REPRESSION OF MATRIX METALLOPROTEINASES AND INHIBITION OF CELL INVASION BY A NUTRIENT MIXTURE, CONTAINING ASCORBIC ACID, LYSINE, PROLINE, AND GREEN TEA EXTRACT ON HUMAN FANCONI ANEMIA FIBROBLAST CELL LINES

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Aim: Fanconi Anemia, an autosomal recessive disorder, is characterized by chromosomal abnormality leading to birth defects, progressive bone marrow failure, and a high probability of developing malignancy at an early age. Head and neck squamous cell carcinoma and myeloid leukemia are the major causes of cancer related morbidity and mortality in Fanconi anemia patients. *Methods*: We investigated the effect of a nutrient mixture on Fanconi Anemia human fibroblast cell lines FA-A:PD20 and FA-A:PD220 on matrix metalloproteinase expression, invasion, cell proliferation, morphology and apoptosis. The cell lines were grown in a modified Dulbecco's Eagle medium and at near confluence were treated with the nutrient mixture at increasing doses: 0; 10; 50; 100; 500; 1000 µg/ml. The cells were also treated with PMA to induce MMP-9 expression. *Results*: Zymography demonstrated MMP-2 and PMA-induced MMP-9 activity. The nutrient mixture inhibited expression of both, MMP-2 and MMP-9, in a dose dependent manner with virtually total inhibition observed at 500 µg/ml. Matrigel invasion was inhibited in both cells lines; with 100% inhibition for FA-A:PD20 at 500 µg/ml and 100% inhibition of FA-A:P220 cells at 100 µg/ml. H&E staining did not indicate any change in cell morphology and causes apoptosis at higher doses. *Conclusion*: Our data demonstrated that the nutrient mixture inhibited matrix metalloproteinase expression, invasion and induced apoptosis, the important parameters for cancer prevention. The results suggest that the nutrient mixture may have therapeutic potential in Fanconi Anemia associated neoplasia.
Key Words: Fanconi Anemia, MMP, invasion, nutrients, apoptosis.

Fanconi Anemia (FA) is a rare autosomal recessive disorder that includes a constellation of clinical symptoms and carries a high risk of developing cancer. It is estimated to affect 1 in 350,000 births. Although FA can be seen across the population, Ashkenazi Jews and Afrikaans in South Africa are more likely to be the carriers of this chromosomal abnormality. Mean age of diagnosis is 7 years [1] with the approximate age at death of 35–40 years. Several common clinically observed congenital defects include short stature, abnormalities of the skin, kidneys, ears and eyes. Additionally, patients with FA may experience vertebral anomalies, anal atresia, cardiac abnormalities, tracheo-esophageal fistula, renal anomalies and radial limb, although 25% patients may not have any physical abnormalities [2]. The hallmark of FA, however, is hematologic abnormality including aplastic anemia and myelodysplastic syndrome in childhood with a 90% probability of bone marrow failure by 40 years [3]. Furthermore, patients with FA have a 20% chance of developing cancers. The highest chance is that of acute myeloid leukemia followed by non-hematologic solid tumors as squamous cell carcinomas of head and neck and ano-genital regions. Other solid tumors include esophageal carcinoma, tumors of the liver, brain, skin and kidney [4].

We have developed strategies to inhibit cancer development and its spread using naturally occurring nutrients such as lysine, proline, ascorbic acid and green tea extract. In our previous studies, this nutrient mixture (NM) has exhibited a broad spectrum biological activity, including synergistic effect and anticancer activity, in vivo in a number of cancer cell lines through inhibition of cancer cell growth, MMP secretion, invasion, metastasis, and angiogenesis [5–7].

FA fibroblasts are different from normal fibroblasts, because they have been shown to have impaired DNA repair process. Additionally, the nuclear extracts have substantially decreased plasmid-rejoining activity [8, 9]. This prompted us to study the effect of NM on FA fibroblast. In the current study, we investigated the effect of our NM on the proliferation, invasion, MMP expression, morphology, and apoptosis of the human FA fibroblast cell lines FA-A:PD20 and FA-A:PD220.

MATERIALS AND METHODS

NM. The composition of the NM and the proportion included the following: vitamin C (as ascorbic acid and as Mg, Ca and palmitate ascorbate) 710 mg; L-lysine 1,000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract (80% polyphenol) 1,000 mg; selenium 30 µg; copper 2 mg; and manganese 1 mg.

Cell line and culture. The FA fibroblast cell lines FA-A:PD220 and FA-A:PD20 were obtained from Fanconi Anemia Research Fund (Eugene, OR, USA). The cells were grown in modified Dulbecco's Eagle medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, Long Island, NY) in 24-well tissue culture plates (Costar, Cambridge, MA). Cells were incubated with 1 ml of media at 37 °C in a tissue culture incubator

equilibrated with 95% air and 5% CO. At near confluence, the wells were treated with the NM, dissolved in the media and tested with the NM at the following doses in triplicate: 0, 10, 50, 100, 500 and 1,000 µg/ml. Cells were also treated with PMA (100 ng/ml) to induce MMP-9 secretion. The plates were then returned to the incubator.

MTT cell proliferation assay. Cell viability was evaluated by MTT assay, as described in the reference [5, 6]. In brief, after 24 h incubation, the cells were washed with phosphate buffered saline (PBS) and 500 µl of MTT (Sigma#M-2128) 0.5 mg/ml in media was added. Following 12 h incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1 ml of DMSO, and absorbance was measured at 570 nm in Bio Spec 1601, Shimadzu spectrometer. The OD570 of the DMSO solution in each well was considered to be proportional to the number of cells. The OD of the control (treatment without supplement) was considered 100%.

Gelatinase zymography. Gelatinase zymography was performed as described in the reference [6, 7]. In brief, the culture media (20 µl) were mixed with sample buffer and loaded for SDS polyacrylamide gel electrophoresis (SDS-PAGE) with tris-glycine SDS buffer. Samples were not boiled before electrophoresis. Following electrophoresis the gels were washed twice in 2.5% Triton X-100 for 30 min at room temperature to remove SDS. The gels were then incubated at 37 °C overnight in $CaCl_2$ at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 min and de-stained; producing clear bands against an intensely stained background. Gelatinase zymograms were scanned using CanoScan 9950F Canon scanner at 1,200 dpi. The intensity of the bands was evaluated using a pixel-based densitometer program Un-Scan-It, Version 5.1, 32-bit (Silk Scientific Corporation), at a resolution of 1 Scanner Unit (1/100 dpi), and expressed as a percentage of control.

Matrigel invasion assay. Invasion studies were conducted as described in the reference [6, 7]. In brief, FA-A:PD220 and FA-A:PD20 cells, suspended in medium, were supplemented with the NM and seeded on the insert in the well. Thus, both the medium on the insert and in the well contained the same supplements. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% room air and 5% CO for 24 h. After incubation, the media from the wells were drawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with hematoxylin and eosin (H&E).

Morphology and apoptosis. For apoptosis studies, FA-A:PD220 and FA-A:PD20 cells, at near confluence, were challenged with NM dissolved in media at the experimental doses and incubated for 24 h. The cell culture was washed with PBS and treated with the caspase reagent as specified in the manufacturer's protocol (Molecular Probes Image-IT Live Green Poly Caspases Detection Kit 135104, Invitrogen). The cells were photographed under a fluorescence microscope and counted. Green-colored cells represent viable cells, while yellow orange represents early apoptosis and red represents late apoptosis.

Statistical analysis. The results were expressed as mean ±S.D. Data was analyzed by independent t-test.

RESULTS

Cell proliferation. NM had no effect on cell proliferation in the both cell lines compared to control (data not shown).

Gelatinase zymography. Zymography demonstrated MMP-2 and MMP-9 expression by untreated and PMA treated FA-A:PD20 and FA-A: PD220 cells. Fig. 1 *a* shows the effect of the NM on the untreated cell line FA-A:PD20 and Fig. 1 *b*, after PMA induction. The NM inhibited expression of both MMPs in a dose dependent fashion with a total virtual inhibition observed at 500 µg/ml. This was further confirmed by densitometry analysis (Fig. 1 *c*, *d*). Similarly, the FA-A:PD220 cell line (Fig. 2 *a*, *b*), both untreated and PMA treated cells exhibited a dose-dependent inhibition of MMP-2 and MMP-9 with virtually total inhibition at 500 µg/ml.
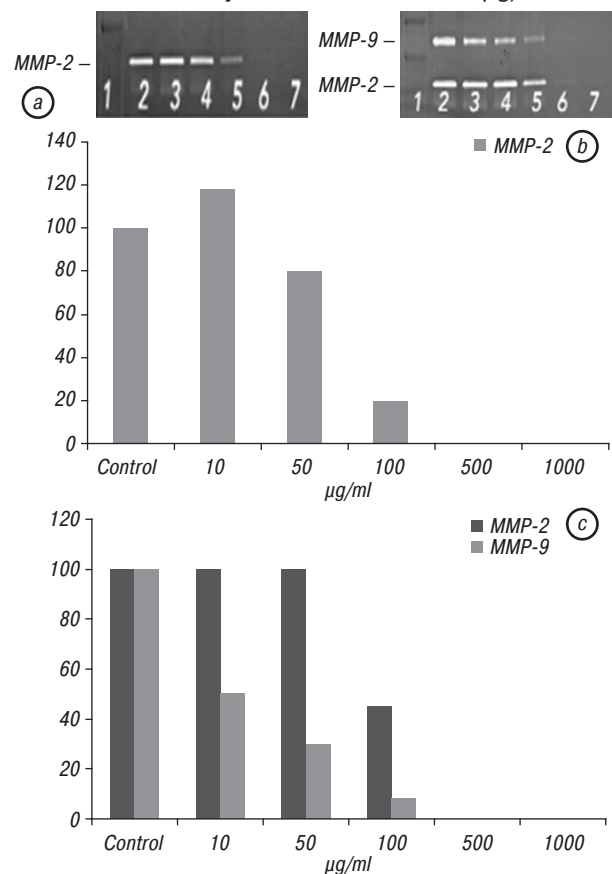

Fig. 1. The effect of the NM on MMP expression by human FA fibroblasts FA-A:PD20. Lane 1 — markers; Lane 2 — Control; Lane 3–7 — 10; 50; 100; 500 and 1,000 µg/ml, respectively. The gel on the right (*b*) was treated with PMA (100 ng/ml) for MMP-9 induction. 1 *c*, *d* — Densitometry analyses of dose dependent decrease of MMP-2 and MMP-9 expression by FA-A:PD20 cells without and with PMA treatment

Fig. 2. The effect of the NM on MMP expression by human FA fibroblasts FA-A:PD220. Lane 1 — markers; Lane 2 — Control; Lane 3–7 — 10; 50; 100; 500 and 1,000 µg/ml, respectively. *a* — without PMA, *b* — with PMA treatment

Matrigel invasion. Fig. 3 reveals a significant dose-dependent inhibition of FA-A:PD20 cell invasion through the Matrigel membrane. For FA-A:PD20 cells, 50; 80 and 100% inhibition was observed at 50, 100 and 500 µg/ml respectively (Fig. 3 *c*, *d*). All the invasion photomicrographs support the extent of inhibition. Similar results were obtained for FA-A:PD220. (p value was <0.01 in both types of cells.)

Morphology and apoptosis. The analysis with live green caspase, revealed a dose-dependent increase in apoptosis at higher doses for the FA-A:PD220 cell line, as seen in Fig. 4 *a-e*. Quantitative analysis of these data (Fig. 4 *f*) showed a 16% rate of apoptosis starting at 250 µg/ml (10% early, 6% late) followed by 48%

at 500 µg/ml (10% early and 38% late) and an 87% rate of apoptosis at 1,000 µg/ml (23% early, 64% late). A similar dose-dependent apoptotic pattern was seen in FA-A:PD20 cell line (data not shown).

DISCUSSION

The results of these in vitro studies of human FA fibroblast cancer cell lines FA-A:PD220 and FA-A:PD20 suggest that the NM is effective in inhibiting invasion through an ECM-like matrix. Indeed a robust decrease in Matrigel invasion was observed with maximal effect observed at high doses for FA-A:PD220 and FA-A:PD20 (100 and 500 µg/ml, respectively). Furthermore, a dose-dependent decrease of MMP-2 and MMP-9 expression was observed in both cell lines, with virtual total inhibition at 500µg/ml. In addition, the NM also induced apoptosis at higher doses.

Live Green Caspase analysis demonstrated significant apoptosis in NM-treated FA fibroblasts. Repression in the MMP expression and inhibition through Matrigel could possibly be due to pharmacological effect of the NM.
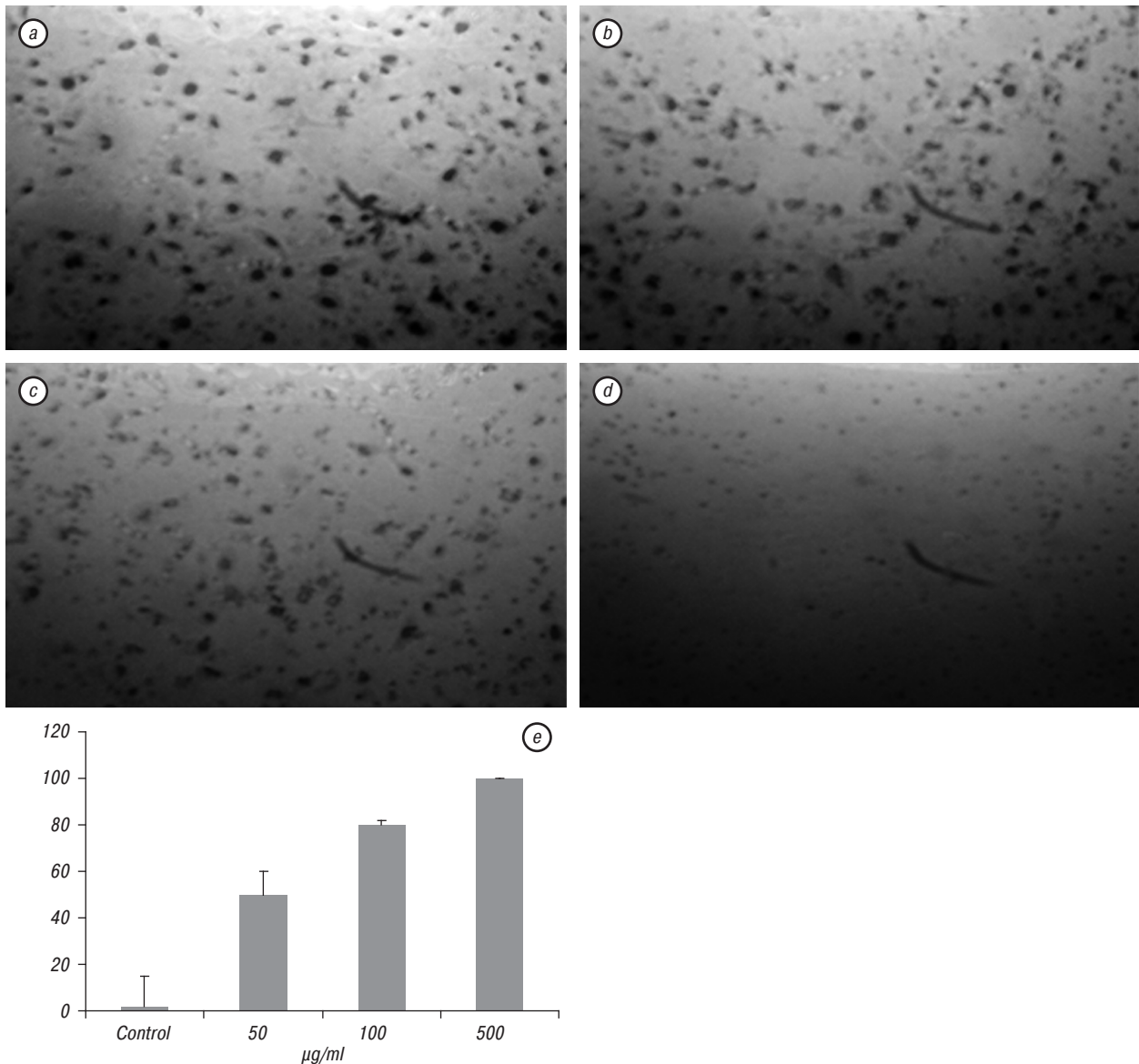

Fig. 3. Effect of NM on Matrigel invasion of FA-A:PD20 cells. *a–e*: Photomicrographs and analytical representation of FA-A:PD20 cells at varying NM concentrations (*a* — Control; *b, c, d* — 50; 100; 500 µg/ml respectively; *e* — Analytical representation, p<0.01)
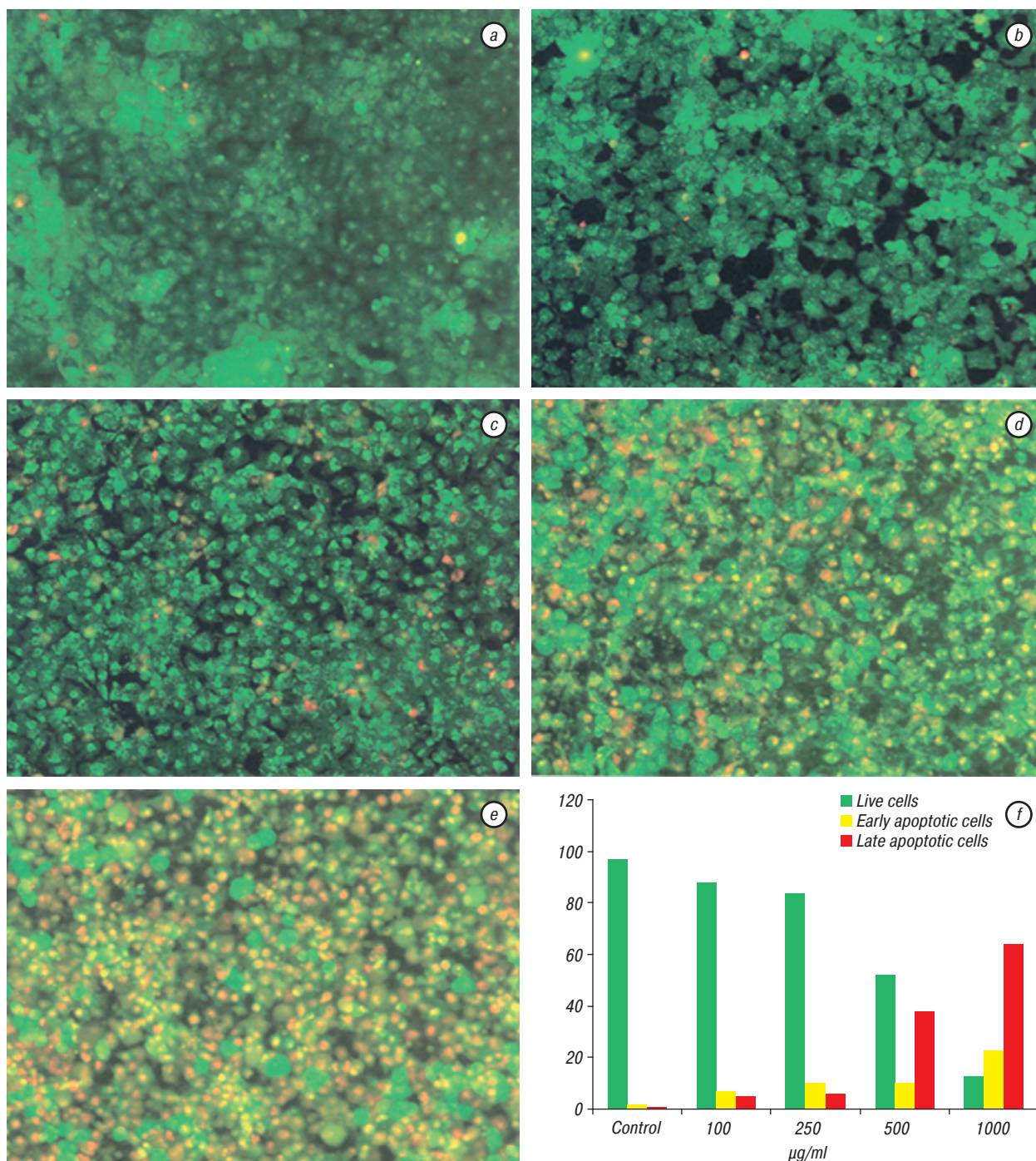
Fig. 4. *a–e:* Photomicrographs of FA-A:PD220 showing increasing apoptosis with increasing NM concentrations using live green caspase (*a* — Control; *b, c, d, e* — 100; 250; 500; 1000 µg/ml respectively). *f*: Quantitative analysis of the cell line FA-A:PD220 showing the increasing rate of apoptosis relative to control conditions

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes that able to degrade connective tissue such as the basement membrane collagen and are associated with cancer metastasis and tumor angiogenesis. Two key gelatinases within the MMP family, MMP-2 and MMP-9, play a key role in the degradation of collagen type IV and gelatin, important components of the extracellular matrix (ECM). As such, high expression of these MMPs may play a role in the malignancy potential of FA-associated cancers.

The NM showed significant inhibition of MMP-2 and MMP-9 expression, important mediators of angiogenesis and metastasis. Extra cellular matrix stability plays a key role in the development of cancer, and lysine is a natural inhibitor of proteolysis of the ECM [10]. Indeed, we have previously shown the NM has inhibited cell invasion and MMP production in multiple cancer cell lines, which suggests an important role of the NM in cancer therapeutics. The NM was formulated by defining the critical physiological targets, such as ECM integrity and MMP activity, in cancer progression and metastasis. The NM shows activity against MMP expression, Matrigel invasion and induces apoptosis at higher doses in human FA fibroblast cell lines. These data suggest a role of NM in the possible prevention and treatment of FA-associated solid tumors, specifically by targeting MMP secretion and thereby

inhibiting migration of cancer within the ECM as well as stabilizing the ECM surrounding an encapsulated tumor. Overall, the NM may offer a therapeutic benefit in FA associated neoplasia, and could play a role in support of FA patients.

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