Lung Cancer 70 (2010) 271–279

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Antiproliferative effect of Aurora kinase targeting in mesothelioma

Stefania Crispi[a,1], Claudia Fagliarone[b,1], Annamaria Biroccio[d], Carmen D'Angelo[d], Rossella Galati[b], Ada Sacchi[b], Bruno Vincenzi[e], Alfonso Baldi[c,*], Alessandra Verdina[b]

[a] Gene Expression Core-Human Molecular Genetics Laboratory, Institute of Genetics and Biophysics, CNR, Naples, Italy
[b] Laboratory D, Department for the Development of Therapeutic Programs, CRS, Regina Elena Institute, Rome, Italy
[c] Section of Pathology, Department of Biochemistry and Biophysics, Second University of Naples, Via L. Armanni, 5, 80138 Naples, Italy
[d] Laboratory of Experimental Chemotherapy, CRS, Regina Elena Institute, Rome, Italy
[e] Section of Oncology, Campus BioMedico University, Rome, Italy

ARTICLE INFO

Article history:
Received 24 December 2009
Received in revised form 14 February 2010
Accepted 9 March 2010

Keywords:
Mesothelioma
Aurora kinase
Related genes
Potential target
Inhibitor
ZM447439

ABSTRACT

The Aurora proteins are a small family of serine/threonine kinase that function in various stages of mitosis. Current interest in Aurora kinase relates to its role in tumours, and its potential as a therapeutic target. In this work we studied the expression of Aurora kinases A and B and related genes in human mesothelioma tissues and in five mesothelioma cell lines. Moreover, we analyzed the effects of ZM447439 (ZM), an Aurora kinase inhibitor, on cellular growth. Results evidenced an over-expression of Aurora kinase A and related genes in human mesothelioma tissues and an over-expression of Aurora kinases A and B in all cell lines. Moreover, we demonstrated that ZM447439 was able to inhibit cell growth in all cell lines and that this inhibition was due to a specific effect as demonstrated by the reduction in the level of Histone H3 phosphorylation. Our findings support a role of Aurora kinase in mesothelioma and the possibility of using Aurora kinase inhibitors in therapeutic modalities.

1. Introduction

Malignant mesothelioma (MM) is a rare tumour related to asbestos exposure that affects serosal surface in humans [1]. It is characterized by rapid local progression, late metastases and a very poor prognosis [2]. Standard chemotherapy and radiotherapy have had limited effectiveness although a survival benefit has been demonstrated with a combination of cisplatin and antifolate drugs and multimodality therapy may increase short-term survival [3]. However, despite aggressive therapies, the low survival rates demand new treatment strategies.

Aurora kinases represent a family of serine/threonine kinases, which plays an important role in chromosome alignment, segregation and cytokinesis during mitosis. In humans three closely related members have been described and termed Aurora A, Aurora B and Aurora C kinases. Aurora A kinase regulates mitotic entry and exit and supports centrosomes maturation process, thus participating in spindle assembly and stability. Aurora B has a crucial role in the regulation of the mitotic checkpoint events and forms a complex with the centromere proteins INCENP, Borealin and Survivin. Both Aurora A and Aurora B activity are modulated by specific substrates or activators, that trigger their activation by autophosphorylation [4].

Aurora C is specifically expressed in the testis and plays a role in spermatogenesis [5].

Aurora kinases have been reported to be over-expressed in many human cancers, including prostate [6], colon [7], pancreas [8], breast [9], and thyroid cancer [10] and elevated expression has been correlated with chromosomal instability and clinically aggressive disease in some instances such as prostate cancer and head and neck squamous cell carcinoma [6,11]. For this reason Aurora kinases have been indicated as appealing target for molecular therapies and a great number of specific inhibitors have been described to date [12] some of which are in clinical trials [13].

Recently, Aurora kinases A and B have been found over-expressed also in MM and this over-expression has been correlated with aggressive behaviour [14]. Moreover, it has been recently demonstrated that the inhibition of survivin and Aurora B kinase sensitizes mesothelioma cells to irradiation by enhancing mitotic arrests [15].

On the ground of these considerations, we have looked to Aurora kinases as potential therapeutic target for MM treatment. To this aim, we have performed bioinformatic analysis on micro array data derived from MM patients and showed that Aurora kinase and related genes are deregulated in MM. The study of Aurora kinase expression in MM tissues and in five human MM cell lines confirmed these data. Moreover, we demonstrated that the specific

* Corresponding author. Tel.: +39 0815666003; fax: +39 0815569693.
E-mail address: alfonsobaldi@tiscali.it (A. Baldi).
[1] These authors contributed equally to this work.

0169-5002/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.lungcan.2010.03.005

Aurora kinase inhibitor ZM447439 significantly reduces proliferation of MM cells.

2. Materials and methods

2.1. Bioinformatics analysis

Functional pathway analysis of the differentially expressed genes was performed using "Ingenuity Pathways Analysis" (IPA, http://www.Ingenuity.com). IPA analysis was performed on mesothelioma tumour samples as previously described [16] retrieving a total of 386 differentially expressed genes and enriching within the set for genes directly or indirectly associated to Aurora kinases and to cell cycle.

2.2. Tumour specimen acquisition and clinical data

All patients were treated at the Second University of Naples between 1980 and 1996. Clinical data were obtained by retrospective chart review. Survival was determined from the date of initial surgery. Follow-up was available for all patients. Two subjects who died of causes other than MM during the follow-up period were excluded from the study. All patients were treated at least with cytoreductive surgery and 13 patients were than treated with radiotherapy or chemotherapy. Tissues from 29 MM specimens (16 epithelioid, 6 sarcomatoid and 7 mixed mesotheliomas) obtained from open biopsies or pleurectomies were collected and fixed in 10% formalin before being embedded in paraffin.

2.3. Histology

The formalin-fixed, paraffin-embedded samples were sectioned at 5 μm and stained with hematoxylin and eosin. The histological diagnosis was reexamined by a pathologist (A.B.) according to the WHO. In addition, the most representative blocks were selected to be cut into new 5 μm-thick sections for immunohistochemical studies.

2.4. Immunoistochemistry

All 29 cases have been assessed by immunohistochemistry for the presence of Aurora kinases A and B. Sections from each specimen were cut at 5 μm, mounted on glass and dried overnight at 37 °C. All sections were then deparaffinized in xylene, rehydrated through a graded alcohol series and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antibody dilution. Endogenous peroxidase activity was blocked by 5% hydrogen peroxide. The mouse monoclonal antibodies for Aurora kinases A and B (BD Transduction Laboratories) were applied at 4 °C for 12 h at the dilution of 1:100 after antigen-retrieval in citrate buffer in a pressure cooken for 5 min. The optimal working dilution was defined on the basis of titration experiments. Then, the sections were immunostained with the streptavidin-biotin system (Dako, Carpintera CA, USA), using diaminobenzidine (DAB) as the final chromogen and haematoxylin as the nuclear counter stain. Negative controls for each tissue section were prepared by leaving out the primary antibodies. A suitable positive control was run with each set of slides. All samples were processed under the same conditions. Given that Aurora kinases A and B are normally undetectable by immunohistochemistry in normal non-mitotic cells, any expression was considered positive, regardless of the number of positive cells [14].

2.5. Drugs

ZM447439 (ZM) was obtained from Tocris biosciences (Missouri, USA). It was dissolved in 100% DMSO to a stock concentration of 10 mmol/L and stored at −80 °C.

2.6. Cell lines

The human MM cell lines MSTO-211H (MSTO), NCI-H2452 (NCI), IstMes1, IstMes2 and MPP89 were cultured as described by Stoppoloni et al. [17]. The human prostate cancer cell line PC3, was obtained from ATCC and cultured in ATCC-formulated F-12K Medium supplemented with 10% FBS and antibiotics.

2.7. Cell treatment with ZM447439 and cell growth

Cells were seeded in complete growth medium and 24 h later were treated with ZM or vehicle (DMSO) at different concentrations and for different times as indicated in each experiment. The expansion of cultures was quantified by manual cell counting at different times after beginning of treatment. Experiments were repeated in triplicate and media values were calculated.

2.8. Protein extraction and Western blot analysis

Cell lysates were prepared by treating cells with ice-cold lysis buffer (Roche Applied Science, Mannheim, Germany) supplemented with protease and phosphatase inhibitors for 20 min followed by centrifugation at 4 °C for 15 min. The proteins were separated on 10% SDS-PAGE gels and then transferred on polyvinylidene fluoride (PVDF) membrane. Membranes were incubated with anti-Aurora kinases A and B monoclonal antibodies (BD Transduction Laboratories), anti-Phospho-Histone H3 (Ser10) polyclonal antibody and anti-PARPp85 fragment polyclonal antibody (Promega). Mouse monoclonal anti-α-tubulin (Calbiochem) and β-actin (Sigma) were used to normalize the samples loading. Antibody reaction was visualized using ECL Western blotting detection reagents. The experiments were done in triplicate with comparable results and electrophoretic bands were analyzed by Scion Image program

2.9. Quantitative real-time PCR

Total cellular RNAs from cell lines were prepared using TRIzol Reagent (Invitrogen Life Technologies, Paisley, UK) according to the manufacturer's protocols and reverse transcribed into cDNA using standard protocols.

Real-time PCR was conducted in a volume of 25 μL containing 40 ng cDNA, 1.25 μL of Aurora kinases A or B primers and 12.5 μL of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as endogenous control, by using GADPH Assay on Demand (Applied Biosystems, Foster City, CA, USA). The conditions for all genes were as follows: denaturation for 10 min at 95 °C followed by 40 cycles of the amplification step at 95 °C for 15 s (denaturation), and then at 60 °C for 60 s (annealing/extension) in 96-well plates with the ABI PRISM 7000 sequence Detection System (Applied Biosystems, Foster City, CA, USA). The standard curves for Aurora kinases A and B genes were constructed using serial dilutions (200-40-8-1.6-0.32 ng) of a pool of cDNA from MSTO, NCI, IstMes1, IstMes2 and MPP8989 cells. Results were analyzed using the Applied Biosystems analysis software and expression levels calculated from a linear regression of the standard curve. Results were given as Aurora kinases A or B expression vs. GADPH expression (Aurora kinase relative expression). All