Radiotherapy and Oncology 121 (2016) 335–341

Contents lists available at ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com

Experimental radiotherapy

Radiosensitization of non-small-cell lung cancer cells and xenografts by the interactive effects of pemetrexed and methoxyamine

Nancy L. Oleinick [a,e,*], Tithi Biswas [a,e], Rutulkumar Patel [a,c], Mingfang Tao [a], Ravi Patel [a], Lachelle Weeks [b], Neelesh Sharma [b,e], Afshin Dowlati [b,e], Stanton L. Gerson [b,e], Pingfu Fu [d,e], Junran Zhang [a,e], Mitchell Machtay [a,e]

[a] Department of Radiation Oncology; [b] Department of Medicine; [c] Department of Pharmacology; [d] Department of Epidemiology and Biostatistics; and [e] Case Comprehensive Cancer Center, Case Western Reserve University and University Hospitals Case Medical Center, Cleveland, USA

ARTICLE INFO

Article history:
Received 15 December 2015
Received in revised form 9 September 2016
Accepted 3 October 2016
Available online 9 November 2016

Keywords:
Radiosensitization
Pemetrexed
Methoxyamine
Non-small-cell lung cancer

ABSTRACT

Background and purpose: The anti-folate pemetrexed is a radiosensitizer. In pre-clinical models, pemetrexed is more effective along with the base-excision-repair inhibitor methoxyamine. We tested whether methoxyamine enhances pemetrexed-mediated radiosensitization of lung adenocarcinoma cells and xenografts.

Materials and methods: A549 and H1299 cells were evaluated for cell cycle distribution by flow cytometry, radiosensitization by clonogenic assay, and DNA repair by neutral comet assay and repair protein activation. H460 cells were included in some studies. Xenografts in nude mice received drug(s) and/or radiation, and tumor growth was monitored by caliper and *in vivo* toxicity by animal weight.

Results: Exposure to pemetrexed/methoxyamine for 24 (H1299, H460) or 48 (A549) hours before irradiation resulted in accumulation of cells near the radiosensitive G1/S border; dose-enhancement factors of 1.62 ± 0.19, 1.97 ± 0.25, and 1.67 ± 0.30, respectively; reduction of mean inactivation dose by 32%, 30%, and 46%, respectively; and significant reductions of SF2 and SF4 ($p < 0.05$). Radiosensitization was associated with rapid DNA double-strand-break rejoining and increased levels of DNA–PKcs. Both tumor-growth rate and tumor-growth delay were significantly improved by adding methoxyamine to pemetrexed pre-irradiation ($p < 0.0001$); no mice lost weight during treatment.

Conclusions: Addition of methoxyamine to pemetrexed and fractionated radiotherapy may improve outcome for patients with locally advanced non-squamous non-small-cell lung cancer.

 Radiotherapy and Oncology 121 (2016) 335–341

Non-small-cell lung cancer (NSCLC) accounts for >80% of all lung cancers, and about 80% of NSCLC are non-squamous. Approximately 30% of lung cancers present with locally advanced but non-metastatic disease [1,2]. With standard platinum-based chemoradiation, overall survival remains poor, with 5-year survival of 15% and median survival of ~20 months [1–4].

Pemetrexed (Alimta®, LY231514) is a multi-targeted antifolate approved for locally-advanced and metastatic non-squamous NSCLC [5,6]. Phase I and II studies show encouraging results with combinations of pemetrexed, a platin and concurrent thoracic radiation therapy (RT) [3,7–13]. Pemetrexed inhibits multiple enzymes, primarily thymidylate synthase (TS) [14], leading to incorporation of uracil into DNA. Subsequent removal of uracil by uracil-DNA-N-glycosylase (UNG) results in apurinic/apyrimidinic (AP) sites, which are repaired by base excision repair (BER). Pemetrexed can also radiosensitize lung cancer cells in culture [15,16].

Methoxyamine (MX) is a strong inhibitor of BER, by reacting covalently with the deoxyribose aldehyde at AP sites to block AP-endonuclease [17]. Preclinical studies at our institution showed that MX augments cell killing and tumor response from agents that cause AP sites, such as bis-chloroethylnitrosourea, temozolomide or pemetrexed [18–21]. MX sensitizes human lung cancer cells to pemetrexed [22], an effect dependent upon the level of UNG [23]. MX also inhibits glycine decarboxylase [24], which transfers single carbons to tetrahydrofolate and is considered an oncogene in lung cancer [25]. A first-in-man Phase I trial of MX with pemetrexed showed safety and efficacy [26].

There is controversy regarding the efficacy of pemetrexed in combined chemoradiotherapy for NSCLC. Therefore, new approaches are needed to enhance the activity of pemetrexed in stage-III NSCLC. Because of established interactions of pemetrexed

* Corresponding author at: Department of Radiation Oncology and the Case Comprehensive Cancer Center, Case Western Reserve University and University Hospitals Case Medical Center, Cleveland, OH 44106, USA.
E-mail address: nlo@case.edu (N.L. Oleinick).

http://dx.doi.org/10.1016/j.radonc.2016.10.007
0167-8140/

with MX [22] and with RT [15,16,27] independently, we reasoned that the triple combination of RT/pemetrexed/MX should increase tumoricidal effects.

Materials and methods

Reagents

Tissue culture media, fetal bovine serum, penicillin/streptomycin and trypsin were from ThermoScientific; pemetrexed from LC Laboratories, Woburn MA; Methoxyamine-HCl and DNase-free RNase-A from Sigma–Aldrich; Matrigel from BD Biosciences, Bedford MA; Propidium iodide from Molecular Probes, Eugene OR.

Cell culture

A549 (p53-wildtype) and H1299 (p53-null) human lung adenocarcinoma cells or H460 human large cell lung carcinoma cells were grown in DMEM and RPMI, respectively, supplemented with 10% fetal bovine serum and penicillin/streptomycin in a 37 °C incubator in 5% CO_2/95% air.

Exposure of cells to drugs and radiation

Pemetrexed was dissolved in water. Methoxyamine was dissolved in water and brought to pH ~7.4 with NaOH. Drugs were further diluted into growth medium. γ-Radiation was provided by a Shepherd Mark-I Cs-137 irradiator (dose-rate 3 Gy/min).

Clonogenic survival

Exponentially growing cultures were treated with 200 nM pemetrexed and/or 3 mM methoxyamine or vehicle (medium) for 24 or 48 h. After 1 h in drug-free medium, cells were trypsinized, collected in complete medium, irradiated, and immediately plated at appropriate densities in triplicate 60-mm dishes to yield ~50–150 colonies/dish. After 10–14 days, colonies were stained with crystal violet. Images of air-dried plates were digitized using a high-resolution scanner, and the plating area was segmented using a custom-written program in Matlab. Images were opened in ImageJ, and a custom macro which thresholded images and utilized built-in macro functions, such as particle count analysis and watershed segmentation, was used. The colony count was obtained as the output segmentation algorithm in ImageJ. Plating efficiency (PE) was calculated as number of colonies divided by number of cells plated. Surviving fraction (SF) was determined by normalizing PE to control PE. Dose enhancement factor (DEF) was calculated as the ratio of the dose reducing SF to 10% without drug to the comparable dose with drug(s). Mean inactivation dose (MID) was also calculated [28].

Cell cycle analysis

Cells were treated with drug(s) as above, then collected, fixed in 90% methanol and washed with ice-cold 1% bovine-serum albumin in phosphate-buffered saline. RNA was removed with 2.5 mg/mL of DNase-free RNase-A, and DNA was stained with 20 mg/mL propidium iodide (PI). The fluorescence of PI-stained cells was measured with an EPIC-XL flow cytometer (Coulter, Miami, FL).

Repair of DNA damage

Cells treated as above were analyzed by neutral comet assay (Trevigen, Gaithersburg, MD) for the presence of DNA double-strand breaks. Comets were analyzed using CometScore software. Levels of UNG and activation (phosphorylation) of DNA–protein kinase catalytic subunit (DNA–PKcs) were monitored by western blotting using the antibodies listed in Supplementary Table 1.

Generation, treatment and responses of xenografts

Animal studies were approved by the CWRU Institutional Animal Care and Use Committee. A549 (5×10^6) or H1299 (0.75×10^6) cells were injected into the right flanks of 6-week-old female athymic NCr (nu/nu) mice. When tumor volumes reached 100–175 mm^3, mice were randomized into 8 arms (Day 0), such that the mean size of each arm did not differ from the overall mean size by more than 7.2%. Mice were treated with pemetrexed (150 mg/kg), MX (4 mg/kg), both drugs, or saline by daily intraperitoneal injection (Days 1–5). Half of the tumors were irradiated on Day 6 (8 Gy). Animals were weighed and tumors were measured twice weekly, and tumor volume was calculated [(length · width · height)/2].

Statistical analysis

Clonogenic survival over radiation dose was summarized by MID, estimated by the area under the survival curve over the dose range [28]. The difference of continuous measurements including MID among treatment groups was tested using analysis of variance (ANOVA) followed by pair-wise comparisons. To estimate tumor growth rate, a mixed-effect model [29,30] was used, assuming tumor volumes measured over time from the same animal are correlated; and unstructured covariance structure was used for inference. Time-to-event analysis defined the event as tumor volume >400 mm^3 (A549 cells) or >600 mm^3 (H1299 cells). Time-to-event was calculated from day 0 (day of randomization) or from Day 7 to the day tumor volume reached 400 mm^3 or 600 mm^3 and was censored for those whose tumor volume did not reach those levels at the end of follow-up. Probability of survival (% of animals whose tumor volume was <400 mm^3 or <600 mm^3) was estimated using the Kaplan–Meier method [31], and the difference of time-to-event among treatment groups was examined using log-rank test. All tests were two-sided, and p-values less than 0.05 were considered statistically significant.

Results

Prior studies of radiosensitization by pemetrexed [15,16,27] and the ability of MX to enhance the cell toxicity of pemetrexed [22] demonstrated inhibition of cell growth with pemetrexed or pemetrexed/MX, due to interference with S-phase progression. We aimed to take advantage of the drug-induced shift toward the radiosensitive G1/S border. Thus, we first evaluated the changes in cell cycle distribution of treated and control populations of A549 and H1299 cells. Results are presented as representative histograms (Supplementary Fig. 1, A–L) and the percentage of cells in each cell cycle phase (Supplementary Fig. 1, M–O). MX itself had no effect on cell cycle distribution compared to non-drug-treated cells. Incubation with pemetrexed for 24 or 48 h caused A549 cells to accumulate near the G1/S border and in early S-phase, and pemetrexed/MX produced a similar or greater redistribution, as indicated by the right-shifted position of the main peak in the treated cells and by the increase in S-phase cells. Similar shifts in cell cycle distribution occurred in H1299 cells after 24 h in pemetrexed or pemetrexed/MX. Thus, cells accumulate toward the G1/S border following pemetrexed exposure, MX may amplify the pemetrexed-induced cell cycle shift, and the effect with or without MX is p53-independent.

Cells are relatively radiosensitive near the G1/S border [32]. Thus, our clonogenic survival studies included pre-treatment with one or both drugs, followed by drug removal, irradiation, then