MARCKS Protein Inhibitors Attenuate Cancer Cell Migration/Metastasis

MARCKS Inhibitors And Metastasis

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Background

Metastasis causes most deaths from cancer. In a number of studies, high expression of the MARCKS protein (Myristoylated Alanine-Rich C Kinase Substrate, a 332-amino acid protein known to play a key role in normal cell motion) in cancer cells has been associated with higher rates of metastasis in breast, lung, prostate, brain, skin and other neoplasias. Recently, Chen et al. showed that the 24-amino acid N-terminal sequence of MARCKS, called the MANS peptide, blocked metastasis of lung cancer cells orthotopically injected into mouse lungs (http: //www.ncbi.nlm.nih.gov/pubmed/23955080).Since the MANS peptide is not practical as an anti-cancer drug candidate, we have identified shorter and far more soluble N-terminal peptide inhibitors of MARCKS that appear to block migration of cancer cells in vitro and metastasis in mice in-vivo at lower concentrations than the MANS peptide, and when administered either IP or as an inhaled aerosol.

Methods

***In vitro* migration assays**

For the in vitro cancer cell migration assays, 1 X 10^5 of three different human lung cancer cell lines (A549, PC-9, and CL 1-5) were cultured in Transwell ®plates (24-well, 8-um pore size). Either PBS (control), MANS, or 3 different N-terminal peptide inhibitor analogs of MANS: BIO-11006, BIO-11002, or BIO-10901 were incubated with the cells for 12 hours, and cells that migrated from the upper to lower surface of the chambers were stained with hematoxylin and counted.

MARCKS siRNA studies

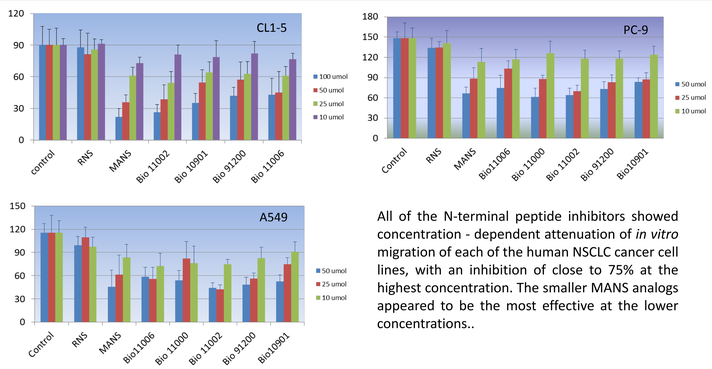
PC-9 and A549 cells were seeded in 6-well plates and cultured until cells reached 70% confluence. Cells were transfected with 100nM of MARCKS siRNA or control siRNA (100nM) from Ambion (Austin, TX) by using the DharmaFECT DuoTransfection reagent (Dharmacon, Lafayette, CO). After 72 hours, cells were harvested and equivalent amounts of proteins separated by SDS/PAGE for immunoblot analysis. Western blot analysis was performed to confirm siRNA-induced down regulation of endogenous MARCKS via immunoblotting with MARCKS antibody. Blots were re-probed with β-actin as a loading control. MARCKS protein was knocked down ~ 50-60% by MARCKS siRNA compared to cells treated with control siRNA.

***In vivo* metastasis evaluations**

For the first in vivo metastasis evaluations, PC-9 cells were orthotopically-injected into the lungs of NOD-SCID mice with or without pre-incubation with either MANS or BI0-11006, and animals were given peptides after 7 days and every 3 days hence, up until day 25, when the mice were harvested and examined. Specifically, 1 x 10^5 cells were suspended in 100 uL PBS containing 0.5 mg/mL Matrigel. Cells were incubated with appropriate peptides for 3 hours. The mice were anesthetized and the cells directly injected into the left lung of mice with an insulin syringe (29 gauge). Another group of orthotopically-injected NOD-SCID animals received treatment with BIO-11006 as an inhaled aerosol starting at day 4 and every day thereafter until day 25. For comparisons, a second group of mice were treated with BIO-11006 via IP injection starting at day 7 post PC-9 cell innoculation. Animals were euthanized at day 25; metastases were assessed macroscopically and microscopically. A third group were treated with inhaled BIO-11006 starting at day 4, and another group starting at day 15.

Finally, A549 cells injected into the tail veins of SCID mice go on to seed in the lung and form tumors. Peptides (MANS or BIO-11006) were administered as an inhaled aerosol either 1 day before or 3 days after the tail vein injection and treated via inhaled aerosol every other day for 7 weeks.

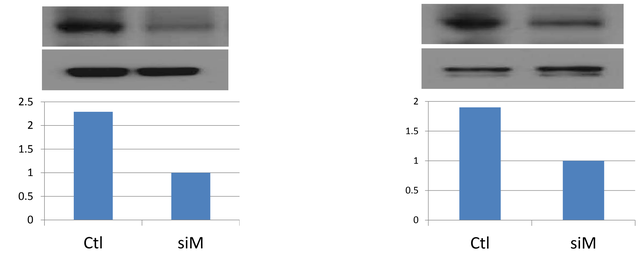
Results



All of the N-terminal peptide inhibitors showed concentration - dependent attenuation of *in vitro* migration of each of the human NSCLC cancer cell lines, with an inhibition of close to 75% at the highest concentration. The smaller MANS analogs appeared to be the most effective at the lower concentrations..

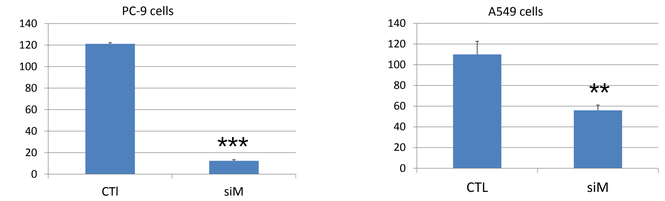
Results

Treatment with MARCKS siRNA decreases expression of MARCKS in both PC-9 and A549 cells



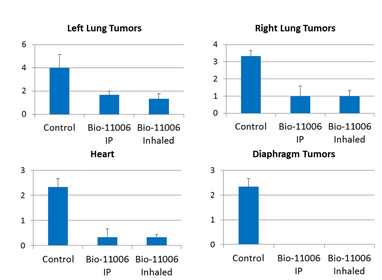
Transfection of PC-9 or A549 cells with MARCKS siRNA knockdown MARCKS protein expression. PC-9 or A549 cells were transfected with MARCKS or control siRNA at 100nM. After 72 hour, Western blot analysis confirmed siRNA-induced down regulation of endogenous MARCKS. MARCKS siRNA decreased MARCKS expression 90% in PC-9 and 50% in A549 cells.

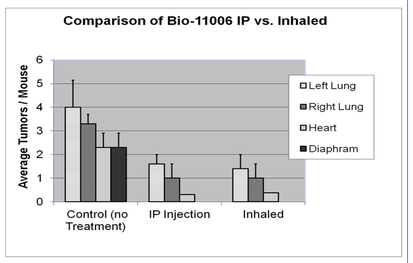
Treatment with MARCKS siRNA inhibits migration of both PC-9 and A549 cells



PC-9 and A549 cells were transfected with MARCKS siRNA or control siRNA at 100nM. After 72h, cells were reseeded for migration studies on Transwell® chambers (24well-8Um port size) at 10,000 cells per well. Cells that migrated from upper to lower chamber for 12 hours were stained with hematoxylin and at least 3 fields per chamber were counted. *** = p<0.001; ** = p<0.05

Comparison of effects on tumor metastasis of BIO-11006 given IP or as an inhaled aerosol

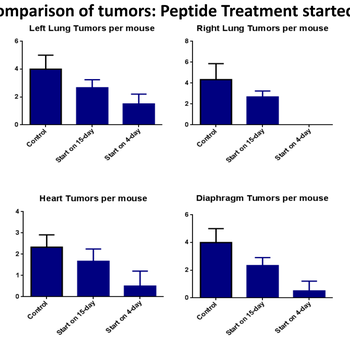


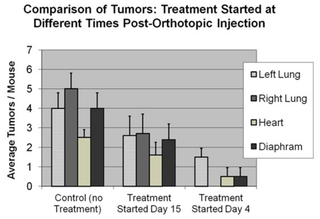


SCID mice were anesthetized and PC-9 cells were directly injected into the left lung via an insulin syringe (29 gauge). BIO-11006 peptide was administered either as an inhaled aerosol every day starting at day 4 post implantation, or injected IP starting at day 7 and every 3 days hence. Mice were sacrificed and examined at day 25.

Both IP and inhaled BIO-11006 significantly inhibited metastasis of the primary tumor to other sites in the lung, heart and diaphragm. Administration of 50 uM BIO-11006 daily via inhaled aerosol had the same inhibitory effect on metastasis as IP injection of 50 uM every three days.

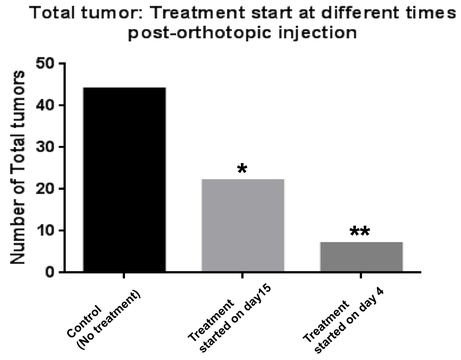
Comparison of tumors: Peptide Treatment started at different times post-orthotopic injection





Treatment started at day 4 showed the least metastatic tumors at all sites; treatment started at day 15 showed more metastatic tumors at all sites than the 4 day start mice, but less than the untreated controls.

Results

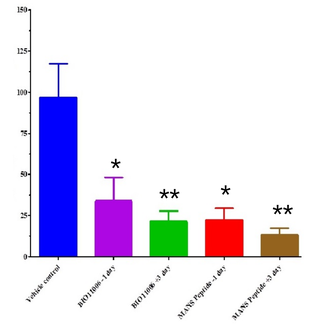


BIO-11006 was administered daily as an inhaled aerosol starting on either day 4 post orthotopic injection or on day 15. Treatment started on day 4 showed less metastasis, when total tumors were counted, than treatment started at day 15; treatment started on either day showed significantly less metastasis than the untreated control mice

Number of macroscopic metastatic tumor nodules in lungs after A549 cells were injected into the tail veins of SCID mice

A549 cells injected into the tail veins of SCID mice go on to seed in the lung and form tumors. Peptides (MANS or BIO-11006) were administered as an inhaled aerosol either 1 day before or 3 days after the tail vein injection and treated via inhaled aerosol every other day for 7 weeks.

Group	No. of metastatic nodules in lungs Mean ± SEM	Remarks
Vehicle Control	97 ± 21	Multi-focal tumor nodules in all animals (8/8) Evidence of distant metastasis in diaphragm & sternum (2/8)
BIO-11006 peptide (-1 Day treatment group)	$34 \pm 14^{*,a,b,c}$	Few focal tumor nodules in (7/8) animals No distant metastasis
BIO-11006 peptide (+3 Day treatment group)	$21 \pm 6^{**,d,e}$	Few focal tumor nodules in (7/8) animals No distant metastasis
MANS peptide (-1 Day treatment group)	$22 \pm 7^{*,f}$	Few focal tumor nodules in (4/8) animals No distant metastasis
MANS peptide (+3 Day treatment group)	$13 \pm 4^{**}$	Few focal tumor nodules in (7/8) animals No distant metastasis



On Day 53 post injection of 2.5 x 10^6 A549 cells, all animals were sacrificed and the lungs and tissues harvested. The number of macroscopic metastatic tumor nodules in lungs were counted & recorded. Values are expressed as Mean ± SEM of 7-8 animals in each group. Both the MANS and BIO-11006, with treatment started at either 1 day prior or 3 days post tail vein injection, significantly inhibited metastasis. * = p,0.05; ** = p<0.01

Conclusions

1. N-terminal peptide inhibitors of MARCKS attenuate migration of human cancer cell lines in vitro in migration chambers in a dose – dependent manner.

2. N-terminal peptide inhibitors of MARCKS significantly attenuate tumor metastasis in the orthotopic injection model of lung cancer, and in the tail vein injection model of lung cancer.

3. N-terminal peptide inhibitors of MARCKS significantly attenuate tumor metastasis in these models when administered either via IP injection or as an inhaled aerosol

4. N-terminal peptide inhibitors of MARCKS may potentially be useful as anti-metastasis therapy in lung and perhaps other cancers.