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Müllerian Inhibiting Substance/anti-Müllerian hormone: a potential therapeutic agent for human ovarian and other cancers

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Abstract

According to the 2008 American Cancer Society statistics, cancer remains the second leading cause of death in American today. Early detection, innovative surgery, new drugs and increased public education regarding avoidable risk factors, such as smoking, have had significant impact on the incidence and survival rates of many cancers, while overall death rates from all cancers have declined a modest 5% over the past 50 years. Ovarian cancer statistics, however, have not been as encouraging. Despite recent advances in the management of this disease, 5-year survival has not improved, and the search continues for rationally designed new treatments. Müllerian Inhibiting Substance is a strong candidate because it addresses many of the deficiencies of existing treatments. Namely, Müllerian Inhibiting Substance has little demonstrated toxicity, it complements the activity of known anticancer drugs, it is highly specific against cancers expressing its receptor and it inhibits the proliferation of drug-resistant tumors.

Keywords

anti-Müllerian hormone; Müllerian Inhibiting Substance; ovarian cancer

For nearly two centuries, the primary treatment of breast and gynecological cancers has been surgical; physically removing a tumor is an obvious goal. However, complete removal of a tumor is not always possible; therefore, adjuvant measures, such as radiotherapy and/or chemotherapy, are often employed. In addition to these approaches, adjuvant hormonal therapy has played a significant role in the management of these cancers, because the majority of them are responsive to endogenous hormones, if not actually dependent upon them. The rationale for manipulating the hormonal environment, of course, is that by ablating endogenous sources of stimulatory hormones or by administering antihormones and other biologicals that specifically inhibit cancer cell proliferation, there should be a

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beneficial effect in reducing tumor burden. This adjuvant endocrine approach to the management of cancer has its origins in the mid-to-late 19th Century. The early strategies that were employed included surgical menopause by ovariectomy, an operation first proposed for breast cancer in 1889 by Albert Schinzinger and later accomplished by George Beatson in 1895 (for a brief review of the history of ovariectomy for cancer see [1]). Subsequently, as gonadal and adrenal steroids, as well as pituitary and hypothalamic polypeptides, were isolated and characterized, it became possible to design hormonal antagonists. As a result, ovariectomy could be replaced, when appropriate, by systemic therapy using these compounds. While considerable success has been attained with breast and most reproductive tract tumors, with anti-estrogens and aromatase inhibitors for example, the results with epithelial ovarian cancers has been disappointing. Unfortunately, results with adjuvant chemotherapy have not been much better. With 5-year survival rates for ovarian cancer hovering around 30% [2], ovarian cancer could be considered a candidate disease for novel therapies.

As suggested by the title of this article, such a new, potentially nontoxic, therapy could be Müllerian Inhibiting Substance (MIS), also termed anti-Müllerian hormone (AMH). This gonadal protein hormone regresses Müllerian structures in fetal males, and it has also been shown to block the proliferation of cancer cells of Müllerian (and other) origin *in vitro* and *in vivo* in animals. Before a review of the preclinical MIS cancer studies can be presented, it is appropriate to provide some relevant MIS biochemistry as useful background information to understand better MIS as a biological response modifier with therapeutic potential.

Müllerian Inhibiting Substance/anti-Müllerian hormone

Background

Mammalian embryos begin development with the capacity to produce both female and male reproductive tracts. The Müllerian ducts will become the upper third of the vagina, the cervix, uterus, Fallopian tubes and the outer lining of the ovaries. The Wolffian ducts evolve into the seminal vesicles, vasa deferens and epididymides. Early in embryogenesis, both ductal systems grow independently of one another. After the genetic sex of the embryo is declared based upon its chromosomal makeup, the undifferentiated gonads become either ovaries or testes in response to the sex-determining region of the Y chromosome in the case of genetic males [3]. As gonads begin to differentiate, at approximately 10 weeks of gestation in the human, one of the reproductive tract primordia must be destroyed and the other will proliferate and differentiate.

Key insights into how this selection is accomplished at a molecular level were provided by Jost [4]. Before Jost's observations, it was believed that if embryonic testes were present, the testosterone secreted would promote male development and destroy the female tract. In the presence of ovaries, the male tract would undergo atrophy without testosterone and the female tract would be spared.

Jost performed experiments that proved testosterone alone could not be responsible for eliminating the embryonic female reproductive tract precursors, that is, the Müllerian ducts. He discovered that some other testicular factor, a 'Müllerian Inhibitor', as he named it, was responsible for ductal regression [4]. After further study, it was determined that Müllerian duct regression was the result of apoptosis, autophagocytosis, disruption of basement membranes and epithelial mesenchymal transformation of cells followed by migration of cells in the direction of the mesonephros [5–10]. In addition, Jost's studies showed that Müllerian ducts persisted and developed even in the absence of an ovary; thus, there seemed to be no ovarian contribution to Müllerian duct development *in utero*.

More than 20 years later, it was determined that MIS was a protein synthesized and secreted by the Sertoli cells of the testes in a number of mammalian species, including rat [11,12], bovine [13–15] and human [16,17]. Subsequently, it has been identified in all species examined, including birds, reptiles and fish [18–19]. Surprisingly MIS expression is not limited to the fetus or to males; it is a sexually dimorphic gene. Transcription of its gene in the testis appears to be regulated by a number of factors, including SF1, SOX9 and WT-1 *in utero*, and postnatal testosterone suppresses MIS secretion [20–25]. MIS inhibits steroid synthesis in ovaries [26] and represses the expression of ovarian aromatase [27] in fetal [28] and adult testes [29–31]. The converse has been observed for males, at least, where testosterone is reported to reduce MIS expression [32].

As stated earlier, MIS is only expressed in significant amounts by males during fetal life; however, postnatally, both granulosa cells in females and Sertoli cells in males produce the protein. In males, serum MIS levels remain at their highest until puberty, dropping to basal levels, thereafter. MIS mRNA and protein have been detected in sheep and mouse ovaries shortly after birth [33,34] and in low levels in prepubertal females by ELISA [35]. After puberty, female serum MIS levels reach those of adult males (Figure 1). The detection of MIS in male and female serum, even after the regression and differentiation of the Müllerian duct in the embryo and after birth [36–38], suggests multifunctional roles for MIS.

The measurement of MIS secretion by both sexes after birth was recognized immediately as potentially useful in a number of clinical circumstances, including ambiguous genitalia and undescended testes and delayed puberty, where serum testosterone is not particularly informative, and in sex cord or granulosa cell tumors. Variations of research assays used to measure MIS in biological fluids [32–34] are now commercially available; thus, serum MIS data in the management of pediatric endocrine, reproductive medicine and gynecologic oncology are becoming more widely used. If this protein is to be used for the treatment of certain cancers (as discussed later), having these reliable and sensitive MIS assays available now is critical for the analysis of human clinical trials.

Serum MIS, for example, is an excellent predictor of the presence of functional testicular tissue in cases of nonpalpable testes [39] and in cases of sexual ambiguity. Normal MIS levels for age predict normal testes, undetectable MIS suggests absent testes, where the cause of ambiguity may be androgen insensitivity, whereas lower than normal MIS is most probably a form of gonadal dysgenesis. Thus, serum MIS data can often be more valuable than hCG stimulation tests in the evaluation of gonadal status.

In adult females, on the other hand, serum MIS is a useful predictor of ovarian reserve [40,41] as well as a marker of response to IVF protocols [42–47], and it appears to be prognostic for pregnancy outcome for infertile couples with advanced female age [48,49]. Abnormally high serum MIS has been detected in patients with polycystic ovary syndrome [50], a finding that has provided a new area of investigation for this complex benign disease. MIS is also elevated in malignant gonadal disease.

Sex cord [51] and granulosa cell tumors [52] secrete MIS, and serum levels predict recurrence and response to therapy as well as tumor volume [53]. These tumors may also express the MIS receptor [54], but the fact that these tumors are unresponsive to the MIS they produce suggests a loss of function downstream from the receptor itself. As a result, these ovarian tumors are not targets for MIS therapy. Serum concentrations of MIS above the upper limit of normal for age is consistent with the presence of either one of these tumors; rising serum MIS reflects tumor growth, and decreases show response to therapy [51,55]. Inhibin B is another marker of these tumors, but MIS appears to be more specific because it is not synthesized elsewhere [56]. In numerous cases, serum MIS rises to

thousands-fold the normal range without any significant adverse reactions; therefore, MIS administered to cancer patients as a therapy may be well tolerated. Whether the recombinant protein is toxic will be the topic of detailed toxicology studies to be completed when clinical-grade MIS is available.

Although the function of MIS in the fetus is well established, its role after birth is the topic of a number of ongoing studies. MIS blocks meiosis II in the ovary [57], inhibits granulosa cell division and progesterone production [26], and modulates follicular development [58]. The concentrations of MIS in follicular fluid are inversely correlated with the granulosa cell proliferative index [59,42]. Similarly, in males, MIS affects Leydig cell development and blocks the steroidogenic enzyme CYP17 [30,31,60,61] and aromatase [27] transcription. Its spermatogenesis stage-specific expression in Sertoli cells in seminiferous tubules indicates that it must also be one of the factors that control that process [62].

The fact that the MIS receptor is expressed in pituitary and certain neurons suggests even more widespread non-Müllerian roles for the hormone [63,64]. The fact that motor neurons express both the MIS receptor and MIS implies a possible autocrine action of this receptor–ligand pair in some tissues [65]. In addition, the prostate gland [66,67] and ductal epithelium of the mammary gland [68], and some of their cancers [69] are possible targets for MIS (see later). The discovery that MIS receptor expression exists beyond Müllerian-origin structures, particularly in tissues with a sexually dimorphic nature, challenges investigators to examine the place MIS has in the growing list of developmental factors; it seems that the protein could have been MIS-named.

MIS/AMH protein

The MIS protein was purified initially from bovine testes [70–74] and later from recombinant human sources [75,76], using a specific rat embryonic urogenital ridge regression bioassay to monitor progress. Initially, rodent fetal and neonatal testes were selected as a source of MIS for purification, but because of availability and larger size, calf testes were later chosen for this important task.

Conventional biochemical approaches were taken to purify MIS from testicular secretions *in vitro* [77] or protein extracts [70–77], including dye and carbohydrate affinity, anion- and cation-exchange chromatography, and, once specific antibodies were produced, immunoaffinity chromatography was employed [78,79].

Compositional analyses revealed MIS to be a 140-kDa glycoprotein of approximately 15% carbohydrate by weight. Western analyses under reducing conditions suggested that MIS was actually a disulfide-linked dimer that was partially cleaved into smaller species, perhaps during the biosynthetic and secretion processes. The significance of these findings became more apparent when the *MIS* gene was cloned [75].

Based on the partial bovine MIS amino acid sequence data, a series of degenerate oligonucleotide primers were designed to facilitate the cloning of the bovine complementary DNA and, later, the human genomic sequence from DNA libraries of bovine testes and human placenta, respectively [75,76]. Sequence analysis of the genes revealed them to be weakly related to the transforming β -family of biological modifiers, with the most striking homology (28%) residing in the carboxy terminal domain. The 2.8-kb human gene contains five exons and four introns and is located on the short arm of chromosome 19 [80]. The deduced protein sequence of this gene contains a 25 amino acid secretion-specific signal peptide and a monomeric protein of 535 amino acids that, upon glycosylation at two putative N-linked sites, has a molecular weight of approximately 70,000 Da (Figure 2). Examination of the primary human MIS sequence showed the presence of a cleavage motif at residue

427, which explains the origins of the major cleavage products of the MIS fragments (12.5 and 55 kDa) appearing on reduced polyacrylamide gels of the purified protein. A weaker motif at residue 229 is also present, but its role in MIS action is unclear. Several studies have clarified the role of MIS carboxy terminal cleavage in the scheme of MIS action. The carboxy terminus results from processing by a biosynthetic protease, most probably a kex-like enzyme such as furin [81,82], into an amino terminal dimer (110 kDa) and a carbohydrate-free carboxy terminal dimer (25 kDa) held together in noncovalent association (Figure 2). It is the carboxy terminal domain that possesses the biological activity [81,83,84]. The amino terminal domain may contribute to proper protein folding and assembly during synthesis and/or it may increase the serum half-life of the carboxy terminus and enhance bioactivity [84]. There is no evidence that the amino terminus interacts directly with either the type I or type II receptors. The carboxy terminal sequence is extremely highly conserved, a fact that explains why MIS from many different species are all active in the rat *in vitro* bioassay used for MIS purification. A recombinant preparation of MIS carboxy terminus is commercially available for study; however, it is not suited for *in vivo* work because of its presumed short half-life in serum given the fact that TGF β -1 carboxy terminus, a gene family member with which MIS has structural homology, has a serum half-life of 2–3 min [85].

The human gene was transfected into Chinese hamster ovary cells, and the conditioned medium secreted from these cells was then used as a source of recombinant human MIS [86,87], as well as for the purification of the MIS in all of the cancer studies summarized in the following sections. MIS as purified from serum-free conditioned media is proteolytically processed, free of potential contaminants from bovine serum, hamster cells, mouse monoclonal antibodies [87] and significant amounts of endotoxins [69]. The bioactivity of the MIS preparations produced in the laboratory was assessed using the *in vitro* urogenital ridge Müllerian duct regression assay [12] before the MIS is used in anticancer assays.

At present, no commercial source of bioactive holo-MIS/AMH exists; therefore, the protein must be acquired from academic laboratories. The increased production for large-scale preclinical, pharmacology, and clinical trials will require commercial partnerships.

MIS/AMH receptor

The biological activity of the protein requires interaction with two very similar receptors, termed type I and II. Each is a single membrane spanning, serine-threonine kinase that, after cross-phosphorylation induced by binding to MIS, initiates a series of intracellular cascades leading to the control of cell-cycle-regulating proteins and the altered transcription of a number of genes, depending upon the target tissues. The type II receptor is responsible for ligand binding and the type I heteromer is the signaling receptor (Figure 3). The MIS type II receptor (MISRII) was cloned first by the Themmen laboratory [88] and subsequently by a number of other investigators [89–91]. The human gene, located on chromosome 12q13 [92], has 11 exons and ten introns.

The MISRII is expressed in Müllerian duct mesenchymal cells surrounding the adjacent ductal epithelium, as one might expect since the fetal mesenchyme directs Müllerian duct regression [93], and in fetal and adult gonadal Sertoli and Leydig cells, as well as granulosa cells [88–90], where MIS regulates testosterone [30,60,61], estradiol and progesterone synthesis [26,27], respectively. Functional MISRIIs have also been found in adult rodent uteri [90,94], the human endometrium [94], breast and prostate tissues [67,95] and, unexpectedly, in motor neurons in the mouse brain [65]. It is not yet understood what receptor-mediated actions MIS has on these tissues in normal adults, but MIS inhibits the proliferation of tumor cell lines derived from them (see later), supporting our hypothesis that MIS may be a useful adjuvant agent in the treatment of these diseases.

Josso and colleagues have studied a large number of patients with Persistent Müllerian Duct Syndrome resulting from loss of function mutations in the MISRII or the MIS molecule itself (for a review see [96]). In so doing, they have defined a number of hot spots in the gene, as well as polymorphisms present in the normal sequence. A common defect in the *MIS* gene is a truncation mutation that produces a protein lacking the carboxy terminal domain and that is, therefore, unable to cause Müllerian duct regression *in utero*. Heterozygotes of either mutation type, that is, receptor or ligand, have normal phenotypes; affected individuals are either homozygous for a given mutation or compound heterozygotes. Our group has added a newly identified MISRII mutant [97] to the list of the known MIS and MIS receptor mutants that contribute to the Persistent Müllerian Duct Syndrome phenotype [92,96,98].

Several different type I receptors have been identified as interacting with the type II receptor (Alk3, Alk2 and Alk6 [99–102]). How each type I receptor interacts with the type II receptor in human tissues is not yet known, although all are expressed in the human cancer cell lines studied thus far.

MIS/AMH & cancer

The concept that a naturally occurring growth inhibitor such as MIS could be an effective adjuvant treatment for cancer is attractive for a number of reasons. First is the issue of possible reduced toxicity. MIS is a naturally occurring inhibitor that has a mechanism of action that is completely dependent upon target cells expressing its specific receptor. Very few tissues express the MIS receptor; therefore, unlike cytotoxic chemotherapeutic agents, MIS should have limited side effects. It is not possible, of course, to predict the toxicity of a recombinant human protein in a clinical setting. This will be assessed in institutional review board-approved clinical trials using MIS that has been prepared to general manufacturing production specification in a commercial US FDA-approved facility. Second, because the mechanism of action of a naturally occurring biological is different from that of a chemotherapeutic drug, the effects of MIS as an adjuvant could be additive or even synergistic, thereby improving outcomes and perhaps also reducing the doses of toxic agents needed for a favorable response. Finally, MIS may be used as a delivery system for more toxic drugs to receptor-positive tumor cells, limiting exposure to nontarget tissues. Covalently attaching cytotoxic agents to MIS with protease-sensitive linkages would allow the drug to be internalized along with MIS after receptor binding, and normally present cytosolic enzymes would cleave the drug from MIS allowing it to function as usual.

The choice to focus initially on a few of the ovarian cancers for treatment arises from the fact that, compared with other potential MIS targets, they have the worst prognosis and might benefit significantly from this novel biological reagent, thereby offering a new approach to augment the efforts to find other useful strategies to employ. In effect, any tumor expressing functional receptors for MIS is a potential target for the material; however, fortunately, the others, listed later, have considerably better prognoses and will be the subject of MIS trials after experience has been gained with ovarian cancer.

Ovarian cancer

The potential for MIS as an anticancer agent for certain ovarian cancers has been a major focus of our laboratory, The Pediatric Surgical Research Laboratory of the Massachusetts General Hospital (MA, USA), since we hypothesized that any cancer of Müllerian origin, such as certain epithelial ovarian cancers [103], could be a target for MIS treatment three decades ago [104,105]. The idea that MIS could be used to treat ovarian cancer is predicted by the fact that the histology of the embryonic Müllerian ducts is recapitulated in the common ovarian adenocarcinomas that arise from the outer ovarian coelomic epithelium,

which, in the embryo, invaginates to form the Müllerian duct [5,6]. Results of more recent experiments, however, identify cervical and endometrial cancers as other potential targets, as well as several non-Müllerian cancers, including breast and prostate (see later). Thus, the original hypothesis can be extended to include any tumor that expresses a functional MISRII.

Before the hypothesis could be tested, several significant obstacles needed to be overcome. Purification of MIS from animal sources was sufficient for research purposes, but to treat patients, it is optimal to use the human protein. The solution was to clone the human gene sequence, and express and characterize the protein it encodes, ideally in sufficient quantity for human trials. Our laboratory has spent considerable effort optimizing purification protocols for the recombinant human protein secreted from mammalian cells and is exploring a number of alternative sources for enhanced production of MIS. The receptor(s) needed to be identified in order to develop reagents for their detection. Patients' tumors needed to be screened for the presence of receptors, because therapy would only be offered to receptor-positive patients. Preclinical trials in both *in vitro* and *ex vivo* systems with cancer cell lines and fresh tumor samples had to be carried out in order to confirm the possibility that the cancers were responsive to MIS. Finally, immunoassays to measure MIS in serum and other biological fluids had to be developed in order to test the pharmacokinetics needed to conduct clinical trials. With the exception of amassing sufficient quantities of clinical-grade recombinant human MIS/AMH for the toxicology, pharmacokinetics and Phase I clinical trials in humans, which have yet to be carried out, all of these intermediate goals have been met.

Epithelial ovarian cancer affects nearly 25,000 Americans each year, and it is the fifth most common malignancy in women, with a 5-year mortality of over 70% and more than 16,000 deaths per year [106]. Although the mortality rate is significantly lower in women with stage Ia or Ib disease, peritoneal seeding and metastatic spread accounts for the fact that fewer than 25% of women are diagnosed early in their disease process. Surgery and cytotoxic drug therapy produces favorable clinical responses in 50–80% of patients; however, unfortunately, the majority will relapse [2].

The earliest experiments began with *in vitro* studies using human ovarian cancer cell lines followed by a series of *ex vivo* experiments on these lines; thereafter, the work progressed to the examination of human ovarian cancer cells in ascites collected from patients with recurrent disease [103].

These initial studies began with partially purified bovine MIS, which suppressed the growth of a human ovarian carcinoma cell line *in vitro* [107] and in nude mice treated systemically with the bovine protein [108]. In addition, a large number of primary tumor cells that had been taken from patients with human ovarian and other reproductive cancers were inhibited *in vitro* in stem cell assays [109]. Importantly, more than 50% of the stage III ovarian patients from whom abdominal ascites were collected had cells that bound biotinylated recombinant human MIS in flow cytometry studies, expressed the type II receptor by PCR, and were growth inhibited when treated *ex vivo* [105] with recombinant human MIS. Highly purified human MIS also inhibited other human carcinoma cell lines of Müllerian origin, including OVCAR 3, 5 and 8, IGROV-1 and HOC-1, *in vitro* [110] and/or *in vivo* [111]. The observations on fresh surgical specimens and ascitic fluid, however, are perhaps the most significant, as they reflect what is true for patients and not cell lines. It is important to stress that MIS is not a cytotoxic agent [112]; MIS inhibits cancer cell growth *in vitro* by causing cell cycle arrest.

The fact that type II receptor expression is common among random samples of ovarian cancer cases is confirmed by two much more extensive studies using immunohistochemistry and/or real-time PCR that show approximately 70% frequency of receptor expression in over 300 cases in total [113,114]. An example of the immunohistochemical evidence of receptor expression has been presented by Bakkum-Gamez *et al.* (Figure 4) [113]. This group presents no evidence of the the MISRII in normal tissues. As predicted by the results of earlier studies, a number of other gynecological cancers also produce the type II receptor (Table 1). In contrast to the Bakkum-Gammez *et al.* study, Song *et al.* did detect MISRII expression in normal ovarian tissues [114] using *in situ* hybridization and real-time PCR techniques (Figure 5 & Table 2). Both studies reported similar frequency data for receptor expression in malignant and benign gynecological diseases.

Müllerian Inhibiting Substance will most probably be administered to patients as an adjuvant in combination with other drugs. Therefore, studying MIS downstream signaling mechanisms proliferation inhibition is necessary before using MIS in combination with commonly used cytotoxic drugs. It is important to test for synergy or additivity between MIS and any drug to make certain that they do not counteract one another. Studies to date show that MIS downstream signal transduction pathways include type I receptor(s), Smads, cyclin-dependent kinase inhibitors (p16) and cytokine-inducible pathways. MIS also increased the expression of p107 and p130 proteins [115]. An inhibitor of cell cycle progression, p16 functions by binding cyclin/cyclin-dependent kinase complexes, thereby preventing nuclear translocation. p16 is mutated in a number of patients with ovarian cancer and, in fact, it is mutated in the SKOV 3 cell line that does not respond to MIS [105]. p107 and p130 are pocket proteins such as Rb, which, incidentally, is mutated in OVCAR 8 cells. Expression of the downstream protein, E2F1, which is associated with apoptosis, was also enhanced by MIS treatment of OVCAR 8 cells [94].

These results suggest mechanisms different from those of most cytotoxic drugs, namely MIS interrupts cell-signaling pathways that stimulate cell division and arrest the cell cycle. If MIS and cytotoxic drugs can function in combination, it may be able to decrease the dose needed for either agent alone, potentially resulting in decreased toxicity and perhaps improved outcomes. Whether this hypothesis is true will require extensive study in clinical trials when a commercial-grade protein preparation is available.

Preclinical *in vitro* and *in vivo* trials for MIS in ovarian cancer were advanced greatly by the development of a new mouse model for the disease [116]. In these animals, the MISRII promoter is used to drive SV40 T antigen expression. Thus, cells expressing transcription factors for the MISRII will also express the Tag oncogene, which inactivates p53 and the pocket proteins, Rb, p107 and p130; as a result, the majority of animals form ovarian cancers [116]. The tumors closely recapitulate the phenotype of the most common human ovarian cancer, serous cystadenocarcinoma.

Cell lines derived from these mouse tumors are known as mouse ovarian carcinoma (MOVCAR) cells, and they are extremely sensitive to MIS for growth inhibition since, by definition, they all express MISRII [117]. MOVCAR cells also grow as explants in immunocompromised mice and they continue to produce MISRII. Tumors appear in a manner proportional to the number of cells injected into the mice; the more cells, the shorter the time to appearance of the tumor.

Intraperitoneal injection of recombinant human MIS into MOVCAR-implanted mice over a period of as long as 11 weeks slowed the time to appearance of the tumors, which were smaller than the tumors seen in vehicle controltreated animals [117]. No apparent toxicity

was noted despite the duration of MIS exposure in the mouse being equivalent to approximately 7 years of continuous cancer treatment in humans.

In reality, ovarian cancers arise from any of several genetic alterations, providing multiple options to resist conventional treatment; in fact, drug resistance is common among long-term survivors of this disease. It is rational, therefore, to attack multiple molecular pathways simultaneously in order to increase tumor inhibition. Such a plan may use MIS in combination with one of the approved ovarian cancer drugs. Having extensively studied the downstream signaling pathways of MIS and knowing, at least partially, the mechanism of action of the effective chemotherapeutic agents that are currently in clinical use, it will be possible to make more rational selections of therapies that MIS could be used in combination with.

The results outlined thus far, which show that MIS has the ability to inhibit cell growth with negligible toxicity, raises the possibility that MIS might also be additive or synergistic with commonly used cytotoxic agents. When this hypothesis was tested *in vitro* with MOVCAR cells, MIS proved to be synergistic with doxorubicin and rapamycin and additive with paclitaxel and cisplatin, and MIS retained its effectiveness against a human ovarian cancer cell line (IGROV-1) that had been made resistant to paclitaxel. Together, these results suggest that MIS can be given in combination with cytotoxic drugs and that it may be a new treatment for drug-resistant disease [118].

Data in support of this possibility come from studies on tumor-initiating cells, also called side population cells, isolated from MOVCAR cell cultures. These cells exhibit stem-cell-like properties in that they are tumorigenic and are resistant to some of the ovarian cancer drugs [119]. For example, MIS is still able to inhibit these cells from proliferating while they are moderately resistant to doxorubicin (Figure 6).

Other Müllerian cancers

The cervix is of Müllerian duct origin; therefore, it is logical to study whether normal cervix and cervical tumors express the MISRII receptor and if its cancers respond to MIS. Three human cervical carcinoma cell lines, CaSki, SiHa and C33A, express the MISRII protein by western analysis and they are all growth inhibited *in vitro* by MIS [115]. The receptor is detected in normal rat cervix by western blot analysis; however, as expected, it was not found in the non-Müllerian lower vagina or in the small intestine [115]. As was seen with the ovarian cancer cells, MIS specifically upregulated p16, p130 and p107, as well as E2F1 and E2F4 in the C33A cell line, without affecting E2F2, 3 or 5. Since p53 is mutated and Rb is absent in C33A, the MIS affect appears to be independent of both. When p16, p130, p107 or E2F1 were overexpressed in these cells, growth was dramatically inhibited compared with vectors alone [115]. These findings in cervical cancer further broaden the targets for MIS.

In addition to the cervix, Fallopian tubes and the ovarian coelomic epithelial lining, the uterus is also a Müllerian structure. The MISRII mRNA is expressed in the Müllerian ducts of the female rat embryo between days 13 and 19 of development and in the uterine mesenchyme after birth. The receptor protein was also found in a human endometrial cancer cell line, AN3CA [94], as well as in nonpregnant and pregnant adult rat uteri, by western analysis using the MIS receptor antibody developed in our laboratory [94] but not in the small intestine [94]. In the human, MISRII mRNA is expressed in human myometrium and endometrium of a postmenopausal uterus, and the protein has been observed by western analysis in endometrial tumors.

Two human cell lines, AN3CA and KLE, that express the receptor mRNA and protein were also inhibited by MIS. AN3CA, a cell line derived from a metastasis, showed significant growth inhibition (57–67% inhibition). KLE, a cell line derived from a poorly differentiated endometrial cancer, also demonstrated significant growth inhibition (44.9%) by MIS. Transient transfection of an MIS cDNA construct into these cancer cells produced growth inhibition of an endometrial cancer cell line. These transfection data support the conclusion that the observed effects are due to the MIS molecule rather than a contaminant in the purified protein preparation. Studies of the MIS molecular mechanism in endometrial cancer cells indicate G1 arrest and some apoptosis, as indicated by an increase in Caspase 3 cleavage products. Unlike the ovarian and cervical cancer cell lines, however, AN3CA cells do not express p16 or p21. MIS treatment increases p107 and p130 mRNA at 48 h, while E2F1 initially increases then decreases at 72 h [94].

Non-Müllerian cancers

Müllerian Inhibiting Substance type II receptor is expressed in locations other than Müllerian tissues and the gonads. It is expressed in the normal rat breast in an inversely proportional manner to the state of proliferation of the breast: the more proliferation, the fewer receptors. MISRII is present in virgin breast tissue but not in the lactating breast. The receptor reappears after weaning [68]. MIS enhanced I κ B-dependent DNA binding of NF- κ B, which resulted in the induction of IEX-1 mRNA [68], an immediate early gene induced by radiation, IFN- γ or TNF- α but not by TGF- β , via an NF- κ B pathway [120,121], regardless of the estrogen receptor status of the tumor cells. When female mice aged 5 weeks were treated with MIS, apoptosis was enhanced in the mammary epithelium [68]. Thus, it is likely that breast cancer could be a target for MIS therapy.

Müllerian Inhibiting Substance treatment of cancer may also include the prostate [66,67]. In addition, because MIS suppresses testosterone production [27–31,60], it might indeed exert a dual effect on prostatic cancer, a direct growth inhibition and an indirect effect of lowering testosterone. The MISRII and two candidate MIS type I receptors [97–102] are expressed in a prostatic cancer cell line as well as in multiple human prostate tumors [66]. MIS inhibits the growth of human prostate cancer cell lines, and it induces IRF1, with IFN- γ dramatically enhancing the inhibiting effects of MIS *in vitro* and *in vivo*. These findings in breast and prostate tissue significantly broaden the target population and the indications for the clinical uses of MIS. Interestingly, a very recent paper has reported a positive correlation between serum MIS and increased breast cancer risk [122]. The data from this prospective study appear to contradict the idea that MIS may suppress breast cancer tumor growth. Clearly, these new data warrant further investigation; however, the serum MIS data were collected when the subjects were premenopausal and before the breast cancers were detected. It would be very interesting to know what the serum MIS levels were at the time of breast cancer diagnosis. If serum MIS was undetectable, for example, the seeming paradox might be easier to understand.

Conclusion

The existing body of work suggests that MIS, a biological response modifier with very little demonstrated toxicity, may be used to treat any tumor expressing the MISRII. It appears that the type I receptor candidates, with which the type II may dimerize, are ubiquitous. The process of type I receptor selection is not known. There are *in vitro* and *in vivo* growth inhibition data for MIS with human and animal cell lines for ovarian, cervical, endometrial, breast and prostate cancers. Potentially, there is a large number of cancer patients who could benefit from MIS therapy. Approximately 25,000 new ovarian cancer patients are diagnosed each year, breast cancer strikes nearly 300,000 women each year, and prostate cancer

another 200,000 cases in the USA alone. This larger target population, without even including endometrial and cervical cancer patients, makes development of MIS as an anticancer therapeutic for more than ovarian cancers more compelling. Clearly, ovarian cancer is the prime target because there are the fewest options for therapy of the disease and it carries the most dire of prognoses.

Perhaps the most significant breakthrough in ovarian cancer treatment that accompanies the future use of MIS, however, is not that it should be highly specific and that it is a naturally occurring human protein that should have minimal adverse reactions. Rather, it is the possibility that MIS could treat drug-resistant disease, particularly that arising from tumor-initiating, stem-like cells. As stated earlier, most patients have a significant response to initial surgery and chemotherapy. It is the recurrent, drug-resistant disease that ultimately defeats the best efforts at eradication. MIS offers a new tool that targets not only drug resistance but also the cell type implicated as source of recurrent disease regardless of drug response status.

Clinical trials await the production of sufficient supplies of clinical-grade recombinant human MIS.

Future perspective

In the next 5–10 years, MIS should reach the clinic in two major areas. First, as an adjuvant treatment for ovarian cancer, the protein would augment, most probably additively, the activity of commonly used chemotherapies such as paclitaxel, cisplatin and doxorubicin. The result will be improved clinical control of the cancer and reduced toxicity of the drugs (i.e., less is needed with MIS as an adjuvant). Patients should have a better quality of life and survive longer. In addition, another use for MIS in the clinic will be as a treatment for a particular type of premature puberty in boys known as testotoxicosis, a problem associated with androgen-secreting tumors and low gonadotropins. MIS can directly inhibit the synthesis of testosterone and provide a new treatment strategy, because the usual hormonal therapies to reduce androgen synthesis do not work in these patients. Finally, the future of MIS in the clinic will center on its measurement in serum rather than as a systemic therapy. Serum MIS levels will continue to be useful in the management of granulosa/sex-cord tumors. The newer uses will be in monitoring ovarian reserve, and it may have predictive value in IVF protocols.

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- of considerable interest

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Executive summary

- Müllerian Inhibiting Substance (MIS)/anti-Müllerian hormone is a naturally occurring, highly specific inhibitor of proliferation.
- MIS's activity is restricted to cells expressing the MIS receptor.
- MIS/anti-Müllerian hormone has no measurable toxicity.
- Drug-resistant tumor cells respond to MIS.
- Tumor-initiating stem-like cells respond to MIS.

Figure 1. The pattern of MIS in human serum is sexually dimorphic and varies with age
 MIS in males is high at birth and, after a transient drop at approximately 2 years of age, levels are maintained until puberty. Serum MIS in females becomes measurable in the prepubertal period and is sustained until the menopause, after which it is no longer produced. The shaded bar demonstrates why serum MIS is useful in the perinatal period for evaluating cases of ambiguous genitalia. MIS is absent in genetic females and positive in cases with testicular tissue. The higher the values, the more normal the tissue. Serum testosterone is undetectable in normal males at this time. Rising serum MIS over normal limits in adult females (dotted line) is consistent with granulosa or sex cord tumors. MIS: Müllerian Inhibiting Substance.

Figure 2. The human *MIS* gene has five exons and four introns and encodes a monomer of 70 kDa with two N-linked glycosylation sites (circles)

The monomer forms a homodimer via disulfide bond formation and is activated by biosynthetic kex-like proteases to produce the 25-kDa carboxy terminal dimer, which is the bioactive domain of the molecule. The 110-kDa amino terminus stays associated with the carboxy terminus via noncovalent forces. MIS: Müllerian Inhibiting Substance.

Figure 3. The human MIS type II receptor gene, with its 11 exons, is larger than the nine-exon type I gene

They share several similar but not identical features, hence the different shadings, including extracellular ligand binding domains, transmembrane spanning regions and intracellular S/T kinase domains. Both types are required for MIS signaling, and mutations in the type II receptor are associated with phenotypic changes in humans.

MIS: Müllerian Inhibiting Substance; S/T: Serine/threonine.

Figure 4. Müllerian Inhibiting Substance type II receptor expression in benign ovarian epithelium and epithelial ovarian cancers

Immunohistochemical analysis shows no receptor expression in benign postmenopausal ovary and strong staining in serous epithelial ovarian cancer. (A) Postmenopausal ovary and (B) surface epithelium. Serous epithelial ovarian cancers at (C) 1.25 $\times$ and (D) 20 $\times$.

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Figure 5. Müllerian Inhibiting Substance type II receptor expression in normal ovarian tissues (A) PCR of Müllerian Inhibiting Substance type II receptor (arrow) in human ovary; lane A: secretory phase; lane B: follicular cyst; lane C: luteal cyst; lane D: borderline mucinous cystadenoma; lane E: borderline malignant cystadenoma; lane F: serous adenocarcinoma; lane G: endometrioid adenocarcinoma. (B) Light microscopy of a proliferative phase human ovary. Granulosa cells of the small antral follicle show expression of the Müllerian Inhibiting Substance type II receptor mRNA (400 $\times$). Adapted from [114].

Figure 6. Mouse ovarian carcinoma (MOVCAR) tumor-initiating cells, also known as SP cells, which can be separated from less tumorigenic NSP cells, respond to MIS *in vitro* (SP: 86%; NSP: 93%)

SP cells are moderately resistant to doxorubicin (30% inhibition) but are still nearly completely inhibited by MIS.

[†] $p < 0.001$.

[‡] $p < 0.0005$. MIS: Müllerian Inhibiting Substance; NSP: Non-side population; SP: Side population.

Müllerian Inhibiting Substance type II receptor expression patterns in malignant and benign gynecologic tissues.

Tissue	MISIIR expression	p-value
Ovarian cancer (all EOCs):	1125 out of 182 (69%)	0.008 ^{†,‡}
– Serous	22 out of 29 (76%)	0.002 ^{†,‡}
– Clear cell	10 out of 25 (40%)	0.14 ^{†,‡}
– Endometrioid	17 out of 31 (55%)	0.011 ^{†,‡}
– Mucinous	3 out of 3 (100%)	0.018 ^{†,‡}
Benign ovarian epithelium	0 out of 5 (0%)	
Dysgerminoma	17 out of 22 (77%)	<0.001 ^{§,¶}
Benign ovarian stroma	3 out of 24 (12.5%)	
Endometrial cancer (all histologies)	82 out of 109 (75%)	<0.001 ^{§,#}
Benign endometrium:	39 out of 139 (28%)	
– Proliferative	11 out of 68 (17%)	<0.001 ^{§,††}
– Secretory	16 out of 47 (34%)	<0.001 ^{§,††}
– Atrophic	12 out of 24 (50%)	0.018 ^{§,††}
Uterine malignant mixed Müllerian tumor	30 out of 51 (59%)	<0.001 ^{§,‡‡}
Leiomyosarcoma	15 out of 29 (52%)	0.004 ^{†,‡‡}
Endometrial stromal sarcoma	4 out of 18(22%)	0.27 ^{†,‡‡}
Benign myometrium	0 out of 11 (0%)	

Histologic subtype was available for 88 of the 182 EOCs.

[†] Fisher's exact test.

[‡] χ^2 test.

[§] Compared with benign ovarian epithelium.

[¶] Compared with benign ovarian stroma.

[#] Compared with overall benign endometrium.

^{††} Compared with endometrial cancer.

^{‡‡} Compared with benign myometrium.

EOC: Epithelial ovarian cancer; MISIIR: Müllerian Inhibiting Substance type II receptor.

Data with permission from [113].

Table 2

Frequency and intensity of Müllerian Inhibiting Substance and Müllerian Inhibiting Substance receptor expression in normal ovary and certain ovarian cancers.

Expression: tumor (number of cases)	MIS/AMHR II		MIS/AMHR II mRNA	
	<i>Frequency (%)</i>	<i>Expression intensity</i>	<i>Frequency (%)</i>	<i>Expression intensity</i>
Benign (n = 11)	45.45	0.64 ± 0.28	45.45	0.82 ± 0.31
Borderline (n = 9)	77.78	1.22 ± 0.32	55.56	1.00 ± 0.37
Malignancy (n = 40)	70.00	1.22 ± 0.16	75.00	1.43 ± 0.17
Epithelial (n = 18)	50.00	0.72 ± 0.21 [†]	55.56	0.72 ± 0.19 [‡]
Nonepithelial (n = 22):	86.36	1.64 ± 0.20	90.91	2.00 ± 0.21 [‡]
– Germ cell (n = 13)	76.92	1.62 ± 0.31	84.62	1.85 ± 0.31
– Sex-cord stromal (n = 9)	100	1.67 ± 0.24	100	2.22 ± 0.31

Values are the mean ± standard error.

[†] p < 0.05, malignant epithelial vs malignant nonepithelial tumors.

[‡] p < 0.001, malignant epithelial vs malignant nonepithelial tumors.

AMHR: Anti-Müllerian hormone receptor; MIS: Müllerian Inhibiting Substance.

Adapted with permission from [114].