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Clinical-translational strategies for the elevation of Nm23-H1 metastasis suppressor gene expression

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Abstract

Interruption of the tumor metastatic process is a new, thought provoking molecular target for the treatment of cancer. The Nm23-H1 metastasis suppressor gene stands as a validated molecular target owing to its reduced expression in many aggressive human tumors, and the reduction in meta-static potential in vivo upon re-expression in multiple cell lines. Several compounds have been identified which elevate Nm23-H1 expression in vitro including indomethacin, γ Linolenic Acid, trichostatin A, 5-aza-deoxycytidine, and high dose medroxyprogesterone acetate. Using a model of lung metastatic colonization by MDA-MB-231 human breast carcinoma cells, we demonstrated that high dose MPA reduced the formation of overt lung metastases by 37–46% and those metastases that formed were statistically smaller. A Phase II clinical trial of high dose MPA, alone or in combination with metronomic chemotherapy has recently opened.

Keywords

Nm23; Metastasis suppressor genes; Gene expression

Clinical rationale

Despite decades of drug identification and clinical testing, mortality from breast and other cancers remains unacceptably high. Much of the drug repertoire in breast cancer focuses on a few selected pathways: Cytotoxic chemotherapy interrupts DNA synthesis and/or induces DNA breaks. The estrogen receptor has been targeted using Selective Estrogen Receptor Modulators (SERMs) and more recently Aromatase Inhibitors. The Her-2 pathway, a receptor tyrosine kinase, has been targeted by the monoclonal antibody trastuzumab and more recently by the small molecule tyrosine kinase inhibitor lapatinib. These therapies were originally described as blocking the growth of the primary tumor and then went on to be used in the metastatic setting. Although control of the primary tumor is effectively accomplished by surgery, radiation, chemotherapy, and therapeutic targets, the majority of mortality comes from the spread of metastasis in patients. We propose that targeting the basic mechanisms of metastasis will identify new targets and drugs with therapeutic value.

Based on Surveillance, Epidemiology, and End Results (SEER) data, only 6% of breast cancer patients have detectable metastatic disease at the time of diagnosis and surgery [1]. For those patients, the process which eventually leads to clinically detectable metastasis has

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been completed. However, for the overwhelming majority of patients, the metastatic process is incomplete to varying degrees at the time of diagnosis of the primary tumor and represents a potential therapeutic target. Current attempts at interruption of the metastatic process include both general mechanisms such as integrin blockade, as well as site specific mechanisms such as Rank-L monoclonal antibodies for bone metastases. These represent a good beginning to target the basic mechanisms of metastasis; however, many important questions remain to be answered: Which pathways are “dominant”? How many redundant pathways can provide resistance to the interruption of a single pathway? What cancer types is this pathway prevalent in? When in the metastatic process does this drug need to be administered?

The most difficult issue for the development of anti-metastatic agents is their clinical testing. Agents are entered into dose finding Phase I trials with metastatic patients who have failed all approved therapies. In brief, we ask an agent to reduce the size of metastatic tumors for a partial or complete response, or to maintain them at their current size for stable disease. It is unlikely that an agent that interrupts the metastatic process will shrink an already established metastasis, thus these agents often “fail” in early clinical testing. Newer trial designs with meaningful pharmacodynamic endpoints are needed.

Restoration of Nm23-H1 expression

Loss of Nm23-H1 protein is observed in a subset of primary breast tumors (and other cancer types), and is generally correlated with poor patient prognosis (reviewed in [2]). Multiple transfection experiments indicate that, when Nm23-H1 expression is forcibly restored, metastases to the lungs, lymph nodes, and other organs are significantly decreased [3]. These data validate Nm23-H1 expression level as a molecular target for cancer therapy.

Several different interacting proteins have been described for Nm23-H1 in the past. One of potential interest for drug targets is its interaction with p53. Indeed, Nm23-H1 has been shown to be up-regulated by p53 [4]. In addition, Nm23-H1 may interact with STRAP, an interaction that is disrupted by p53 signals releasing both partners to bind to Mdm2 [5]. This frees p53 to induce apoptosis and cell cycle arrest.

The question is what aspect of Nm23-H1 is important? Multiple types of alterations have been reported including reduced protein and mRNA expression levels, allelic deletion, and occasional mutation. Cropp et al. examined a panel of human breast carcinomas for several of these characteristics. Allelic deletion of the Nm23-H1 gene did occur, but did not correlate with poor patient prognosis; and no mutations in the Nm23-H1 coding sequence were found. Only reduced Nm23-H1 protein expression, regardless of allelic deletion status, significantly correlated with poor patient survival [6]. The data suggested that, while *nm23-H1* mutations are rare and that allelic deletions occur, Nm23-H1 protein levels were most important. This suggested that Nm23-H1 expression was simply “turned off” and that a compound could be identified to turn gene expression back on with potential therapeutic activity.

Using cell lines in vitro, a number of compounds have been reported to stimulate Nm23-H1 expression (Table 1). Many of these compounds are nonspecific, which may represent an advantage or a disadvantage depending on the balance of other therapeutic versus stimulatory effects. The experience with the DNA methylation inhibitor 5aza dC provides a useful caution in the interpretation of cell line data. While this agent reversed the DNA methylation pattern of a CpG island in the *nm23-H1* promoter in two metastatic breast carcinoma cell lines, examination of multiple CpG islands in 20 human breast carcinomas found no differences in their DNA methylation status—regardless of whether the tumor cells expressed high or low Nm23-H1 protein levels.

While down regulation of Nm23-H1 protein is associated with poor prognosis in a wide variety of tumors, it is not without its exceptions. Increased levels of Nm23-H1 were shown to be associated with worse prognosis in neuroblastoma [7, 8] and hematopoietic tumors such as lymphomas [9, 10]. Therefore, while increased levels of Nm23-H1 protein are desirable in most solid tumors, in childhood tumors, and hematopoietic tumors, the opposite effect would be desirable.

Identification and validation of medroxyprogesterone acetate (MPA) as a lead compound

A promoter analysis was carried out to rationally identify compounds that could lead to elevated Nm23-H1 expression. To accomplish this, a promoter fragment was cloned onto a reporter gene and deletion constructs tested for activity. A 248 bp region was identified that regulated reporter activity by two-to fivefold. The region contained a cassette of transcription factor binding sites present in the MMTV-LTR. Comparison of these sites to other breast cancer promoters confirmed their potential importance. The cassette was also found in the Wap promoter, and the promoters of milk genes. Deletion of these sites reduced reporter expression and confirmed their functional involvement in regulating *nm23-H1* expression [11].

In the MMTV-LTR, this cassette of transcription factors is regulated by glucocorticoid response elements (GREs). Based on this evidence, dexamethasone was chosen and elevated the Nm23-H1 expressions of MDA-MB-435 and -231 metastatic breast carcinoma cells when cultured in a corticosteroid-free medium. Unfortunately, dexamethasone was ineffective at increasing Nm23-H1 expression when the endogenous levels of corticosteroids in fetal bovine serum were present. Thus, dexamethasone was active in physiologic but not pharmacologic ranges.

Further investigations revealed a possible new use for an old drug, Medroxyprogesterone acetate (MPA), which binds the progesterone, androgen, and glucocorticoid receptors (GR) [12]. This compound has a long clinical history, and at low doses it is used as a contraceptive. For many years it was combined with estrogen in hormone replacement therapy (HRT), where epidemiological studies have shown that HRT is deleterious as it elevates the risk of breast cancer.

However, the effect is different at higher doses, where MPA exerted suppressive effects on breast cancer in animal models. Several clinical trials were conducted largely before the development of the SERMs. High-dose MPA was tested as a single agent and in combinations in advanced breast cancers as a hormonal treatment (rev. in [13]). Although some responses were found, an optimal dose and schedule was never established, favoring tamoxifen. However, two of the older trials that used long-term MPA dosing reported 12- and 13-year follow-up data, and a retrospective subset analysis suggested a benefit in post-menopausal patients. A total of 950 patients were randomized to chemotherapy with or without a six-month course of MPA, given in a one-month induction, five-month maintenance protocol [14, 15]. In both trials, the MPA-treated subsets of patients had improved disease-free survival ($P = 0.01$ in one trial; in the other, $P = 0.06$ for node-negative and $P = 0.002$ for node-positive patients). Longer overall survival was noted in the postmenopausal arm ($P = 0.02$) in one trial indicating that in post-menopausal subset, high dose MPA was beneficial.

While the separation from the post-menopausal subset may be a statistical artifact, biology suggests not so. Estrogen combined with MPA was demonstrated to be deleterious in the form of HRT, so it is reasonable that MPA should not be administered to patients that are

pre-menopausal. Intriguingly, patient responses were not well-correlated with progesterone receptor expression [14, 16, 17], which suggested that more recent data showing that interaction of MPA with the GR may be relevant.

MPA, at high doses, elevated the Nm23-H1 expression of PR-metastatic MDA-MB-231 and -435 breast carcinoma cell lines in vitro and inhibited their anchorage-independent colonization [18]. An interaction with the androgen receptor was ruled out using inhibitors, leaving the GR as the target. In order to determine if Nm23-H1 induction or alternatively other effects were responsible for the inhibition of anchorage-independent growth by MPA, the MDA-MB-231 breast carcinoma cell line was transfected with an antisense *nm23-H1* construct so that MPA could not elevate Nm23-H1 expression. Approximately 90% of the colonization-inhibitory effect of MPA was abrogated in the antisense transfectants, which indicated that elevation of Nm23-H1 expression was a significant factor in this phenotypic effect of MPA [19]. Other studies have reported that MPA can advance experimental mammary cancers [20]. These effects are mediated through the progesterone receptor and use, at least, a log higher concentration of MPA. Thus there is a defined window of MPA concentration where it appears to be inhibitory.

In order to test the hypothesis that high-dose MPA could stimulate the Nm23-H1 expression of breast cancer cells through the GR and inhibit metastatic colonization, we used a mouse model of experimental lung metastasis by the ER and PR-negative (GR positive) MDA-MB-231 breast cancer cell line. Because of the window of MPA inhibitory activity, care was taken to administer this compound to mice at doses that were clinically relevant. We conducted a dose and schedule study of MPA, administering it to mice by different routes and in different doses, and determined the serum concentration of MPA at multiple timepoints using a HPLC assay. These data were compared to pharmacokinetic data published for the early high dose MPA trials [21]. Two doses were identified that produced serum MPA levels in mice comparable to those achieved in humans that responded to high dose MPA therapy [22].

Using a mouse model we attempted to target metastatic colonization of the lung by an aggressive breast cancer cell line in mice. Estrogen-and progesterone receptor-negative MDA-MB-231 breast cancer cells were injected via tail vein into immunocompromised mice [22]. Micrometastases formed in the lungs 4 weeks later, at which point mice were randomized to vehicle or MPA, given on a 1-month induction and thereafter maintenance schedule. MPA reduced the number of gross pulmonary metastases by 33–62%. We noted that the size of metastases from the MPA-treated mice was smaller compared to the vehicle control. To quantify this effect, all of the surface lung metastases were measured in their largest single dimension. A threefold reduction in large metastases (>3 mm) was noted in the MPA arms. The expression of Nm23-H1 was high in only 13% of the control mice compared to 43% of mice in the MPA-treated arm. Side effects in the mice were limited to weight gain, which was significant, although there was no difference in bone density, mammary histology, or lean/fat tissue ratio [19].

Clinical trial of high dose MPA

Previous studies have reported a potential anti-angiogenic effect of high dose MPA [23, 24], although at other doses MPA may play a widely different role in angiogenesis regulation [25]. Based on these data, as well as the Nm23-H1 studies, a Phase II trial has been initiated to test this new potential application of MPA (Kathy D. Miller, PI, Indiana University). The primary objective is to determine the clinical benefit of MPA montherapy and MPA + low-dose oral cyclophosphamide and methotrexate (“metronomic therapy”, IdoCM) in postmenopausal patients with refractory hormone receptor-negative metastatic breast cancer.

A starting daily oral dose of 1 g MPA will be administered and increased to 1.5 g if serum concentrations are <50 ng/ml. In a second cohort, “metronomic” IdoCM will be administered based on its reported anti-angiogenic activity [26]. Preclinical studies suggested greater activity when metronomic chemotherapy is combined with a second anti-angiogenic agent [27, 28].

Several pharmacodynamic measurements will be performed to determine whether the levels of Nm23-H1 are affected in this trial. The formalin-fixed, paraffin-embedded block from the patient’s primary tumor will be stained for Nm23-H1 expression to determine if those patients that responded to therapy had low Nm23-H1 expression. Optimally one would like to determine that the metastatic lesions have low expression of Nm23-H1, but this is impractical to accomplish. However, multiple studies indicate that metastases express Nm23-H1 levels that are either comparable or lower than that of the matched primary tumor [29–33]. In addition, multiple skin biopsies will be obtained from consenting patients in order to determine if Nm23-H1 expression is elevated. An elevation in the Nm23-H1 expression of the basal skin layer was observed in preclinical studies (Steeg, unpublished data). For the potential anti-angiogenic effects of MPA, plasma samples will be taken from each patient and levels of thrombospondin and PAI-1 will be studied.

Although MPA has fallen out of use for the treatment of breast cancer in favor of tamoxifen, it is still used for treating endometrial cancer. A recent multicenter study enrolled 45 young patients with endometrial cancer or atypical hyperplasia, using a daily oral dose of 600 mg MPA with low-dose aspirin with the objective to preserve fertility. Either estrogen–progestin therapy or fertility treatment was provided to responders following MPA therapy. The primary endpoint, pathologic complete response, was obtained in 55% of endometrial carcinoma cases and 82% of atypic hyperplasia cases. Two of the enrolled patients had grade 3 body weight gain, and one patient had grade 3 liver dysfunction. During a three-year follow-up period, 12 pregnancies and 7 normal deliveries were achieved. Fourteen recurrences were found in 30 patients between 7 and 36 months [34]. Similar results were reported in a second trial using 400 mg daily [35]. These data provide additional supporting evidence for a beneficial effect of high dose MPA.

Although a handful of substances have been shown to elevate Nm23-H1 expression levels in vitro, only MPA is currently in use in a clinical setting. Future studies, involving high throughput screening, will be directed at identifying other compounds with the ability to up-regulate levels of Nm23-H1. Currently we plan to use the Nm23-H1 promoter sequence linked to a reporter gene construct in these drug screening studies.

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Table 1

Compounds reported to elevate Nm23-H1 expression in vitro

Compound	Model system
Acetylsalicylic acid	Upregulated Nm23 and down-regulated Bcl2 and CD44v6 expression in SW480 colon carcinoma cells, with anti-proliferative and anti-invasive effect
Indomethacin	Elevated Nm23 expression in normal mammary epithelial and MCF-7 ER+ cancer cells, but not in metastatic MDA-MB-231 or -435 metastatic breast cancer cells
γ Linolenic acid	Elevated the Nm23 expression of HT-115 colon and MDA-MB-231 breast cancer cells and reduced their invasion
All-trans retinoic acid (ATRA)	Increased the Nm23-H1 expression of the 7721 hepatocellular carcinoma cell line and reduced its migration and invasion
Trichostatin A	Upregulated the Nm23-H1 expression of the MKN-1 and -28 gastric cancer lines [36], but failed to upregulate Nm23-H1 in metastatic breast cancer cells [37]
5-aza-deoxycytidine (5aza dC)	Elevated the Nm23-H1 expression of two breast cancer cell lines with hypermethylated CpG islands in the *nm23-H1* promoter
Medroxyprogesterone acetate (MPA)	High doses elevated Nm23-H1 expression of MDA-MB-435 and -231 cell lines via the glucocorticoid receptor