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| 12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited | | | 12b. DISTRIBUTION CODE | |
| 13. ABSTRACT (Maximum 200 Words) Taxol resistance is an important issue in relapsing ovarian cancer. Two approaches to address this resistance include the use of drug copolymers and drug targeting. In this proposal, paclitaxel covalently coupled to backbones of poly(L-glutamic) acid (non-targeted) or hyaluronic acid (CD44 targeted) as prodrugs will be evaluated in CD44 over-expressing human ovarian carcinoma i.p. (orthotopic) xenografts. We have documented the high CD44 expression levels of several such models (HEY, NMP-1, SKOV-3 and SKOV-3i.p.). Further, all but the former present as multi-focal tumors in the peritoneal cavity, most relevant to an initially surgically debulked or relapsing patient. All lines were sensitive in vitro to Taxol and to PGA-TXL, and Taxol caused rapid activation of caspases. Further, we have shown that dimethyl-sphingosine, a potent inhibitor of sphingosine kinase that catalyzes the formation of pro-survival/anti-apoptotic sphingosine-1-phosphate, causes supra-additive cytotoxic interactions with Taxol on these lines. We will compare the antitumor efficacy of PGA-TXL and HA-TXL in these CD44 over-expressing ovarian tumor models, and also determine whether dimethyl-sphingosine can further reduce the apoptotic threshold in these tumors. These approaches may be important new tactics in addressing ovarian tumor chemoresistance. | | | | |
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INTRODUCTION

Although Taxol has proven to be a most worthy addition to the chemotherapeutic regimens which can be offered to Stage III/IV ovarian cancer patients following surgical debulking, as with other drugs, evidence for resistance to taxanes has emerged. New agents and strategies are urgently needed to address Taxol-resistant ovarian cancer. Among the approaches to overcome drug resistance and to enhance the therapeutic index are the use of drug copolymers and drug targeting.

Drug copolymers are high molecular weight conjugates that can be actively transported to the endosome, where they are then cleaved to release free drug at this organelle. For DNA-targeting drugs, this may afford superior nuclear access compared to import via diffusion as occurs with free drug. Further, it restricts the gradient of export of conjugate-released drug via membrane-localized drug efflux mechanisms, e.g., P-gp170, that are clearly operant on free drug. This approach has proven to be successful for doxorubicin *in vitro*.

In vivo, other considerations may be more relevant, including distribution to tumor vs. normal tissue. High molecular weight drug copolymers may, on the one hand, 1) restrict diffusion-controlled uptake by normal tissues that occurs with free drug; but, on the other, they may 2) enhance extravasation across the abnormal tumor endothelium, thereby enhancing tumor localization compared to free drug.

In these studies, two paclitaxel copolymers will be evaluated. The first copolymer to be employed, poly(L-glutamic acid)-paclitaxel (PGA-TXL), has shown both reduced toxicity and greater tumor localization compared to Taxol in animal models, thereby fulfilling two expectations of copolymer behavior. PGA-TXL should be considered non-targeted, as there is no known receptor for its uptake, and most like is endocytosed by pinocytosis. The other paclitaxel copolymer is designed to be targeted to CD44 expressed on the tumor cell surface. It will be based on a hyaluronic acid (HA) backbone that will also serve as a ligand for receptor-mediated uptake by CD44. In this proposal, we will establish the toxicity, pharmacokinetics and anti-tumor efficacy of these paclitaxel copolymers in human ovarian adenocarcinoma xenograft models in nude mice; comparison of the efficacy of these copolymers with each other and to Taxol will provide evidence by which to judge the merits of tumor targeting of novel copolymers of established drugs.

BODY

Task 1 Synthesis and characterization of hyaluronic acid-paclitaxel (HA-TXL) conjugates with ester or acid-labile linkages

Since the original submission of this grant, we subsequently learned that our proposed synthetic schemes for HA-TXL were very similar to some reported just prior to our submission (1). Subsequent to becoming aware of this, we have tried to develop novel HA-TXL constructs using alternative linker strategies and other techniques. We also attempted to couple to a different backbone, chondroitin/chondroitin sulfate, both also serving as ligands for CD44. The intent was to develop an intellectual property position, as our long-term goal is also drug development; a patent position would greatly facilitate this endeavor.

After more than a year at these efforts, we are reluctantly coming to the conclusion that the prior reported chemistry for the synthesis of HA-TXL is quite appropriate and comprehensive, as we have so far been unsuccessful in supplanting it. Further, for reasons we cannot yet ascertain, the attempts to couple paclitaxel or paclitaxel adducts to chondroitin/chondroitin sulfate simply do not proceed. We do not believe that it is due to the presence of the sulfate group.

Hence, we are now returning to essentially our original plans: to couple HA to the NHS ester of paclitaxel via a hydrazide linkage, following the general procedures of Prestwich and coworkers (1). These efforts have an extremely high probability of success and we are completely confident that they will be so. In fact, Dr. Prestwich's group has engaged in a collaboration with a group at George Washington University and they presented an abstract at the 2003 AACR meeting on this. Their study did NOT deal with any human ovarian xenograft models, however, so our main work was in NO way pre-empted.

Obviously, our efforts to circumvent this prior art has been costly in time and effort, and has delayed our progress in the HA-TXL related Tasks. We regret this and are now poised to remedy this. Further, we recognize that Prestwich has synthesized an HA-TXL formulation with a modestly-sized HA backbone, ~11kD, with the reported intent to exploit renal clearance of this relatively small conjugate. However, we will focus on a much larger HA backbone as the pilot formulation, ~100kD. Since a major interest of ours as articulated in this proposal is the application of HA-TXL to i.p. therapy of ovarian carcinoma peritoneal xenografts, we propose that a larger conjugate should have a longer clearance time from the peritoneal cavity. Further, we propose that these favorable pharmacokinetic characteristics should lead to greater antitumor efficacy. If our formulation proves superior to his in this regard, we might be able to generate a proprietary position despite his prior art.

Task 2 Mechanistic Studies: Effects on Cell Cycle Distribution/Apoptosis and RAF-1 Kinase Activation

In these studies, we evaluated the responses of four human ovarian carcinoma cell lines, each of which is capable of growth as an i.p. xenograft model, to a concentration range of Taxol and PGA-TXL, with assessment of HA-TXL to follow. These lines were NMP-1, established via CDDP-selection *in vitro* from OVCAR-3 and subsequent i.p. passage, HEY, SKOV-3 and its

i.p.-selected line, SKOV-3i.p. We anticipated that the responses to the prodrugs would be more protracted than the response to Taxol, due to more immediate availability of free paclitaxel with the latter.

We observed that the losses in survival (as determined by dye assays) were time- and paclitaxel concentration-dependent, with generally low negative slopes on the concentration curves. For the NMP-1 cell line (Fig. 1), the IC_{50} at 120 hr for its response to Taxol was between 125 and 250 ng/ml, with even much lower concentrations still capable of causing significant losses of survival. For PGA-TXL, the IC_{50} was >500 ng/ml (Fig. 2). This difference in IC_{50} values in favor of greater sensitivity to Taxol is the opposite from the sensitivity of NMP-1 tumors to these drugs *in vivo* (2), PGA-TXL was active against NMP-1 and HEY tumor models, whereas they were highly Taxol-resistant. This underscores the favorable tissue pharmacokinetics of PGA-TXL in this setting.

With HEY cells, the pattern was quite similar to that observed with NMP-1, although these cells were slightly more sensitive (Figs. 3 and 4); the IC_{50} for Taxol was between 31 and 62.5 ng/ml, and for PGA-TXL it was 500 ng/ml. As noted, the HEY model also demonstrated much greater sensitivity to PGA-TXL than Taxol *in vivo* (2).

With SKOV-3 cells, the pattern was quite similar to that observed with NMP-1 and HEY, although these cells were less sensitive (data not shown); the IC_{50} at 120 hr for Taxol was between 250 and 500 ng/ml, and for PGA-TXL, a concentration of 500 ng/ml reduced survival to only 75%. SKOV-3i.p. cells, previously selected for i.p. implantation efficiency, were only slightly more sensitive to Taxol, with the IC_{50} at 120 hr for Taxol being between 125 and 250 ng/ml, and for PGA-TXL, a concentration of 500 ng/ml reduced survival to only 70% (Figs. 5 and 6).

To begin to define the mechanism(s) of paclitaxel-induced loss of survival, we treated NMP-1 cells with a low concentration (5 nM) of Taxol and evaluated caspase activation using a flow cytometric assay (CaspasTag). We anticipated that caspases might be activated by Taxol in these cells, and with much more rapid kinetics than was observed for loss of survival, as caspase activation should precede cell death as measured by dye uptake assays. Control cells displayed low levels of constitutive caspase activation (Fig. 7; 1.3%, lower right panel). By four hr, this caspase(+)/PI(-) population increased slightly to 3.4% (Fig. 8; lower right panel). At 24 hr, this population had increased substantially, to 7.6% (Fig. 9; lower right panel); some of the caspase(+) population had also become PI(+) (Fig. 9; upper right panel). We take this as preliminary evidence that caspase activation by Taxol could play a role in the death pathway. More studies are needed to confirm and extend this notion and to link it to other downstream regulators, e.g., Raf kinase.

We have recently become aware of and begun to develop evidence for the so-called "sphingolipid rheostat" model (3-5); in this model, the balance between pro-apoptotic sphingolipids, ceramide and sphingosine, and the anti-apoptotic or pro-survival sphingolipid, sphingosine-1-phosphate, determines cell fate. A key modulator of this rheostat is sphingosine kinase, which phosphorylates sphingosine to sphingosine-1-phosphate; the sphingolipid, dimethyl-sphingosine (DMSP), is the most potent known lipid inhibitor of this kinase. DMSP is also apoptogenic by itself. We have begun to evaluate the possible role of this rheostat in determining the apoptotic sensitivity of human ovarian carcinoma cell lines to paclitaxel *in vitro*. This may have *in vivo* relevance, as well; since we have been able to induce antitumor effects using liposomal-DMSP as monotherapy in a human breast adenocarcinoma orthotopic xenograft

model (6), we should be able to evaluate the effects of combination targeted paclitaxel/DMSP regimens in our i.p. human ovarian carcinoma xenograft models.

In the first studies to evaluate the responses to sphingolipids, we determined whether caspases were activated in NMP-1 cells by DMSP treatment alone. Based on the same control cells as shown in Figure 7, four hr treatment with 30 uM DMSP resulted in approximately a four-fold increase in caspase(+)/PI(-) cells: 5.1% (Fig. 10, lower right panel). After 20 hr of treatment, this population increased only slightly (6.3%), whereas the number of caspase(+)/PI(+) cells increased sharply (from 0.6% to 14.5%), as did the entire PI(+) population (2.3% to 26.5%; Fig. 11). This suggests that the initial activation of caspases by DMSP results in a phenotype that is quite rapidly converted to PI(+) cells; hence, the caspase(+)/PI(-) population remains at a near steady state, whereas the PI(+) population continues to increase. Further, it suggests that in this model, a primarily caspase-dependent apoptotic death process is induced by DMSP.

In the next phase, we began to test the effects in a survival assay of combinations of paclitaxel with DMSP, with each agent alone at minimally apoptotic concentrations. DMSP was added one hr prior to Taxol to attempt to blunt any rapid and concomitant activation of survival responses induced by the latter. In a representative experiment with HEY cells, 12.5 nM Taxol alone reduced survival to 63.4%, and 5 uM DMSP alone reduced survival to 75.1%; with their combination, survival dropped to 8.6%. Similar DMSP-mediated potentiation was observed with higher and lower Taxol concentrations.

With NMP-1 cells, this interaction was also evident: 5 uM DMSP alone also reduced survival to similar levels as in HEY cells, 75.9%. When combined with 2 nM Taxol, survival was reduced to 37.3%, whereas this level of Taxol alone brought survival to 71.3%.

This pattern was generally upheld with SKOV-3 and SKOV-3i.p. cells, as well. For example, with the former, 5 uM DMSP reduced survival to 86.7%; its combination with 12.5 nM Taxol increased the effectiveness of the latter, from 54.8% survival to 25.2%.

In light of these results, as an additional arm in the antitumor efficacy studies, we intend to explore whether concomitant administration of DMSP (e.g., as a liposomal formulation) might enhance the activity of the paclitaxel formulations against human ovarian xenografts.

Task 3 Pharmacokinetics: Cellular and IP Administration

These studies have not yet been undertaken, in large part because of the delays in the initial synthesis of HA-TXL.

Task 4 Efficacy Studies: Her-2/neu- and CD44- high and low expression models

To characterize the human ovarian carcinoma cell lines in which we will attempt to determine the validity of using CD44 as a target for paclitaxel copolymer internalization, we determined the distribution and magnitude of CD44 expression in NMP-1, HEY, SKOV-3 and OVCAR-3 cell lines. Both flow cytometric and immunoblotting techniques were employed. The results from flow cytometry are shown in Figures 12-14.

NMP-1 cells demonstrated significant and quite uniform expression of CD44, with the control mean channel fluorescence of 9.8 (Figure 12, top panel) rising to 39.2 after adding the antibody (Figure 12, bottom panel). Both distributions appeared symmetrical as plotted on the

cells demonstrated a substantial left shoulder (Figure 13). Further, these patterns of strong CD44 expression were also observed with SKOV-3 cells (Figure 14); there was a ~four-fold rise in CD44 mean channel fluorescence (from 9.6 for controls to 41.0 for stained cells) following antibody staining. In SVOV-3i.p. cells, this increase was more modest, ~three-fold (data not shown).

These results were generally paralleled by anti-CD44 immunoblotting of cell lysates (Fig. 15). Strong bands were observed for all the cell lines: NMP-1, HEY, and both of the SKOV-3 cell lines (5 ug of cell lysate shown). OVCAR-3 cells presented a complex behavior, which has previously been reported (7). Early passage cells were negative for or low expressors of CD44, but with continued culture, increased expression was detected (data not shown). Therefore, from these results, NMP-1, HEY and either of the SKOV-3 lines (the latter, Her-2/neu-high expression models) should all be appropriate CD44 high expression models for *in vivo* evaluation in the antitumor efficacy studies of PGA-TXL and HA-TXL. Further, of the ovarian carcinoma cell lines and models we have so far characterized, there does not appear to be an ideal CD44(-) control with which to evaluate the specificity of HA-TXL activity. For this reason, we will most likely 1) rely on pre-blocking with a CD44 ligand prior to HA-TXL administration to establish the role of the HA/CD44 interaction in antitumor efficacy, and 2) compare the activity of targeted HA-TXL to non-targeted PGA-TXL.

KEY RESEARCH ACCOMPLISHMENTS

- Documented the sensitivity of four human ovarian carcinoma cell lines, NMP-1, HEY, SKOV-3 and SKOV-3i.p., to Taxol and PGA-TXL
- Documented Taxol-induced caspase activation in NMP-1 cells
- Documented DMSP-induced caspase activation in NMP-1 cells
- Documented the supra-additive sensitivity of four human ovarian carcinoma cell lines, NMP-1, HEY, SKOV-3 and SKOV-3i.p., to Taxol and low concentrations of DMSP
- Documented the expression of CD44 by four human ovarian carcinoma cell lines, NMP-1, HEY, SKOV-3 and SKOV-3i.p., by flow cytometry and immunoblotting

REPORTABLE OUTCOMES

An abstract entitled "MRI for Serial, Non-invasive Monitoring of Human Ovarian Carcinoma Growth in Murine Xenograft models" by J. Klostergaard, E. Auzenne, J. Bankson and R. Price was presented at the 2003 AACR meeting in Washington, D.C.

CONCLUSIONS

We have demonstrated that four human ovarian carcinoma cell lines that grow as i.p. xenograft models are high CD44 expressors, that all are inherently paclitaxel-sensitive, that caspases are activated in response to paclitaxel treatment, and that their apoptotic sensitivity can be enhanced by inhibition of the sphingosine-1-phosphate pro-survival pathway. We will next establish the activity of HA-TXL in these models in vivo.

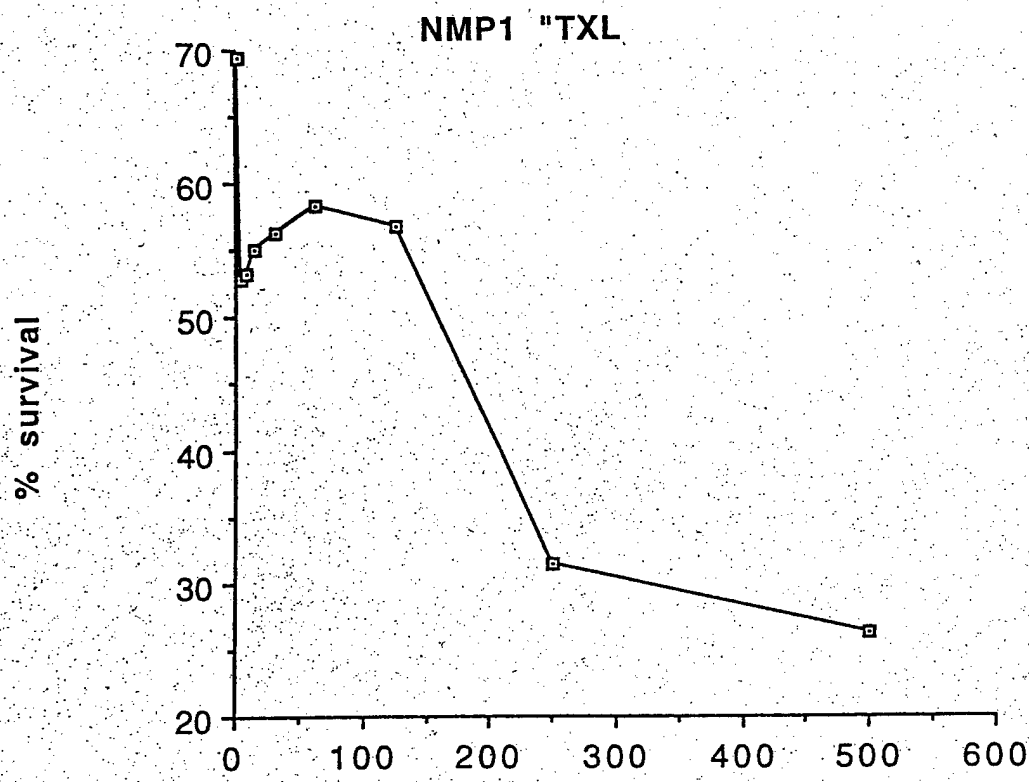
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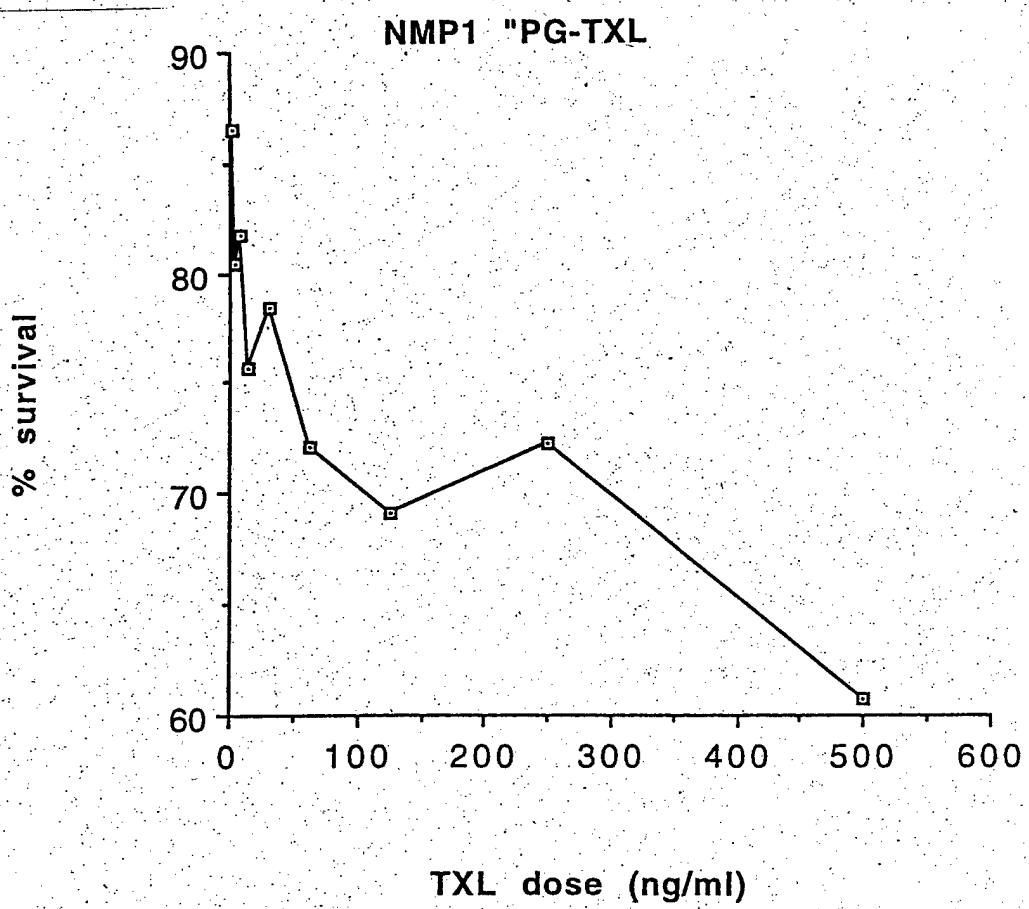
APPENDICES

MRI for Serial, Non-invasive Monitoring of Human Ovarian Carcinoma Growth in Murine Xenograft models. Klostergaard, J., Auzenne, E., Bankson, J.A., and Price, R.E. *The University of Texas MD Anderson Cancer Center, Houston, TX 77339*

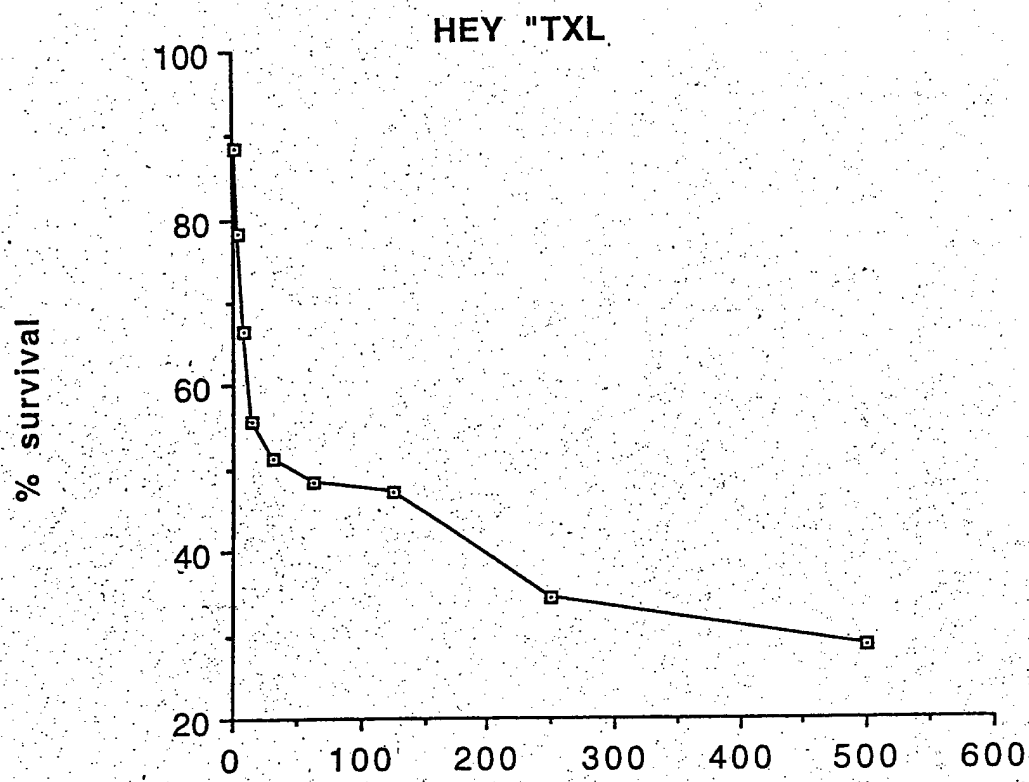
Characterization of the intraperitoneal growth of human ovarian carcinomas in nude mouse xenograft models by non-invasive means would allow longitudinal studies with serial monitoring of individual growth patterns and the use of real-time, response-guided treatment protocols. Female mice were inoculated i.p. with human ovarian carcinomas, HEY and NMP-1. Coronal T₂-, T₁- and post-contrast T₁-weighted images of the abdomen were acquired on Days 7, 14 and 21 post-inoculation using a Bruker 4.7 T, 40 cm *Biospec* MR system. Ascites formation with the NMP-1 model (Day 7) was detectable earlier by MR imaging than by other techniques; dispersed, multifocal solid tumor growth (Day 14) was evident by MR imaging and confirmed by necropsy examination. Solitary HEY tumors were detectable by MR imaging on Day 14 in the needle-track. MR data on Day 21 was correlated with block faced imaging of frozen tissue slices that correspond to the MR imaging planes. MR and other imaging modalities will be used in these mouse models to serially evaluate early tumor growth and treatment responses to standard and investigational agents. Supported in part by DOD Ovarian Cancer Research Program Grant OC000036 (JK).



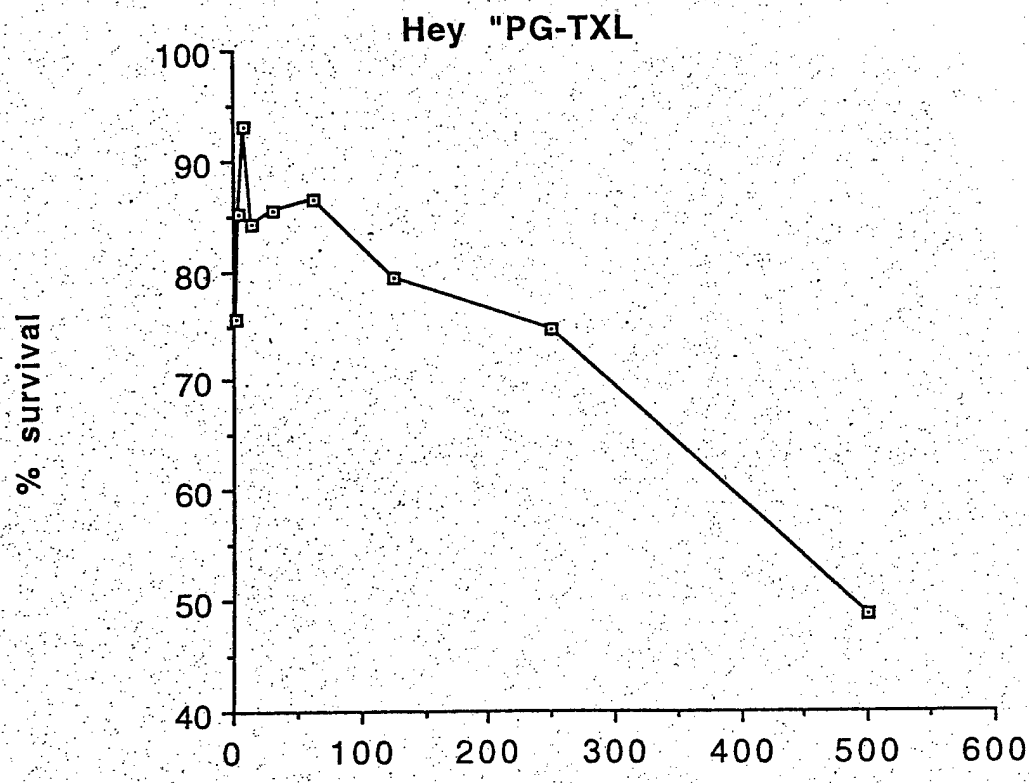
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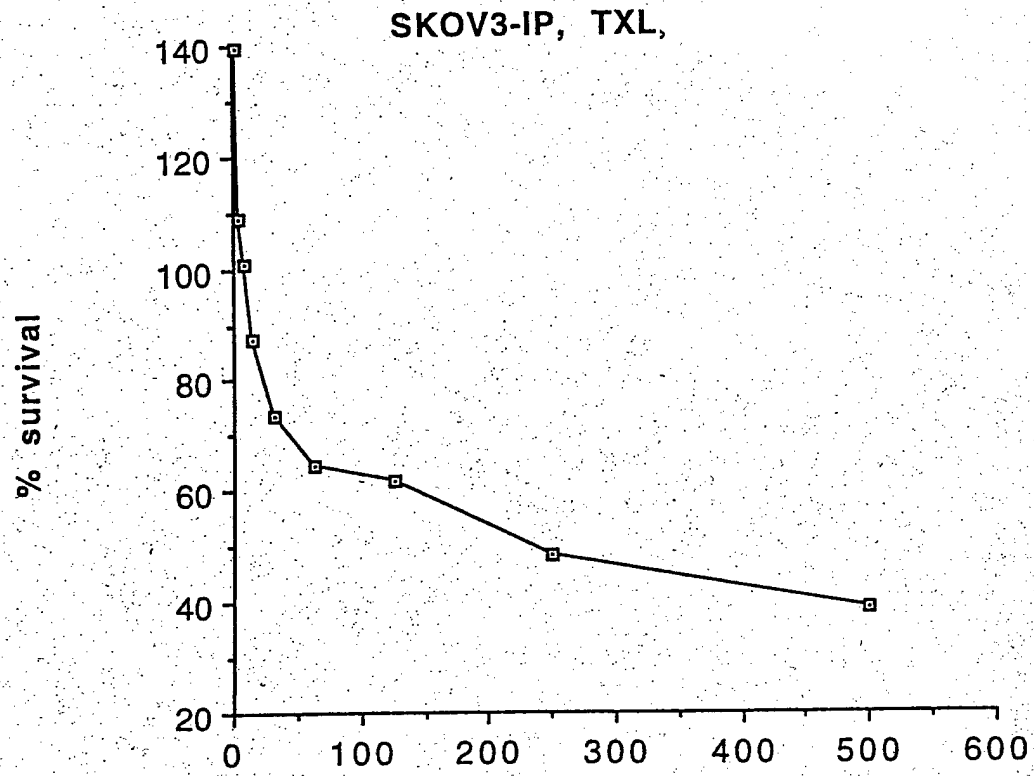


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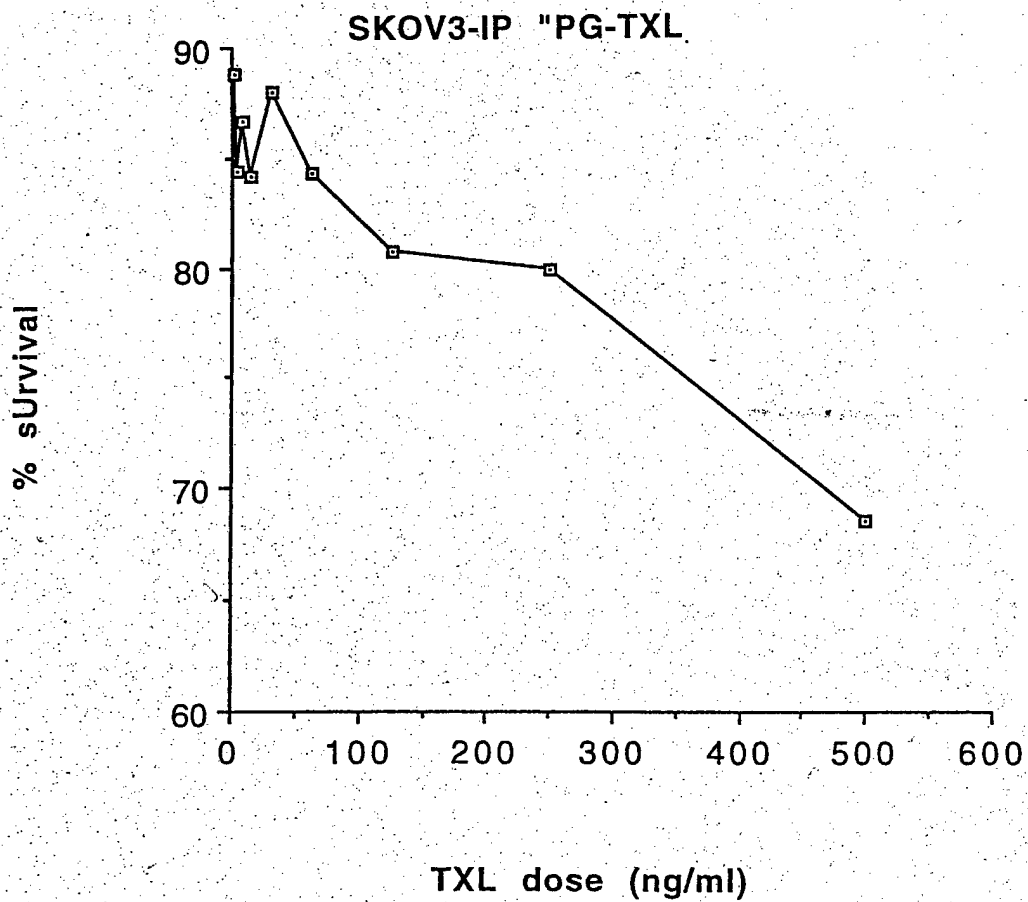


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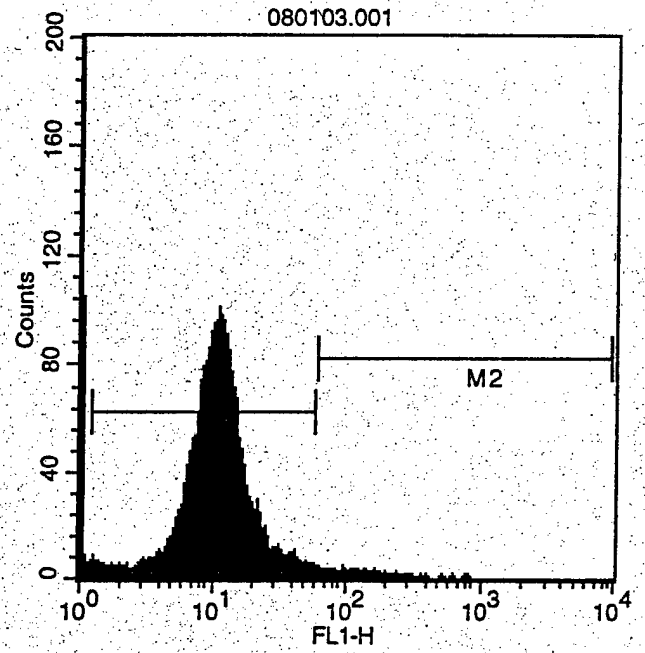
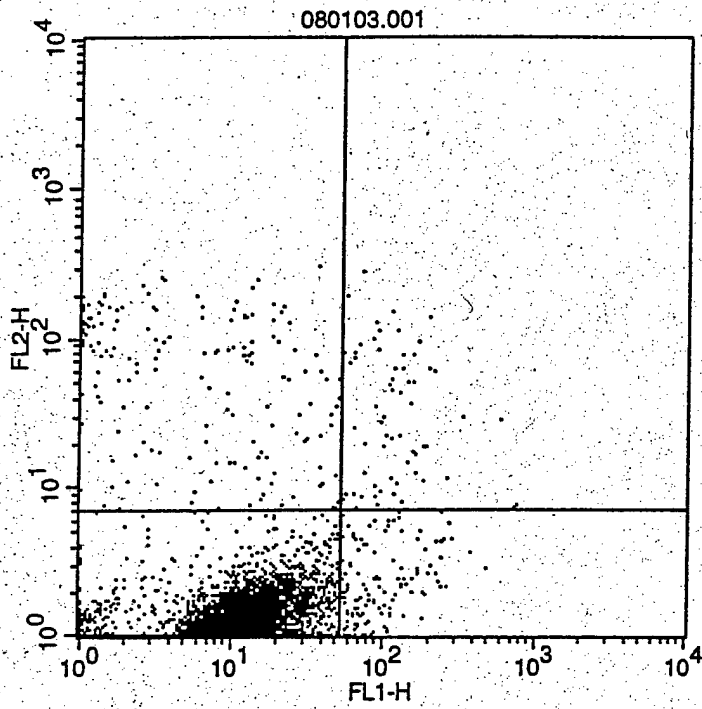
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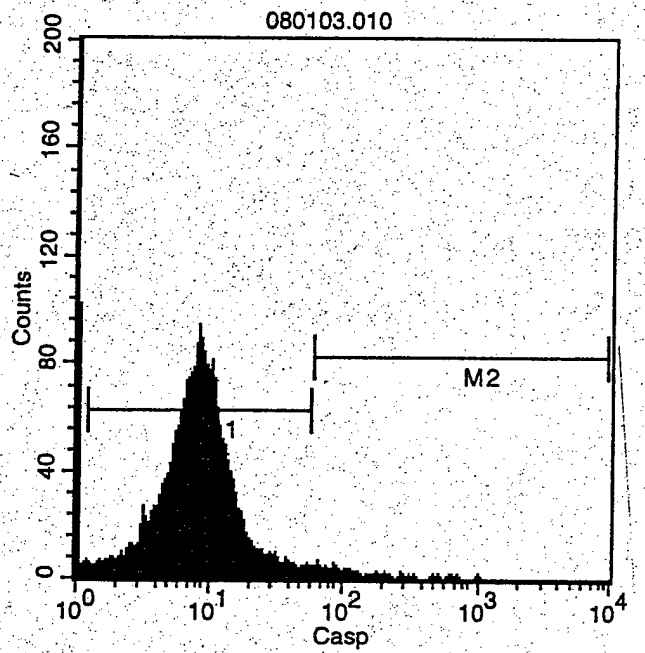
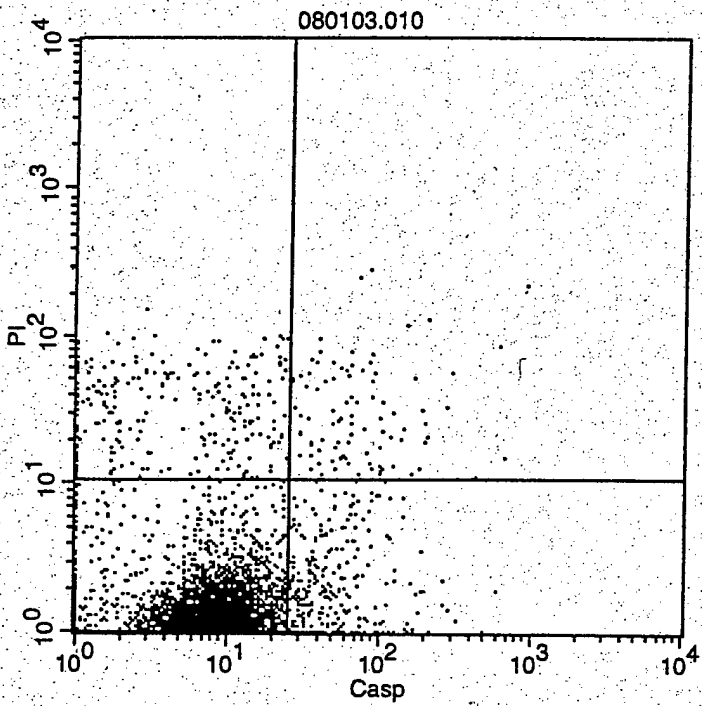
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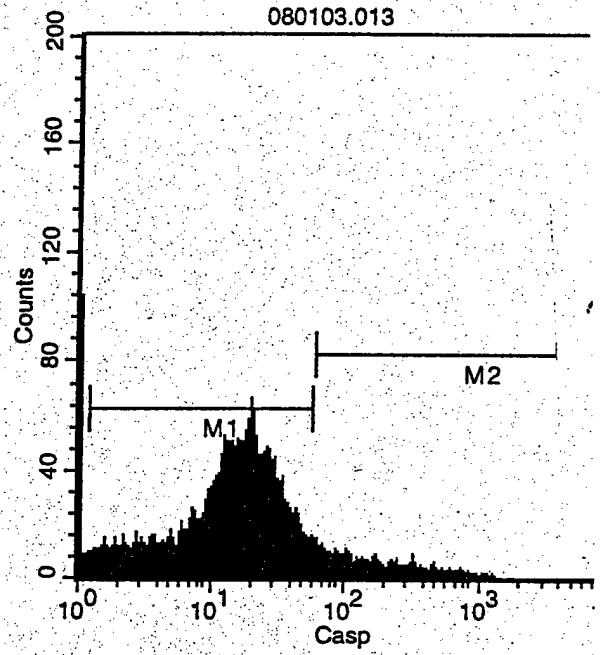
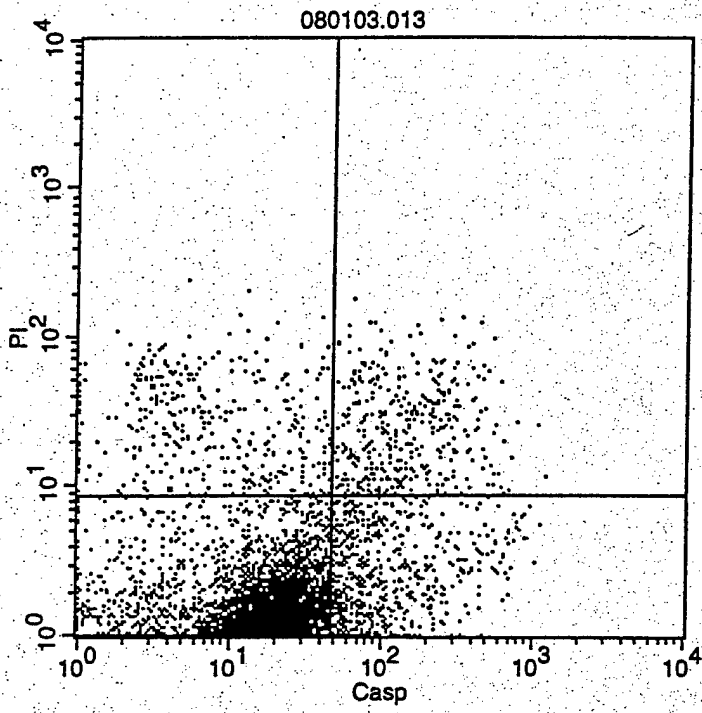
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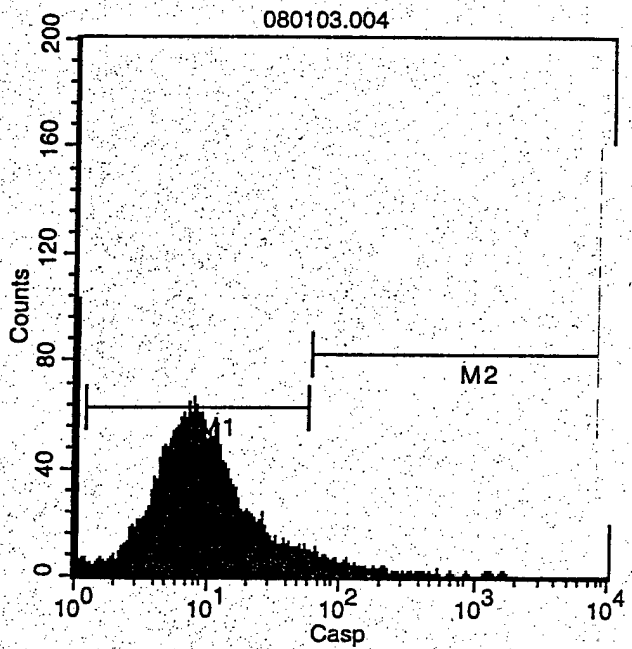
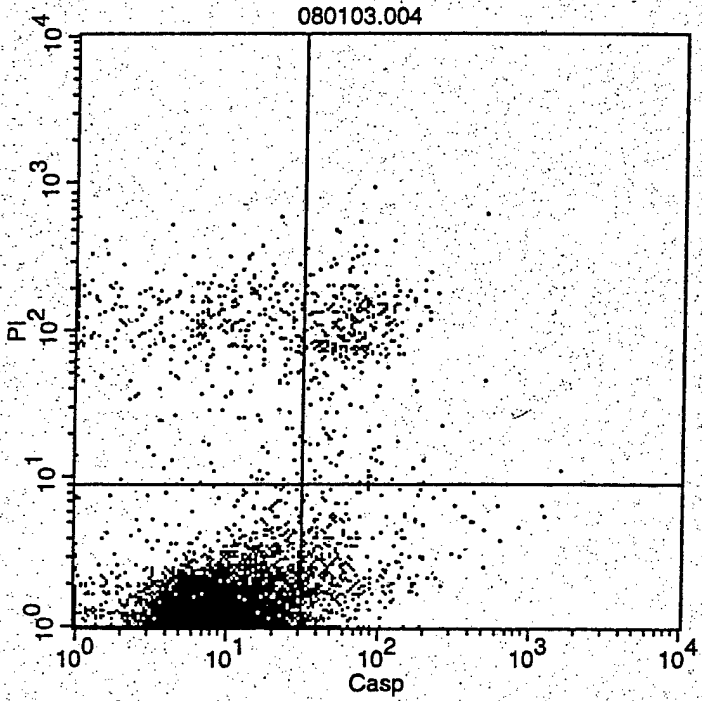
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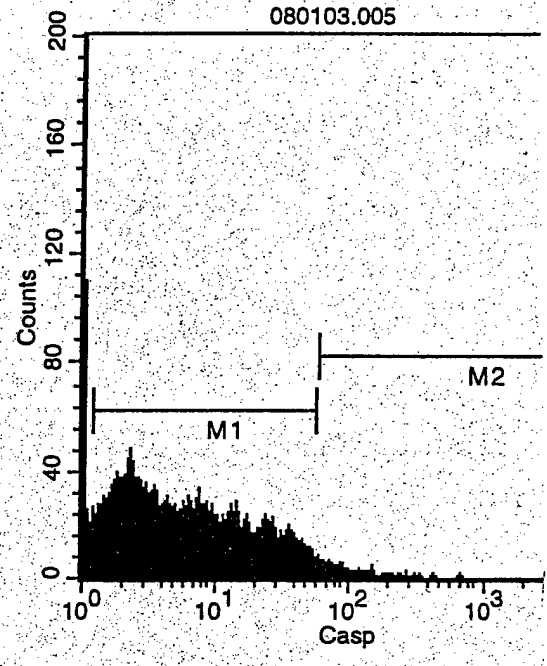
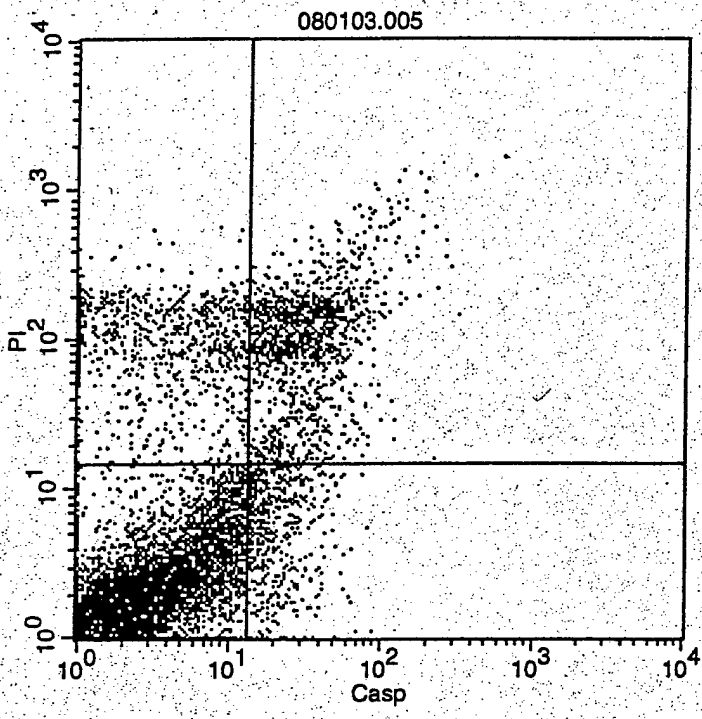
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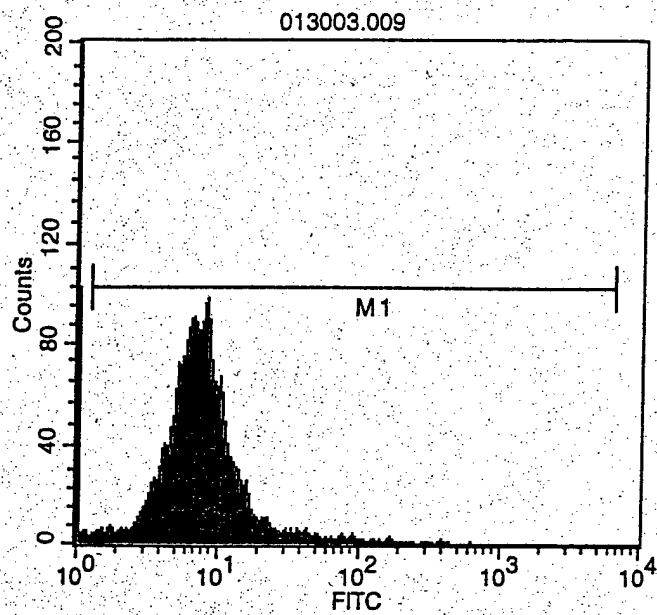
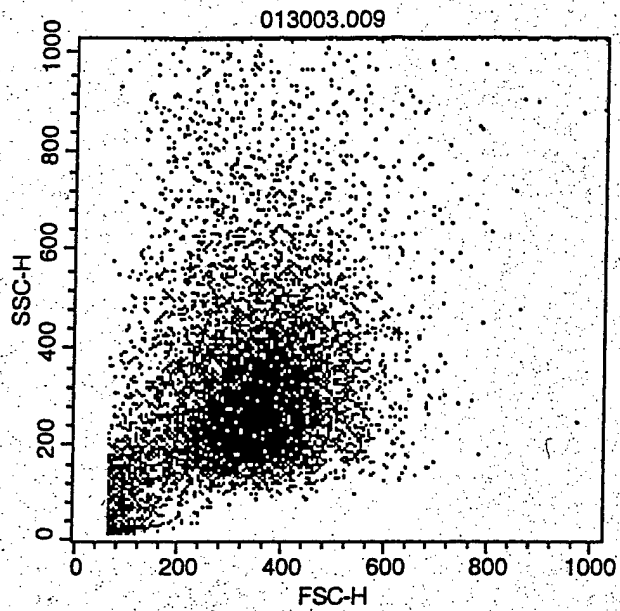


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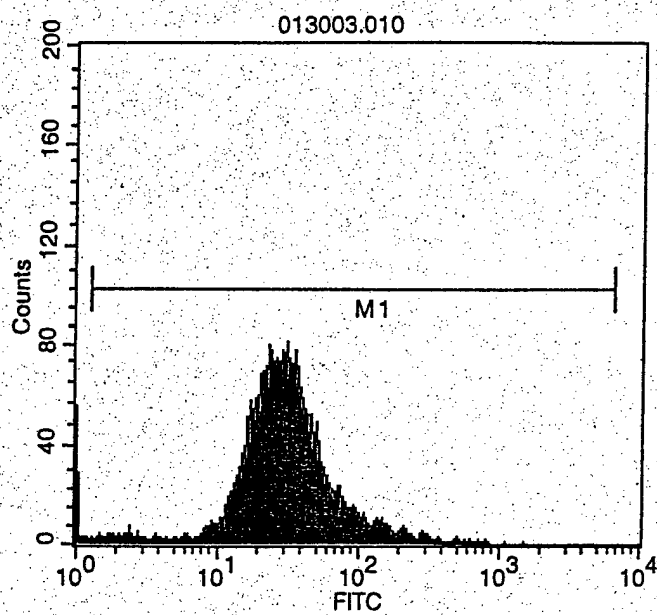
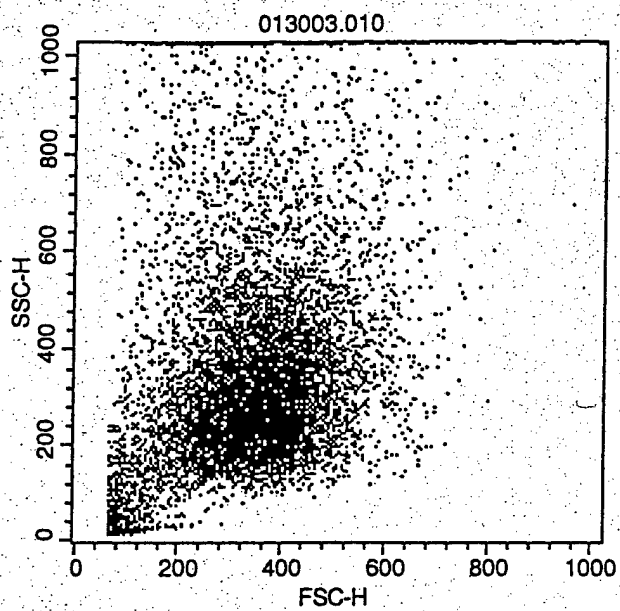


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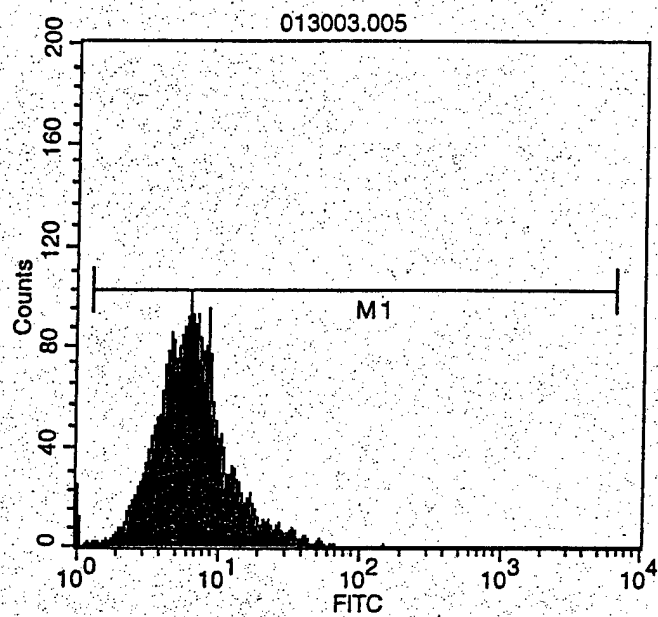
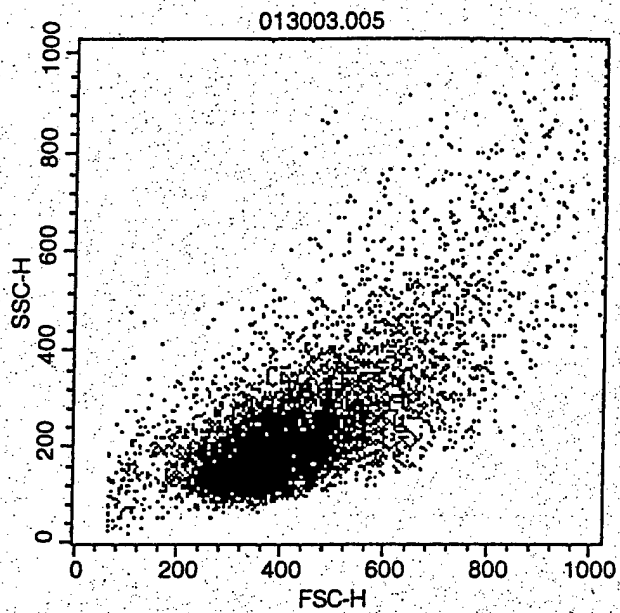




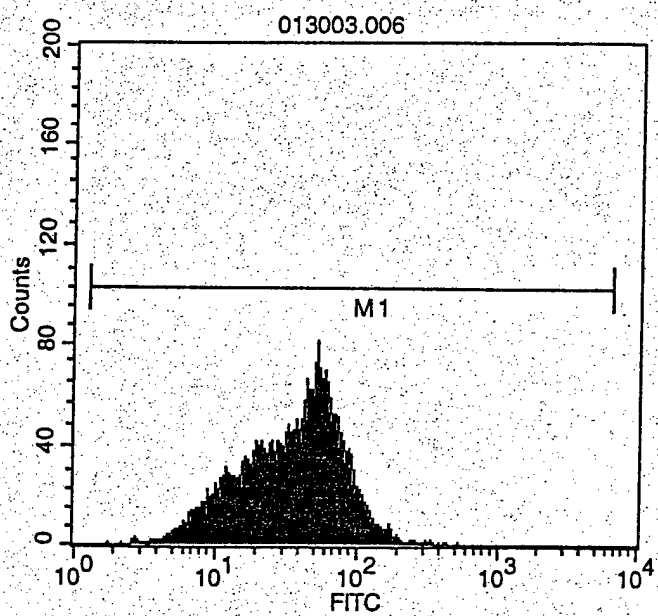
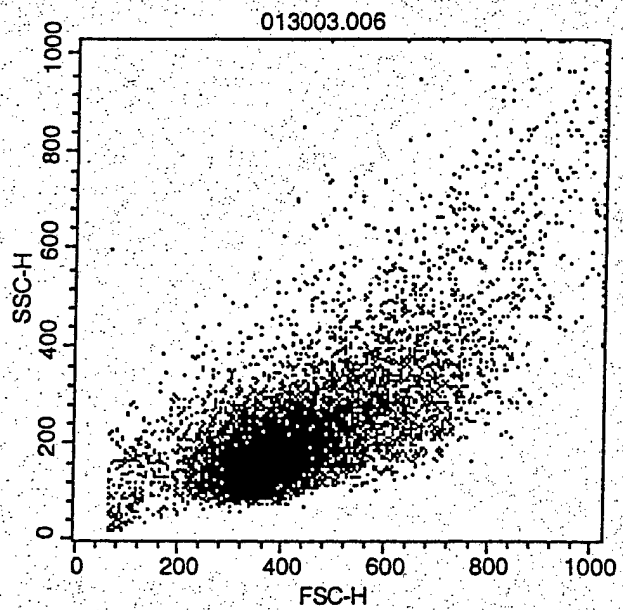
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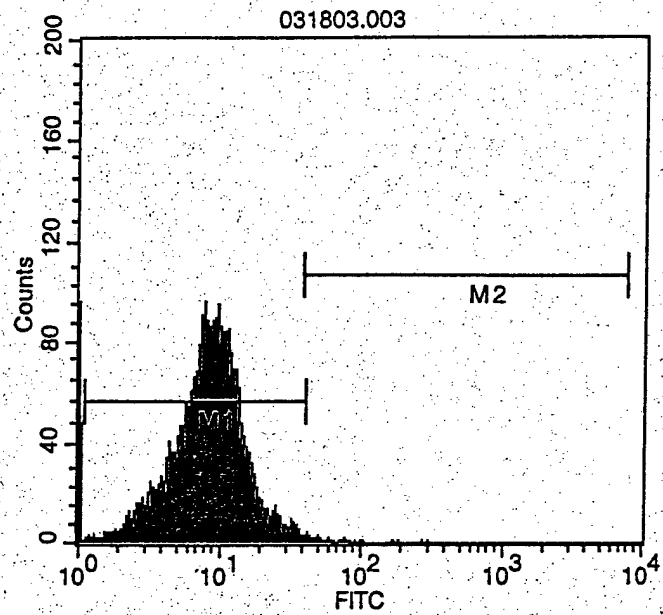
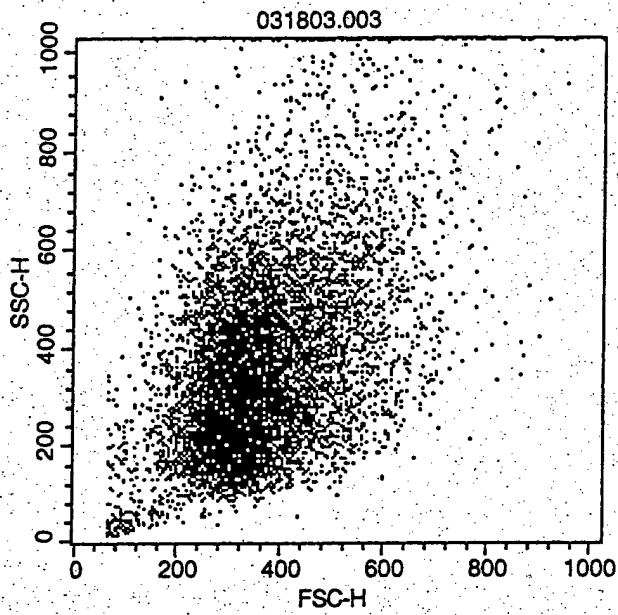
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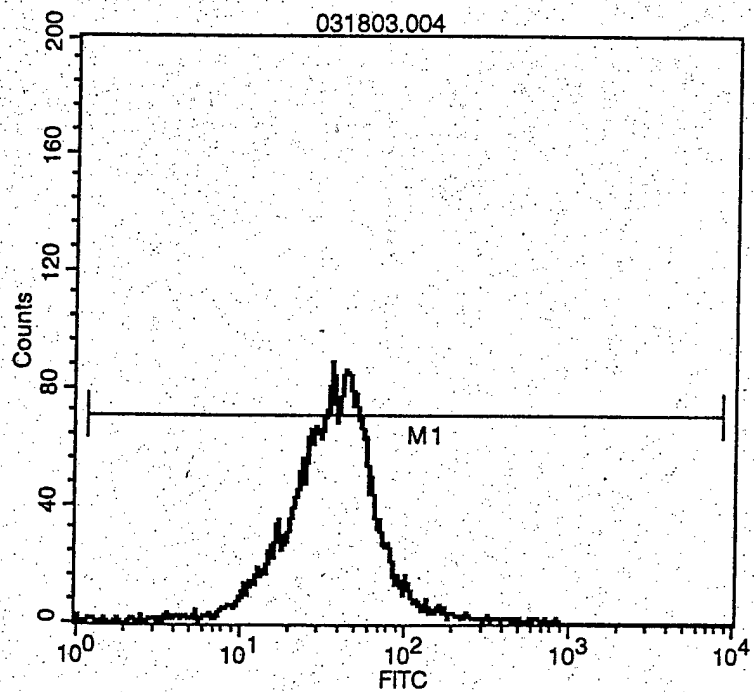
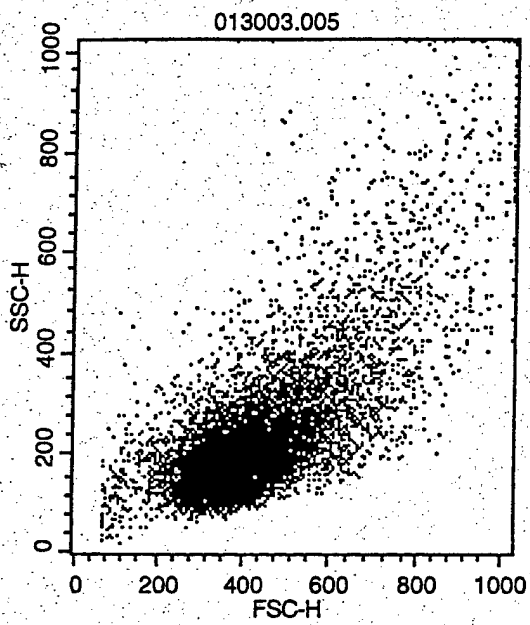
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1. NMP-1

2. HEY

3. SKOV3

4. SKOV3-IP