides. This preparation was passed through a 70 micron cell strainer (Fisher, Pittsburgh, PA) and red blood cells were lysed by hypotonic shock. Splenocytes from SH-SY5Y tumor-bearing mice were stimulated *in vitro* with 100 units/ml of IL-2 (NCI BRB preclinical repository, Fisher BioServices, Rockville, MD) for 3 days.

2.5 Proliferation assays

2.5.1 Thymidine incorporation assay—Cells were placed in 96-well plates in quadruplicates (Fig. 1) or triplicate (Fig. 5) wells with dilutions of RV. Dimethyl sulfoxide (DMSO), the vehicle for maintaining solubility of RV, was used as control. For tumor cell lines and A/J splenocytes, 1×10^5 cells/well and for hPBMC, 3×10^4 cells/well were used. Tumor cells were incubated for 24 hours. Human PBMC were stimulated with 0.1% phytohemagglutinin (PHA) for 3 days or 100 units/ml of IL-2 for 6 days. Murine splenocytes were stimulated with 100u/ml of IL-2 for 5 days or 5mcg/ml ConA for 2 days. RV was added at the beginning of the assay. The plates were incubated at 37°C/5% CO₂ and pulsed with 1mCi/well of ³H-thymidine (³H-[TdR]) for the last 6 hours of the incubation period. The cells were harvested and analyzed as previously described [30].

2.5.2 CFSE assay—Human PBMC and murine splenocytes at 1×10^6 cells/ml in phosphate buffered saline (PBS) were labeled with 3mM Carboxyfluorescein succinimidyl ester (CFSE) at 37 °C for 5 minutes. The labeling reaction was stopped with an equal volume of ice cold FCS. CFSE-labeled cells were placed in 12-well plates, 4×10^6 cells/well for hPBMC and 6×10^6 cells/well for murine splenocytes, with dilutions of RV and 0.1% PHA for 3 days (hPBMC) or 100units/ml of IL2 for 5 days (splenocytes) at 37°C/5% CO₂. DMSO was used as the control. Following incubation cells were harvested, labeled with propidium iodine (PI) and analyzed by flow cytometry using a BD-FACSCalibur (Becton Dickinson, San Jose, CA). Viable cells (20,000) were collected and the data were analyzed using ModFit LT™ (Verity Software House, Topsham, ME) to determine CFSE distribution.

2.6 Cell cycle analysis

Human PBMC and murine splenocytes, were placed in 12-well plates and stimulated as in the CFSE assay above. M21 and NXS2 cells were placed in 12-well plates and incubated at 37°C/5% CO₂ for 24hrs. Upon incubation, cells were washed with PBS and $1-0.5 \times 10^6$ cells were fixed in 0.1ml with 0.9ml absolute methanol overnight at -20°C. Cells were then washed with PBS and stained with 0.5ml of PI solution (1mg/ml RNaseA, 33mcg/ml PI, 0.2% NP-40 in PBS). Cells (20,000) were collected using a BDFACSCalibur and the data were analyzed using ModFit LT™ to determine cell cycle distribution.

2.7 ADCC or NK assay

These assays were run as previously described [28]. The effector to target ratio (maximum of 100:1) was achieved by plating 5×10^5 effector cells/well and 3 serial 2 fold dilutions of effectors were included in each assay. For ADCC, IC as a source of antibody for GD2-positive targets (M21, NXS2 and SH-SY5Y) was added to the wells at 0.5mcg/ml (hPBMC effectors) or 10mcg/ml (murine splenocyte effectors). Rather than IC, IL-2 was added at 100 units/ml in the case of murine splenocytes killing the NK-sensitive YAC-1 (non-GD2⁺) cells. Results are expressed as percent cytotoxicity or as lytic units where 1 lytic unit is the number of effector cells necessary to achieve 20% lysis of the 5×10^3 targets cells.

2.8 Annexin-V assay

NXS2 or M21 cells (0.5×10^6) were cultured in a 12-well plate with media alone or with RV (50 and 1mcM) or DMSO at 37°C/5%CO₂ for 24hrs. The cells were harvested, counted and stained with Annexin-V-FITC according to the protocol for the BD-apoptosis kit. Cells (20,000) were collected using a BD-FACSCalibur and analyzed using FlowJo 7.5 software (Ashland, OR).

2.9 RV *in vivo* anti-tumor study

Female, 6–8 week old A/J mice were injected with 2×10^6 NXS2 cells subcutaneously (s.c.) on day 0. Treatment with 50 or 100 mg/kg RV given by i.g. or i.p. was started on day 4 or 7 following tumor injection. Mice were treated daily for 33–35 days. Neobee M5 oil (Spectrum Chemical Manufacturing Corp.) was used as the RV vehicle. Tumors were measured every 3–4 days and mice were sacrificed according to the animal facility policy if the tumor ≥ 15 mm in diameter. Tumor volume was determined using the formula: $V = (\text{width} \times \text{length} \times \text{width}/2) = \text{mm}^3$. Mean of tumor volume per group was calculated using the last determined volume prior to sacrifice carried forward for the sacrificed mice.

2.10 RV *in vivo* immunomodulatory study

Female 4–5 week old athymic mice bearing SHSY5Y tumors were treated with RV (50mg/kg, i.g., for 5 weeks) as previously described [12]. Thirty minutes after the last dose, blood, peritoneal exudate cells (PEC) and splenocytes were harvested. To collect blood the mice (n=4) were anesthetized using isoflurane. Blood was collected from the axillary plexus and dispensed in an EDTA-coated 0.5ml tube. Mice were immediately euthanized by CO₂ asphyxiation. Samples were sent to the clinical laboratory of the UW Hospital and Clinics for complete blood cell (CBC) and differential counts. PECs were harvested as previously described [30], from naïve, vehicle- or RV-treated *nu/nu* mice carrying SH-SY5Y. Peritoneal macrophages were then tested for nitrite production and ability to inhibit proliferation of SH-SY5Y tumor cells with or without the addition of 100 units/ml of interferon gamma (IFN- γ) and 10ng/ml of lipopolysaccharide (LPS). Nitrite production and tumor cytostasis assays were performed as previously described [30]. Splenocytes were harvested and used as effector cells in ADCC and NK assays as described above.

2.11 Cell microscopy

M21 and NXS2 cells (2.5×10^5 cell/wells) were cultured in 12-well plates with RV for 3 days at 37°C/5% CO₂. On day 3, pictures were taken in bright field 10X using a Leica DM IRB inverted microscope (Leica Microsystems, Wetzlar, Germany).

2.12 Statistical analyses

Each of the *in vitro* experiments presented here were repeated multiple (≥ 3) times, except for the *in vivo* immunomodulatory experiments which were done twice. A one-way Analysis of Variance (ANOVA) was used for comparison of multiple experimental groups. When

ANOVA yielded significance, pairwise comparisons using t-test with the Bonferroni adjustment were conducted between two groups. Bars on graphs represent the standard deviation (Figures 1, 3, 4, 5, 7 and 8). Tumor volume presented in figure 9 summarizes the results of three experiments and the data were analyzed using a mixed model for repeated measures. Results were considered statistically significant if a two-tailed p-value is <0.05 .

3. Results

3.1 RV interferes in a concentration dependent manner with the proliferation and cell cycle of NXS2 and M21 cells *in vitro*

The *in vitro* effect of RV on the proliferation of NXS2 (Figure 1A) and M21 (Figure 1B) was assessed in a ^3H -thymidine incorporation assay. Results from this experiment showed a dosedependent anti-proliferative effect within 24hrs. Fifty mcM RV significantly inhibited the proliferation of NXS2 and M21. Concentrations of 25 and 12.5 mcM also inhibited NXS2. The inhibitory effect of RV was confirmed by visible differences in the cell density achieved after *in vitro* exposure to RV for 3 days. Fewer cells were present in the wells incubated with 50 mcM RV (Figure 2C,D), while cells incubated with 1 mcM RV (Figure 2E,F) expanded similarly to the cells in control wells (Figure 2A,B).

The effect of 24 hours exposure to RV on cell cycle of tumor cells was determined. Results revealed that 50mcM RV arrested both NXS2 and M21 cells in G1 phase ($p < 0.05$), while 1mcM did not affect the cell cycle when compared to the control. Seventy-eight % of the M21 and 89% of the NXS2 cells were in G1 phase after exposure to 50 mcM RV compared to 48–64% for 1mcM RV or medium alone (Figure 3B). Representative individual histograms are provided in Figure 3A. These results show that RV prevented some cells from entering S-phase. Annexin-V staining was used to determine if RV induced apoptotic changes in the tumor cell lines. When both M21 and NXS2 are exposed to 50mcM RV for 24 hours a modest number of the cells appear to be in early and late apoptosis, but this concentration did not affect the viability of the majority of these tumor cells (over 90% for M21 and over 84% for NXS2 (Figure 4A and 4B). Lower concentrations of RV did not affect the viability of the tumor cells (Figure 4B).

3.2 RV interferes with the proliferation and cell cycle of murine splenocytes and hPBMCs *in vitro*

The *in vitro* effect of RV on the proliferation induced by IL-2, ConA or PHA, as measured in murine splenocytes and hPBMCs, was assessed by ^3H -thymidine incorporation and CFSE distribution assays. 25–50mcM RV inhibited the proliferation of hPBMC, and 12.5–50 mcM RV inhibited murine splenocyte proliferation, while concentrations of 6.25 mcM or less of RV did not inhibit the proliferative response (Figure 5).

The murine splenocytes and hPBMC were labeled with CFSE, and incubated under the same conditions shown in Figure 5. Table 1 shows that over 90% of these CFSE-labeled cells were retained in the parental generation when treated with 50mcM RV, compared to 44–55% in the parental generation for the same stimulated cells, but exposed to 1mcM RV or no RV. Representative individual histograms are provided in Figure 6. These data indicate that 50mcM RV blocks immune cell division *in vitro*.

Cell cycle distribution analysis demonstrated that 50mcM RV also arrested PHA stimulated hPBMC and IL-2 stimulated murine splenocytes in G1 phase, 89% and 86% respectively compared to stimulated cells cultured without RV, 64% and 78% respectively ($p < 0.05$) (Figure 7B). Representative individual histograms are provided in Figure 7A. Thus RV prevents immune cells from entering S-phase when stimulated with PHA or IL-2.

3.3 RV interferes with the cytotoxic activity of immune effector cells *in vitro*

We investigated the effect of RV on the ADCC activity of immune effector cells in combination with IC as the source of antibody *in vitro*. Fresh hPBMCs and IL-2-stimulated murine splenocytes were incubated with radiolabeled M21 or NXS2 cells and IC in the presence of RV. Results show that 25 or 50 mcM RV inhibited the effector cells ability to lyse the target cells via ADCC (Figure 8). Samples with 50mcM RV yielded less than 10% cytotoxicity compared to over 30% cytotoxicity for cultures with lower concentrations of RV using hPBMC and the M21 melanoma target cells (Figure 8A). RV by itself did not induce significant release of ^{51}Cr (Figure 8A), indicating that RV interfered with the effector cell population rather than the target cells in this assay. Similar suppression of cytotoxicity was obtained when NK sensitive YAC-1 cells were used as target cells for IL-2 activated murine splenocytes (data not shown).

3.4 *In vivo* RV treatment slows NXS2 tumor growth

In order to assess the anti-tumor activity of RV in our model of neuroblastoma, mice bearing NXS2 tumors were treated with 50 mg/kg given i.p. or i.g. or 100mg/kg given i.g. The combined results of these 3 experiments show that the tumors in RV-treated mice (n=26) grew more slowly than the tumors in vehicle-treated mice (n=16; $p<0.01$, Figure 9). These results confirmed prior studies with human neuroblastoma xenografts in immune deficient mice, which also demonstrated that a similar daily regimen of systemic RV inhibited tumor growth *in vivo* [12].

3.5 RV is not immunosuppressive *in vivo*

The effects of the systemic RV regimen on the innate immune system were assessed in athymic nude mice. SH-SY5Y neuroblastoma bearing-mice were treated i.g. with 50mg/kg of RV daily for 5 weeks. Thirty minutes after the last dose, blood, spleens and PEC were collected. Naïve mice were also used as the no-treatment control group. CBC and differential counts were assessed (n=4) as well as cytotoxic activity of splenocytes (naïve n=2, vehicle and RV n=3). Cytotoxicity of target cells in both ADCC and NK assays by naïve, vehicle and RV treated mouse splenocytes were similar, suggesting that RV when given at 50mg/kg i.g. does not affect the *ex-vivo* activation and activity of splenic NK cells (Table 2); these results represent one of two identical experiments.

Automated complete and differential blood cell counts for all treated and control groups were comparable, suggesting that RV did not have a negative affect on circulating red blood cell, platelet and leukocyte numbers(data not shown).

PEC collected from all groups (naïve, vehicle and RV treated) were co-cultured *ex vivo* with SHSY5Y in the presence or absence of IFN- γ and LPS. Suppression of tumor cell growth *in vitro* by these PECs was measured by ^3H -thymidine incorporation; nitrite production was measured as an indication of Nitric Oxide (NO) production and macrophage activation. Tumor cell growth inhibition and nitrite production were similar for all groups indicating that peritoneal macrophage function was not affected by the *in vivo* administration of RV (data not shown). Overall, these results indicate that systemic RV administration does not have a negative impact on the bone marrow and murine innate-immune effector function under conditions that result in tumor inhibition.

4. Discussion

This study first evaluated the effect of RV on tumor cells and immune effector cell function *in vitro*. We assessed cell proliferation and cell cycle regulation following RV exposure. RV at high concentrations (> 25mcM) inhibited cell proliferation and affected the cell cycle

distribution, inducing G1 phase arrest. These *in vitro* data using GD2-expressing tumor cells are in agreement with the effect of RV previously reported with other cell lines [4, 12, 31]. The molecular targets of RV on our cells were not assessed; however, RV has been reported by others to upregulate the expression of the tumor suppressor *p53* gene and decrease cell cycle-related molecules in cells showing growth arrest *in vitro* [32, 33, 34]. After the upregulation of *p53* expression, Cipl/p21, which is downstream to *p53*, is able to inhibit G1 cyclins/CDKs activity leading to the G1 growth arrest phenotype. RV may also interfere with the function of the proteasomes in the cell [5, 35]. The proteasome helps the cell to eliminate damaged and unneeded proteins by proteolysis. A nonfunctional proteasome may lead to cell cycle arrest as the cell will be unable to remove proteins from each phase of the cell cycle, which prevents the cell cycle from progressing.

Previously, RV was shown to slow tumor growth *in vivo*; peak RV serum levels in those treated mice were approximately 1mM after oral administration [7]. When we tested this concentration of RV *in vitro*, it did not inhibit tumor cell proliferation or proliferation of immune cells.

Some studies examining the combination of RV with other anti-cancer drugs suggest that RV, in addition to being a tumor cell growth inhibitor, may also enhance the antitumor activity of certain chemotherapy regimens [22, 23]. *In vitro* studies reported previously by others found RV to synergize with agents such as paclitaxel [22]. In addition, a study combining RV with 5-FU *in vivo* demonstrated better *in vivo* anti-tumor effects on liver cancer tumors than seen with either agent alone [23]. To our knowledge, the possibility of combining RV and immunotherapy for cancer treatment has not been experimentally addressed.

We thus tested RV *in vitro* for any possible immunomodulatory effect when combined with our novel form of immunotherapy, hu14.18-IL2 that is being tested pre-clinically and clinically. The anti-tumor effect seen with IC is mainly executed through ADCC by NK cells [28], although the role of macrophages cannot be excluded. In our *in vitro* ADCC assays RV prevented immune effector cells from lysing their target cells in a dose-dependent manner with complete abrogation of killing at concentrations > 25mM. Lower concentrations of RV had no measurable effect. RV at any of the concentrations tested did not enhance or augment the cytotoxic activity when combined with IC, *in vitro*. Treatment with RV *in vivo* did not have a negative effect on the immune system, as measured by the impact on the bone marrow function and *ex vivo* functions of splenocytes and peritoneal macrophages.

These findings demonstrate that RV used *in vitro* has dose-dependent anti-proliferative effects on tumor and immune cells. RV at 50mg/kg showed anti-tumor activity, without negative effect to the immune system *in vivo*, and *in vitro* studies with 1mM RV (the peak level achieved *in vivo*) did not interfere with IC-mediated tumor destruction, making RV a good candidate for future studies combining it *in vivo* with IC immunotherapy. As both RV and IC immunotherapy induce anti-tumor effects, our next step has been to investigate whether RV, when combined *in vivo* with IC will have an enhanced anti-tumor effect in our pre-clinical syngeneic model of neuroblastoma (NXS2) [36].

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Figure 1. Proliferation of NXS2 and M21 cells in the presence of RV

NXS2 (A) and M21 (B) cells were incubated with DMSO or dilutions of RV for 24hrs and then pulsed with ³H-[TdR] for the last 6 hours of incubation. Fifty mcM RV prevented the M21 cells from incorporating ³H-[TdR] while 50- 12.5mcM inhibited ³H-[TdR] in NXS2. *p<0.05 vs. DMSO.

Figure 2. Microscopy of NXS2 and M21 cells in the presence of RV
M21 cells (A, C, E) and NXS2 cells (B, D, F) were incubated with DMSO (A, B), 50mM RV (C, D) or 1mM RV (E, F) for 3 days. Bright field microscopy (10X) was performed. Fifty mM RV had a visible cytostatic effect on the cells whereas 1mM RV did not have any effect, yielding results similar to the DMSO control.

Figure 3.

Cell cycle distribution of NXS2 and M21 incubated with RV. NXS2 or M21 cells, 1×10^6 cells, were seeded into 12-well plates and incubated with media, DMSO, 50 mcM or 1mcM RV for 24hrs (n=2). At the end of the incubation period, cells were harvested, stained with PI and changes in the cell cycle analyzed by flow cytometry. Representative histograms (A) and numerical data (B) of DMSO and 50mcM RV treatments indicated that 50mcM RV arrested the cells in G1 phase of their cycle compared to DMSO (*p<0.05), while lower concentrations yielded a cell cycle distribution similar to control. These results represent one of multiple experiments yielding similar results.

Figure 4.

Effect of RV on viability of NXS2 and M21 cells. Cells, 0.5×10^6 , were seeded onto a 12-well plate in the presence of media, DMSO, 50 mcM or 1 mcM RV for 24hrs (n=2). Cells were harvested, stained with Annexin-V and analyzed by flow cytometry. Representative dot plots (A) and numerical percentage data (B) of DMSO and 50 mcM RV treatments show that a modest percentage of the cell population was in early apoptosis (Annexin-V single positive population) and late apoptosis (Annexin-V/PI double positive population), and the viability of the majority of both NXS2 and M21 tumor cell lines was not compromised.

*p<0.05 vs. DMSO. These results represent one of multiple experiments yielding similar results.

Figure 5. Proliferation of hPMBC and murine splenocytes in the presence of RV
Human PBMC were incubated with PHA (A) or IL-2 (B) and murine splenocytes with IL-2 (C) or 5mcg/ml ConA (D); and with DMSO or with dilutions of 1.5 – 50 mcM RV. Cells then were then pulsed with ^3H -[Tdr] for the last 6 hours of incubation. * $p < 0.05$ vs. DMSO.

Figure 6.

CFSE distribution in cells incubated with RV. CFSE-labeled human PBMC and murine splenocytes were incubated with media, DMSO, 50 and 1mcM, and PHA (hPBMC) or IL-2 (murine splenocytes). Fifty mcM RV prevented cell division as determined by the retention of CFSE in the parental cell population. CFSE distribution within 1mcM RV-treated cells was similar to that of DMSO-treated cells.

Figure 7.

Cell cycle distribution in hPBMC and murine splenocytes incubated with RV. Cells, 4×10^6 , were seeded into 12-well plates, incubated with 0.1% PHA (hPBMC) and 100u/ml IL-2 (splenocytes) and with media, DMSO, 50 and 1mM concentrations of RV for 3 days or 5 days, respectively. At the end of the incubation period, cells were harvested, stained with PI and changes in the cell cycle were analyzed by flow cytometry. Representative histograms (A) and numerical data (B) indicated that 50mM RV arrested the cells in G1 phase of the cell cycle, while lower concentrations yielded a cell cycle distribution similar to control [$p < 0.05$ vs. DMSO]. These results represent one of multiple experiments yielding similar results.

Figure 8. Effect of RV on ADCC assays

M21 (A) and NXS2 (B) target cells were incubated with effector cells, hPBMCs (A) or murine splenocytes (B) in the presence of DMSO or with various concentrations of RV for 4hrs. Hu14.18-IL2 (IC) was used, where indicated, as the source of antibody for the ADCC assays at 0.5mcg/ml (A) and 10mcg/ml (B). * $p < 0.05$ vs. DMSO

Figure 9. Growth of NXS2 tumors in mice treated with vehicle or RV (i.g./i.p)

A/J Mice bearing NXS2 tumors were treated i.p. or i.g. with 50 or 100 mg/kg of RV once daily for 35 days, starting on day 4 or 7 post tumor implantation. Tumor volume was determined every 3–4 days during treatment. The combined results of the 3 experiments are shown. The control mice developed larger tumors than the RV-treated mice over time ($p < 0.01$). Bars represent 95% confidence intervals of the means.

Table 1
CFSE distribution in cells incubated with RV

CFSE-labeled human PBMC and murine splenocytes were incubated with media, DMSO, 50 or 1mM RV and PHA (hPBMC, n=4) or IL-2 (splenocytes, n=3). Fifty mM RV prevented their division as determined by the retention of CFSE in the parental cell population. CFSE distribution within 1mM RV-treated cells was similar to that of the RV-free control treated-cells.

	Treatment	Cell in this generation (%)		
		Parent	2-5	6-10
hPBMC	Media no PHA	98.42 (± 1.46)	1.20 (± 1.19)	0.38 (± 0.31)
	Media + PHA	44.72 (± 17.25)	54.45 (± 17.31)	0.83 (± 0.52)
	DMSO + PHA	52.27 (± 20.39)	46.73 (± 20.39)	1.00 (± 0.26)
	50uM RV + PHA	93.48 * (± 1.75)	6.28 (± 1.89)	0.25 (± 0.18)
	1uM RV + PHA	45.39 (± 19.02)	53.74 (± 19.27)	0.88 (± 0.57)
Murine splenocytes	Media no IL-2	81.89 (± 8.14)	15.96 (± 7.92)	2.30 (± 0.44)
	Media + IL-2	55.24 (± 10.90)	42.35 (± 11.72)	2.41 (± 0.87)
	DMSO + IL-2	56.41 (± 1.24)	39.38 (± 2.18)	4.21 (± 2.51)
	50uM RV + IL-2	90.75 * (± 3.35)	8.56 (± 3.24)	0.68 (± 0.45)
	1uM RV + IL-2	52.59 (± 5.58)	42.46 (± 3.34)	4.95 (± 2.72)

Data are mean % $\pm$ SD

*
p- <0.05 vs. DMSO + IL-2 or PHA (Parent generation).

Table 2
ADCC of SH-SY5Y and killing of NK-sensitive YAC-1 cells by splenocytes from mice treated with RV

Splenocytes from RV-treated athymic mice were stimulated *in vitro* with IL-2. Stimulated splenocytes were incubated with ^{51}Cr -labeled SH-SY5Y, +/- IC at 10mcg/ml or with YAC-+/- IL-2 in the assay. Naïve n=2, Vehicle and RV n=3. Results are expressed in lytic units as mean $\pm$ SD.

Group	Lytic units			
	SH-SY5Y		Yac-1	
	media	IC	media	IL-2
Naive	10.86 ($\pm$ 2.81)	39.24 ($\pm$ 9.28)	55.56 ($\pm$ 12.74)	88.09 ($\pm$ 4.86)
Vehicle	12.37 ($\pm$ 10.75)	47.01 ($\pm$ 25.96)	80.94 ($\pm$ 58.6)	119.79 ($\pm$ 85.53)
RV	7.23 ($\pm$ 6.54)	32.71 ($\pm$ 19.22)	47.89 ($\pm$ 45.13)	78.59 ($\pm$ 56.25)