Ceftriaxone, an FDA-approved cephalosporin antibiotic, suppresses lung cancer growth by targeting Aurora B

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Introduction

Drug development is a time-consuming and costly process, moving from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval.

Materials and methods

Reagents

Ceftriaxone was purchased from the University of Minnesota Boynton Health Services Pharmacy (Minneapolis, MN). Dulbecco’s modified Eagle’s medium, basal medium eagle and other supplements were from Life Technologies, Inc. (Carlsbad, CA). CNBr-Sepharose 4B beads were purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). The human recombinant Aurora B and histone H3 proteins and the antibody used to detect phosphorylated histone H3 were purchased from Millipore Corp. (Billerica, MA). Antibodies used to detect Aurora B, phosphorylated Aurora B and histone H3 were from Cell Signaling Technology, Inc. (Danvers, MA). The small hairpin RNA (sh-RNA) construct against aurkb (sense sequence for human: CCTGCGTCCTACACTTATT, sense sequence for mouse: CCACAGAGGTCTACTTAAT) used in this study was from the University of Minnesota BioMedical Genomics Center.

Abbreviations: ATP, adenosine triphosphate; EDTA, ethylenediaminetetraacetic acid; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Cell culture

Mouse epidermal JB6 P+ cells were cultivated in Eagle's minimum essential medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 25 μg/ml of gentamicin. H1975, H1650 and H520 cells were cultivated in RPMI-1640/10% FBS. Calu-3 and MRC-5 cells were maintained in Dulbecco's modified Eagle's medium/10% FBS. A549 cells were grown in F-12K medium/10% FBS. All cells used in these studies were purchased from the American Type Culture Collection (Manassas, VA) and maintained with antibiotics at 37°C in a 5% CO2, humidified incubator.

Kinase profiling. Kinase profiling was conducted by Millipore's KinaseProfiler according to the protocols detailed at http://www.millipore.com/drugdiscovery/dd3/KinaseProfiler. The ceftriaxone concentration for each specific kinase assay was set at 100 μM and adenosine triphosphate (ATP) concentration was set at 10 μM.

Molecular modeling. Computer modeling of ceftriaxone and Aurora B was performed using the crystal structure of Aurora B, which was obtained from the RCSB Protein Data Bank (PDB entry 2VRX (17)). The structure was prepared using the Protein Preparation Wizard in Maestro v9.2. Hydrogen atoms were added consistently with a pH of 7 and all water molecules were removed. Then the structure was minimized with an RMSD cutoff value of 0.3 Å. The 3D structure of ceftriaxone was drawn and optimized in Maestro v9.2, whereas the chemical structure of ceftriaxone was prepared using the program LigPrep v2.5 and then assigned AMSOL partial atom charge. Ligand docking was accomplished using the program Glide v5.7 (18). The receptor grid was created with the centroid of the crystal ligand as the center of the grid. Flexible docking was performed with extra precision mode. The number of poses per ligand was set to 10 in postdocking minimization and at most 5 poses would be output. The other parameters were kept as default.

Western blot analysis

Cellular proteins were extracted using Radioimmunoprecipitation (RIPA) lysis buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid (EDTA) and protease-inhibitor cocktail). Proteins were then separated by dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by Protein Preparation Wizard in Maestro v9.2. Hydrogen atoms were added consistently with a pH of 7 and all water molecules were removed. Then the structure was minimized with an RMSD cutoff value of 0.3 Å. The 3D structure of ceftriaxone was drawn and optimized in Maestro v9.2, whereas the chemical structure of ceftriaxone was prepared using the program LigPrep v2.5 and then assigned AMSOL partial atom charge. Ligand docking was accomplished using the program Glide v5.7 (18). The receptor grid was created with the centroid of the crystal ligand as the center of the grid. Flexible docking was performed with extra precision mode. The number of poses per ligand was set to 10 in postdocking minimization and at most 5 poses would be output. The other parameters were kept as default.
transfer onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). The membranes were incubated with the appropriate specific primary antibody and a horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized using an enhanced chemiluminescence reagent (Amersham Biosciences Corp.).

**In vitro kinase assay**

A histone H3 protein was reacted with active Aurora B in the presence of different concentrations of ceftriaxone, 100 µM ATP and x1 kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.01% Brij 35; Cell Signaling Technology). The reactions were carried out at 30°C for 30 min and stopped by adding x6 SDS sample buffer. The proteins were boiled and then resolved by SDS-PAGE. Total histone H3 and phosphorylated histone H3 were detected by Western blot analysis using specific antibodies.

**Cytotoxicity and anchorage-independent growth assay**

For the cytotoxicity assay, JB6 P+ cells were seeded (2 × 10⁴ per well) into 96-well plates. After incubation for 24 h, cells were treated with different concentrations of ceftriaxone and incubated for another 24 or 48 h. CellTiter96 Aqueous One Solution (20 µl; Promega) was then added and cells were incubated for an additional 1 h at 37°C in a 5% CO₂ incubator. Absorbance was read at an optical density of 492 nm. For the anchorage-independent growth assay, cells (8 × 10⁵ per well) were suspended in 1 ml basal medium eagle/10% FBS/0.33% agar with different concentrations of ceftriaxone and plated on a layer of solidified basal medium eagle/10% FBS/0.5% agar with the same concentration of ceftriaxone as the bottom agar. The cultures were maintained at 37°C in 5% CO₂ incubator for 1 to 2 weeks, and colonies were counted under a microscope using the Image-Pro Plus software (v. 4) program (Media Cybernetics). All experiments were repeated three times.

**In vitro ATP competitive binding assay and ex vivo pull-down assay**

For the in vitro ATP competitive binding assay, a recombinant human Aurora B protein (200 ng) was first incubated with different concentrations of ATP (10 or 100 µM) for 1 h at 4°C and then incubated with ceftriaxone-conjugated Sepharose 4B (or Sepharose 4B only as a control) beads (50 µl, 50% slurry) in a 1 x reaction buffer (50 mMTris pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% NP40, 2µg/ml bovine serum albumin and 0.02 mM phenylmethanesulfonylfuoride, protease inhibitor cocktail). For the ex vivo pull-down assay, A549 cell lysates (500µg) were incubated with ceftriaxone-conjugated Sepharose 4B (or Sepharose 4B only as a control) beads (50µl, 50% slurry) in a 1 x reaction buffer. After incubation with gentle rocking overnight at 4°C, the beads were washed five times with x1 wash buffer (50 µM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% NP40, 0.02 mM phenylmethanesulfonylfuoride), and proteins bound to the beads were boiled and resolved by 10% SDS-PAGE followed by western blot analysis.

**In vivo xenograft mouse model**

Athymic nude mice (Crl:NIH(S), NIH Swiss nude, 6–9 weeks old) were purchased from Charles River. Animals were maintained under ‘specific pathogen-free’ conditions according to guidelines established by Research Animal Resources, University of Minnesota. Mice were randomly divided into three groups: (i) vehicle group (n = 8); (ii) 100 mg/kg ceftriaxone-conjugated group (n = 8); (iii) no cells-injected group (n = 8). Each group was treated with 100 mg/kg ceftriaxone. Group 3 was maintained as a negative control to observe any possible side effects of ceftriaxone. The experiment was repeated with A549 and H520 cell lines and resolved by 10% SDS-PAGE followed by western blot analysis.

**Immunohistochemistry analysis**

Tumor tissues from the mice (vehicle- and 100 mg/kg ceftriaxone-treated groups) were prepared for immunohistochemistry analysis of phosphorylated histone H3. Sections (5 µm) from 10% neutral formalin solution–fixed paraffin-embedded tissue were cut on silane-coated glass slides and then deparaffinized with xylene and dehydrated through a graded alcohol bath. The slides were incubated with 1:100 dilution of an antirabbit antibody in 10% normal goat serum at 4°C overnight to detect phosphorylated histone H3. Normal goat serum was used as a negative control. After washing three times with PBS, the slides were incubated with a 1:200 dilution of a biotinylated antirabbit antibody for 1 h in the dark. The slides were developed in diaminobenzidine and counterstained with a weak solution of hematoxylin stain. Images were captured and analyzed using the Image-Pro-plus software (v. 4) program.

**Results**

**Ceftriaxone inhibits EGF-induced anchorage-independent growth of JB6 P+ cells**

In clinical use, 2g of ceftriaxone produces an average peak concentration in serum of 262 µg/ml (19), which is equal to 396 µM. The chemical structure of ceftriaxone is shown in Figure 1A. To determine the cytotoxicity of ceftriaxone, different concentrations of the drug were used to treat JB6 P+ mouse epidermal cells (JB6 P+ cells) for 24 or 48h. Cytotoxicity was measured by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay and the results indicated that ceftriaxone had no cytotoxicity toward JB6 P+ cells up to 2000 µM at either 24 or 48h (Figure 1B). Next, we investigated whether ceftriaxone had an effect on anchorage-independent growth of JB6 P+ cells. Anchorage-independent growth ability is a hallmark of *in vitro* transformed cells and cancer cells (20,21). Our results revealed

| Kinase profiling results indicate that Aurora B is a potential target of ceftriaxone |
|---------------------------------------------|-------------------|-------------------|
| Ceftriaxone, 100 µM | Ceftriaxone, 100 µM |
| Abb(h) | 130 | GSK3β(h) | 89 |
| AMPKα1(h) | 95 | IGF-1R(h) | 106 |
| ASK1(h) | 115 | IKKα(h) | 105 |
| Aurora-A(h) | 87 | IKKβ(h) | 103 |
| Aurora-B(h) | 45 | JNK1α1(h) | 107 |
| CDK1/cyclinB(h) | 99 | JNK2α2(h) | 97 |
| CDK2/cyclinA(h) | 95 | MAPK1(h) | 108 |
| CDK3/cyclinE(h) | 110 | MAPK2(h) | 103 |
| CHK2/R1545(h) | 100 | MEK1(h) | 100 |
| CSK(h) | 104 | Met(h) | 90 |
| c-Raf(h) | 106 | MKK3(m) | 111 |
| c-Src(h) | 138 | MKK6(h) | 105 |
| DYRK2(h) | 75 | MKK7(h) | 101 |
| EGFR(h) | 101 | MSK1(h) | 108 |
| EGFR(L858R)(h) | 85 | MSK2(h) | 78 |
| EGFR(L861Q)(h) | 83 | mTOR(h) | 92 |
| EGFR(T790M)(h) | 100 | p70S6K(h) | 94 |
| EGFR(T790M,L858R)(h) | 93 | PDK1(h) | 109 |
| EphB1(h) | 114 | PKB(h) | 94 |
| FAK(h) | 112 | PKCα(h) | 73 |
| FGFR1(h) | 103 | Src(h) | 107 |
| Flt3(D835Y)(h) | 88 | T341M(h) | 97 |
| Flt3(h) | 103 | Yes(h) | 103 |
| Fyn(h) | 100 | ZAP-70(h) | 105 |
| GSK3α(h) | 103 | P13 Kinase (p110/p85)(m) | 101 |
that JB6 P+ cells treated with ceftriaxone formed fewer colonies compared with control cells treated with only epidermal growth factor (EGF) (Figure 1C). The magnification of representative photographs for the anchorage-independent growth assay is 25X. U0126, a well-known MEK (Mitogen activated protein kinase kinase) inhibitor (22), was used as a positive control. These results showed that ceftriaxone could attenuate EGF-induced anchorage-independent cell growth and was not cytotoxic at clinically relevant doses.

**Kinase profiling identifies Aurora B as a potential target of ceftriaxone**

Based on the previous data, the next step was to identify the potential cellular targets of ceftriaxone. In the EGF-induced signaling pathway, several molecules downstream of the epidermal growth factor receptor (EGFR) are activated by EGF, including the Ras/Raf/MEK and the phosphoinositide 3-kinase (PI3-K), Akt and mammalian target of rapamycin (mTOR) signaling pathways (23). To determine potential targets of ceftriaxone, 50 candidate kinases were selected from different kinase families. Kinase assays were conducted by Millipore’s KinaseProfiler according to their established protocols. Scores (Table I) represent the percent of control, which was derived from the following formula: % of control = [(sample − mean no enzyme)/(mean plus enzyme − mean no enzyme)] × 100. This means that ceftriaxone would enhance kinase activity if the score was higher than 100, whereas if the score was lower than 100, the activity would be suppressed. The results revealed (Table I) that Aurora B was the most relevant potential target of ceftriaxone compared with the other kinases tested. Accumulating evidence supports a critical role for Aurora B in cancer development, and therefore targeting Aurora B is regarded as a useful strategy in chemoprevention and cancer therapy (24). Our data suggested that ceftriaxone could inhibit Aurora B activity and Aurora B was a more potent target of ceftriaxone than other kinases.

**Ceftriaxone specifically binds with Aurora B in vitro and ex vivo**

After identifying the potential target of ceftriaxone, the next question was whether Aurora B could bind with ceftriaxone. To confirm this prediction, we constructed a computer docking model with Aurora B and ceftriaxone. Our results showed that ceftriaxone bound with Aurora B and fit into the ATP-binding site very well (Figure 2A). In the docked model of ceftriaxone and Aurora B (Figure 2B), ceftriaxone forms hydrogen bonds with ALA173 and LYS101 and maintains van der Waals contacts with the side chain of PHE172. To further validate this prediction, we performed an in vitro ATP competitive binding assay using ceftriaxone-conjugated Sepharose 4B beads. Results revealed that recombinant Aurora B was able to bind with ceftriaxone-conjugated Sepharose 4B beads and the binding affinity was decreased as ATP concentration was increased, which meant ceftriaxone could bind with Aurora B in an ATP competitive manner (Figure 2C). We also confirmed the ex vivo binding of ceftriaxone with endogenous Aurora B in A549 cells (Figure 2D). These results indicated that ceftriaxone could bind with Aurora B in vitro and in A549 cells, which clearly supports our hypothesis that Aurora B is an ‘off’ target of ceftriaxone.

**Fig. 2.** Ceftriaxone specifically binds with Aurora B in vitro and ex vivo. (A) Proposed binding mode of ceftriaxone and Aurora B. Ceftriaxone is shown in stick representation and surrounded by a semitransparent surface, which occupies the ATP-binding site between the N and C terminals. (B) Interaction between ceftriaxone and Aurora B. Ceftriaxone forms hydrogen bonds with ALA173 and LYS101 and maintains van der Waals contacts with the side chain of PHE172. Carbons of ceftriaxone are colored magenta, whereas oxygen, nitrogen and sulfur are shown in red, blue and yellow, respectively. The in vitro ATP competitive binding (C) and ex vivo binding (D) of ceftriaxone with Aurora B were confirmed using a pull-down assay. A recombinant human Aurora B protein (200ng) or A549 cell lysates (500 µg) were incubated with ceftriaxone-Sepharose 4B (or Sepharose 4B only as control) beads. Proteins bounded to the beads were boiled and resolved by 10% SDS-PAGE followed by western blot analysis. Data shown are representative of triplicate experiments.
Ceftriaxone suppresses Aurora B activity in vitro and in cells

Based on our previous data showing that ceftriaxone directly binds to Aurora B, we performed an in vitro kinase assay as described in section ‘Materials and methods’. The expression level of phosphorylated histone H3 (Ser10; arrow) was confirmed by western blot analysis using an antibody against phosphorylated histone H3. Total histone-H3 was used as a loading control. Data are representative of results from triplicate experiments. (A) Ceftriaxone inhibits Aurora B activity in vitro. The inhibitory effect of ceftriaxone on Aurora B was determined by an in vitro kinase assay as described in section ‘Materials and methods’. The expression level of phosphorylated histone H3 (Ser10; arrow) was confirmed by western blot analysis using an antibody against phosphorylated histone H3. Total histone-H3 was used as a loading control. Data are representative of results from triplicate experiments. (B) Ceftriaxone inhibits Aurora B activity in JB6 P+ cells. Different concentrations of ceftriaxone were incubated with JB6 P+ cells for 2 h and cells were then treated with 10 ng/ml EGF for 30 min. Cells were harvested and total ERKs, RSK, Aurora B, histone H3 and phosphorylated ERKs, RSK, p38, JNKs, Aurora B and histone H3 proteins were detected by western blotting using specific antibodies. Data are representative of results from triplicate experiments. (C) Expression level of Aurora B in JB6 P+ cells is decreased by knockdown of Aurora B. JB6 P+ cells were transiently transfected with sh-mock or sh-Aurora B and cell lysates were analyzed by western blotting. (D) Ceftriaxone has less effect on anchorage-independent cell growth of sh-Aurora B cells than that of sh-mock cells. JB6 P+ cells were grown in soft agar with ceftriaxone (0, 200, 500, 1000 µM) for 7 days and colonies were counted. Data are represented as mean ± standard deviation from triplicate experiments. The asterisk (*) indicates a significant decrease compared with sh-mock cells (P < 0.05).

Ceftriaxone suppresses Aurora B activity in vitro and in cells

Based on our previous data showing that ceftriaxone directly binds to Aurora B, we performed an in vitro kinase assay with histone H3 as substrate to further confirm that ceftriaxone inhibits Aurora B activity. The results showed that phosphorylation of histone H3 (Ser10) was substan-
tially attenuated after treatment with 10 µM ceftriaxone (Figure 3A). Reversine, a novel Aurora B inhibitor, was used as a positive control (25). In addition, we examined whether ceftriaxone could suppress Aurora B activity in JB6 P+ cells. Data indicated that phosphorylation of histone H3 (Ser10) was decreased by treatment with ceftriaxone in a dose-dependent manner (Figure 3B). In contrast, phosphorylation of histone H3 (Ser10) was increased slightly and phosphorylation of ERKs and JNKs showed no obvious change. To investigate whether the effects of ceftriaxone are mediated directly through Aurora B, we compared the effects of JB6 P+ cells transfected with an sh-mock or sh-Aurora B plasmid (Figure 3C). Ceftriaxone suppressed anchorage-independent growth in sh-mock cells but had less effect in sh-Aurora B cells (Figure 3D). Previous studies showed that phosphorylation of histone H3 (Ser10) is an essential regulatory mechanism for EGF-induced neoplastic cell transformation (26). Based on this finding, our results suggested that ceftriaxone suppresses anchorage-independent growth by inhibiting phosphorylation of histone H3 (Ser10).

Aurora B is highly expressed in lung cancer cell lines and ceftriaxone inhibits anchorage-independent growth of lung cancer cells

A large number of studies revealed that Aurora B is highly expressed in several human cancers including ovarian, colon and prostate (27–29). To determine whether Aurora B is highly expressed in human lung cancer cells, expression was compared in several human lung cancer cell lines and normal lung cells. The results revealed that
compared with normal lung cells, Aurora B expression was higher in five different lung cancer cell lines compared with normal lung cells. Aurora B expression level was confirmed by western blot analysis using an antibody against Aurora B. α-Tubulin was used as a loading control. Data are representative of results from triplicate experiments. (B) Ceftriaxone inhibits anchorage-independent growth of A549 cells. Cells were cultured with different concentrations of ceftriaxone for 10 days and then colonies were counted. Data are shown as mean ± standard deviation from triplicate experiments. The asterisk (*) indicates a significant (P < 0.05) decrease in colony formation with ceftriaxone compared with the untreated control group. Reversine was used as a positive control. Ceftriaxone also inhibits anchorage-independent growth of H520 (C) and H1650 (D) cells. Cells were cultured with different concentrations of ceftriaxone for 7 days (H520) or 14 days (H1650) and then colonies were counted. The magnification of representative photographs for the anchorage-independent growth assay is 25X. Data are shown as mean ± standard deviation from triplicate experiments. The asterisk (*) indicates a significant (P < 0.05) decrease in colony formation with ceftriaxone compared with the untreated control group. Reversine was used as a positive control.

Aurora B is required for the inhibitory effects of ceftriaxone

In addition, to examine the mechanism explaining the inhibitory effect of ceftriaxone on anchorage-independent cell growth, western blot analysis was used to evaluate different molecular targets. As expected, phosphorylation of histone H3 (Ser10) was inhibited in a dose-dependent manner (Figure 5A), which suggested that ceftriaxone attenuated anchorage-independent growth of A549 cells by suppressing Aurora B activity. To investigate whether the effects of ceftriaxone are mediated directly through Aurora B, we compared the effects of A549 cells transfected with an sh-mock or sh-Aurora B plasmid (Figure 5B). Ceftriaxone suppressed anchorage-independent growth in sh-mock cells but had less effect in sh-Aurora B cells (Figure 5C). These results suggest that Aurora B is a direct target for ceftriaxone to suppress lung cancer cell growth.

Ceftriaxone suppresses tumor growth by inhibiting Aurora B activity in vivo

Based on our previous data, the next step was to determine whether ceftriaxone could suppress tumor growth in vivo. After injecting A549 or H520 cells into nude mice, the first measurable tumors were observed on day 12 or 14, respectively, and then mice were divided into two matched groups. Vehicle or ceftriaxone treatment was started when the tumors reached a mean tumor volume of 100 mm³. Results indicated that the tumors treated with 100 mg/kg ceftriaxone grew significantly more slowly and the sizes of the tumors were smaller compared with the vehicle group (Figure 6A, B).
Supplementary Figure 1A and B, available at Carcinogenesis Online). On day 56, the average tumor volume per mouse treated with 100 mg/kg ceftriaxone was 116 mm³ compared with 360 mm³ in the vehicle group (Figure 6A). Similar differences were observed for H520 tumors—on day 32, the average tumor volume per mouse treated with 50 mg/kg ceftriaxone was 130 mm³ compared with 370 mm³ in the vehicle group (Supplementary Figure 1B, available at Carcinogenesis Online). The average body weights of each group were similar throughout the study (Figure 6B, Supplementary Figure 1C, available at Carcinogenesis Online), which indicated that the dose of ceftriaxone used for the experiment had no toxicity to the mice. To further determine whether the antitumor effect of ceftriaxone was associated with its inhibition of Aurora B activity, tumor extracts from each group were prepared and analyzed for phosphorylation of histone H3. Immunohistochemistry analysis results showed that expression of phosphorylated histone H3 was substantially decreased in the ceftriaxone-treated group compared with the vehicle group (Figure 6C). The magnification of representative photographs for the immunohistochemistry staining is ×100. Overall, our results indicated that ceftriaxone suppresses tumor growth by inhibiting Aurora B activity in vivo.

Discussion

Lung cancer is the most common cause of cancer-related death in men and women worldwide, with an estimated 222,520 new diagnoses and 157,300 deaths in the United States in 2010 (30). The most common symptoms are shortness of breath, coughing (including coughing up blood) and weight loss (31). Several treatment options are available for lung cancer including radiation therapy, chemotherapy, surgery and targeted drug therapy. Some of the treatments, such as chemotherapy, weaken the immune system and make the body more prone to infection (32). Ceftriaxone, an FDA-approved third-generation cephalosporin antibiotic, which was used primarily for treatment of pneumonia, herein is reported to exert antitumor activities. Our data clearly show a role of ceftriaxone as a chemotherapeutic agent for lung carcinoma and strongly suggest that Aurora B as a potential therapeutic “off” target of this drug.

Aurora kinases, a collection of highly related serine/threonine kinases, are comprised of Aurora A, B and C and are key regulators of mitosis performing vital functions in chromosome alignment, segregation and cytokinesis (33). As a member of Aurora kinase family, Aurora B is a chromosomal passenger protein critical for...
accurate chromosomal segregation, cytokinesis and regulation of the mitotic checkpoint (34). According to the literature, Aurora kinases have been shown to play important roles in regulating cell division. Inhibition of Aurora B kinase resulted in cell-cycle arrest or even cell death (35). Azzariti et al. (36) reported that AZD1152, a specific Aurora B inhibitor, suppressed chromosome alignment and segregation in both colon and pancreatic cancer cell lines, resulting in increased chromosome number and cellular apoptosis. Recently, Lee et al. found that reversine, a well-known Aurora B inhibitor, suppressed oral squamous cell growth by interfering with the progression of the cell cycle (37). To determine whether ceftriaxone has a similar effect in A549 cells, different concentrations of ceftriaxone were used to treat A549 cells for 12h, and changes in cell-cycle distribution were examined. Consistent with the report by Lee et al., our data show that the percentage of G2/M cells slightly increased after treatment with high doses (500 µM) of ceftriaxone, which suggests that ceftriaxone induces G2/M arrest of A549 cells (Supplementary Figure 2), available at Carcinogenesis Online). Previous studies showed that Aurora B directly phosphorylates histone H3 not only at Ser10 but also at Ser28, resulting in mitotic chromosome condensation (38). However, accumulating evidence indicates that Aurora B is highly expressed in several malignancies. Progressive increases in the nuclear expression of Aurora B is observed in prostate cancers with increasing Gleason grades compared with normal prostate (39). Inappropriate phosphorylation of histone H3 in the entire cell cycle enhances proliferation of liver

Fig. 6. Ceftriaxone suppresses tumor growth by inhibiting Aurora B activity in vivo. (A) Ceftriaxone treatment suppresses tumor volume compared with the vehicle-treated group. Tumor volume was measured and recorded as described in section ‘Materials and methods’. The asterisk (*) indicates a significant increased tumor size (P < 0.05) in the vehicle-treated group compared with the ceftriaxone-treated group as determined by one-way analysis of variance. (B) Ceftriaxone has no effect on the body weight of mice. The body weight of each mouse was measured and recorded once a week. Data are shown as mean ± standard error. (C) Ceftriaxone inhibits expression of phosphorylated histone H3 in vivo. Immunohistochemistry analysis was used to determine the level of phosphorylated histone H3 in tumor tissues. Top, representative photos from each group; phosphorylated histone H3 is indicated by arrows. Bottom, percentage of phosphorylated histone H3 expression with ceftriaxone treatment compared with the vehicle group. The magnification of representative photos for the immunohistochemistry staining is ×100. Data are shown as mean ± standard deviation from triplicate experiments. The asterisks (**) indicate a significant (P < 0.01) decrease in phosphorylated histone H3 expression in the 100 mg/kg ceftriaxone-treated group compared with the vehicle-treated group. The duration of this animal study was 57 days. It was terminated when most untreated tumors reached 1 cubic centimeter per University of Minnesota IACUC guidelines.
cancer cells (40), which suggested aberrant expression of Aurora B might be involved in hepatocarcinogenesis. Previous studies provided immunohistochemical evidence showing that Aurora B is overexpressed in lung cancer tissues compared with normal adjacent tissues (41). Consistent with this report, our results also showed that Aurora B expression is higher in several lung cancer cell lines, including A549, Calu-3 and H520 cells, compared with MRC-5 normal lung cells (Figure 4A). Moreover, inhibiting Aurora B by ceftriaxone suppressed anchorage-independent growth of A549, H520 and H1650 cells (Figure 4B–D). These results suggest Aurora B might play a significant role in lung cancer cell growth.

Aurora B is only expressed during mitosis and most normal cells in the human body do not proliferate rapidly. Thus, a number of Aurora B inhibitors, including JNJ-7706621, Hesperadin and PHA-739358 (34,42,43), were selected because they could have a broader therapeutic effect than non-specific cytotoxic agents. A number of Aurora B inhibitors have demonstrated antitumor activity and some, such as AZD 1152, have subsequently entered clinical trials. However, neutropenia was observed in a Phase I trial (44), suggesting that this agent had antiproliferation toxicity on the bone marrow. Therefore, finding Aurora B inhibitors, which are highly effective in suppressing Aurora B activity with low toxicity, is urgent and beneficial. Here we found that ceftriaxone, an FDA-approved cephalosporin antibiotic, specifically bound with Aurora B (Figure 2A–D) and suppressed its activity (Figure 3A and 3B) in vitro and in cells. Moreover, knockdown Aurora B decreases the inhibitory effects of ceftriaxone in anchorage-dependent growth of JB6 P+ (Figure 3D) and A549 (Figure 5D) cells. Importantly, an in vivo xenograft animal model showed that ceftriaxone suppressed A549 and H520 lung cancer tumor growth by inhibiting Aurora B activity (Figure 6, Supplementary Figure 1, available at Carcinogenesis Online). These results suggest that Aurora B is a secondary or ‘off’ target protein of ceftriaxone, which suppresses lung carcinoma progression.

In conclusion, we showed that Aurora B is highly expressed in lung cancer cell lines. Moreover, we provided evidence showing that ceftriaxone effectively suppresses anchorage-independent cell growth of lung cancer cells and in vivo tumor growth of A549 cells by inhibiting Aurora B activity. Overall, our findings support the anticancer efficacy of ceftriaxone through its targeting of Aurora B for the treatment of lung carcinomas.

Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/.

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