A Preliminary Study on Combination Therapy of Artemisinin Dimer Oxime and Topotecan against Nonsmall Cell Lung Cancer in Mice

Mohammad K. Ashfaq, Mohamed Sadek Abdel-Bakky*, Mir Tahir Maqbool, Waseem Gul*a, Mahmoud A. ElSohly1,3,6

*National Center for Natural Product Research, School of Pharmacy, University of Mississippi, University, MS 38677. 1Department of Pharmaceutics, School of Pharmacy, University of Mississippi, University, MS 38677. 2ElSohly Laboratory Incorporation, Industrial Drive, Oxford, MS 38655, USA, 3Faculty of Pharmacy, Al-Azhar University, Cairo, 11884, Egypt

Abstract

Background: Artemisinin dimer oxime – dimer molecule synthesized from artemisinin possesses high bioavailability and marked in vitro anticancer activities against solid tumor-derived cell lines, endothelial cell proliferation, migration, and angiogenic processes. Numerous marine models have been developed to study human cancer. The most widely used models are the human tumor xenograft mouse model.

Materials and Methods: In this study, human tumor cells (NCI-H640, 1 × 10⁶ in 100 µL) are implanted subcutaneously, or 1 × 10⁶ in 50 µL in the thoracic cavity, in athymic nude mice (nu/nu). The implanted cells were allowed to grow for 10 days before initiation of drug treatment (dimer oxime and topotecan, ip). Tumor volume and thoracic/body weight ratio were recorded.

Results: We successfully established subcutaneous and thoracic xenografts with human nonsmall cell lung cancer cell line xenografts in athymic nude mice in only 10 days. Using these models, we attempted treatment of xenografts with topotecan – a known anticancer drug and artemisinin dimer oxime or combination of these two drugs. Combination therapy showed a significant reduction in tumor volume and tumor/body weight. Treatments with combination of topotecan and dimer oxime resulted in the reduced mortality rates in comparison with untreated mice.

Conclusions: Xenograft tumor models are useful for preclinical screening of new pharmacophores. From this preliminary study, it appears that combination of dimer oxime and topotecan may be used as chemotherapeutic agents against nonsmall cell lung cancer. Further studies are needed to evaluate other combination treatment regimens as well as the mechanism(s) of action.

Keywords: Artemisinin, cancer, dimer oxime, topotecan, xenografts

INTRODUCTION

Artemisinin is a natural product present in the Artemisia annua. In the 4th century, this plant was prescribed as a natural remedy for treating fever by Chinese physicians. In the succeeding centuries, this natural remedy was commonly prescribed for hemorrhoids and malaria. However, the active agent – artemisinin – was identified and isolated in the 1970s. At present, numerous derivatives of artemisinin are being used for treating various ailments. Several in vitro studies have demonstrated that artemisinin and its derivatives possess promising anticancer, antiproliferative, and antiapoptotic properties in tumor cell lines of colon, breast, and lung.1 Dihydroartemisinin has shown therapeutic activity against gallbladder cancer metastasis.2 Artemisinin dimer oxime (Ox) – dimer molecule synthesized from artemisinin possesses high bioavailability and marked in vitro anticancer activities compared to its monomers.3 In a recent study, the antitumor activity of five chemically synthesized dihydroartemisinin dimers against human colon cancer cell lines, HT29, HCT116 and the nontumorigenic colon cell line, DR14, was investigated.4

Address for correspondence: Dr. Mohammad K. Ashfaq, National Center for Natural Product Research, School of Pharmacy, University of Mississippi, Oxford, MS 38655, USA. E-mail: mikashfaq@olemiss.edu

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

© 2018 World Journal of Traditional Chinese Medicine | Published by Wolters Kluwer - Medknow

Received: 26-05-2017, Accepted: 08-01-2018

How to cite this article: Ashfaq MK, Abdel-Bakky MS, Maqbool MT, Gul W, ElSohly MA. A preliminary study on combination therapy of artemisinin dimer oxime and topotecan against nonsmall cell lung cancer in mice. World J Tradit Chin Med 2018;4:8-14.
and FHC were compared. Dimer oxime showed remarkable selective toxicity in HT29 and HCT116 cells. In vitro studies have shown anticancer activity of artemisinin dimer oxime against selected cell lines of nonsmall cell lung cancer, colon cancer, leukemia, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer.

Nonsmall cell lung cancer accounts for approximately 80% of entire lung cancer and is the leading cause of cancer-related deaths for both men and women worldwide. Statistical estimates by the American Cancer Society for 2017 indicate 222,500 new lung cancer cases and 155,870 associated deaths in the United States alone, suggesting that more individuals are likely to die of lung cancer than of colon, breast, and prostate cancers combined. The pathogenesis of lung cancer remains highly intangible due to its aggressive biologic nature and considerable heterogeneity, as compared to other cancers. It has been observed that despite having aggressive local and systemic therapies, most patients die of the progressive metastatic disease. These circumstances markedly impede the study of this disease in humans and necessitate the use of experimental models that can be used under more uniform, controlled conditions than that achievable in the clinical situations.

According to the American Cancer Society, combination therapy has been ascertained to be an effective strategy for reducing nonsmall cell lung cancer burden and metastasis. Several compounds have been in use including the analogs of topotecan, cisplatin, and paclitaxel singly or in combination to reduce tumor burden. Several reports indicate that natural products enhance the efficacy of known antitumor agents. Theanine – an amino acid enhances antitumor activity of doxorubicin – a known antitumor drug, against ovarian sarcoma. Similarly, quercetin has been shown to act synergistically with triazofurin and carboxytriazole (known anticancer drugs) against human ovarian carcinoma cells OVCAR-5 and human breast carcinoma MDA-MB-435 cells, respectively. Furthermore, genistein a phytoestrogen enhances the antiproliferative activity of eicosapentaenoic acid in human breast carcinoma. Topotecan alone or in combination with other anticancer drugs has shown some promising activity in the treatment of nonsmall cell lung cancer. A recent study has also shown that combination of dimer oxime and irinotecan analog of topotecan has potent anticancer activity against colon cancer cells in vitro.

Currently, several animal models are widely used for evaluating novel therapeutic strategies against lung cancer. These include chemically induced lung tumors, transgenic mouse models, and human tumor xenografts. To understand lung tumor biology, facilitate novel therapies and diagnostics, development of appropriate animal model for lung cancer is necessary. Thus, any drug that has a potential in vitro anticancer effect goes through a screening process in preclinical cancer models. In the present study, we have developed a tumor model with human tumor cell line NCI-H640, which exhibits similarity to human disease. Although xenograft subcutaneous tumor models have served a useful purpose for the preclinical efficacy of anticancer drugs, the recent trend has diverted attention toward developing orthotopic tumor models. To this end, urinary and digestive tract tumors have been constructed in immune-deficient SCID mice and nude mice making use of corresponding malignant tumor cells. McLemore et al. established the first orthotopic model of human lung cancer in nude mice through endobronchial injection. Later on, researchers adopted injection of tumor cells through the tail vein and intrapulmonary inoculation of the tumor mass.

In the present study, we have used xenograft subcutaneous and intrathoracic tumor model in mice for assessing in vivo antitumor activity of artemisinin dimer oxime (Ox) alone and in combination with topotecan (Tp) – a known anticancer drug.

**Materials and Methods**

Topotecan was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and dissolved in injectable water. Artemisinin dimer oxime was synthesized at ElSohly Laboratories Inc., Oxford, MS, USA. Matrigel was purchased from BD Biosciences, (Billerica, MA, USA).

**Synthesis of the dimer oxime**

Dihydroartemisinin dimer ketone, (50 mg, 0.08 mmol), sodium acetate (0.48 mmol), and hydroxyl amine (0.1 mmol) were taken in 5 ml of dichloromethane (freshly distilled) and refluxed for 4 h under argon. TLC indicated the completion of the reaction [Figure 1].

The resulting reaction product was stripped to dryness, the residue dissolved in 6 ml of ethyl acetate, washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was chromatographed on silica gel column (300 mg) and eluted with hexane with polarity increasing to 60:40 hexane: ethyl acetate. Fractions were collected and combined according to TLC similarities to give one major fraction having the desired product (44.1 mg), with spectral data consistent with structure of dimer oxime.

**Cell lines**

Human nonsmall cell lung cancer cell lines (NCI-H640) were purchased from ATCC (Bethesda, MD). These cell lines were grown in RPMI 1640 with 10% fetal calf serum supplemented with 1% PenStrep. When the monolayers were 90%–95% confluent, the cells were harvested and suspended to the desired concentration (~1-2 × 107 cells/100 µL) in RPMI1640 with equal volume of Matrigel 1 mg/ml (Corning Life sciences).

**Mice**

Athymic nude mice (nu/nu) were obtained from Harlan Lab Inc. (Indianapolis, IN). These mice were housed in filter-top microisolator cages and provided autoclaved corn cob bedding and sterilized animal chow. They were housed in a room with...
12 h night and day cycles. This tumor model protocol was approved by the University of Mississippi, IACUC.

**Preparation of topotecan and oxime for animal dosing**

Mice were injected ip with Ox at a dose of 10 mg/kg body weight (dissolved in ethanol 5%, cremophore 5%, and injectable water 90%) and Tp at a dose of 2 mg/kg body weight (dissolved in injectable water).

**Subcutaneous and thoracic tumor models**

In a volume of 100 µL, 1 × 10^7 NCI-H640 cells were injected subcutaneously in the neck region. For intrathoracic injections, animals were anesthetized with isoflurane, placed in lateral recumbency and 50 µL of the cell suspension was injected into the right thoracic cavity. The implanted cells were allowed to grow for 10 days before initiation of treatment.

Mice bearing subcutaneous tumors were randomized and divided into four groups with four mice in each group. Similarly, mice given intrathoracic injection of NCI-H640 cells were also divided into four groups. Group I, as untreated negative control. Group II, as positive control given Tp (2.0 mg/kg, intra-peritoneal) thrice a week. Animals of the Group III were given Ox (10 mg/kg) thrice a week and Group IV was given Tp (2.0 mg/kg) and dimer oxime (10 mg/kg) thrice a week, ip. Tumor size was recorded daily and tumor volume was calculated as:

\[
\text{Tumor volume} = \frac{1}{2} \times a \times b^2, \quad \text{where} \quad a = \text{length of tumor mass} \quad \text{and} \quad b = \text{width of tumor mass}
\]

For assessment of anticancer activity of these treatment regimens in mice implanted with NCI-H640 cells in the thoracic cavity, thoracic/body weight ratio was determined.

**RESULTS**

**Establishment of xenograft model in nude mice**

Subcutaneous injection of human nonsmall cell lung cancer cell line (NCI-H640) (~1–2 × 10^7 cells/100 µL) produced solid tumors in mice within 10 days. Figure 2a showing tumor size in untreated mice and those treated with topotecan (Tp). Figure 2b showing percentage increase in the tumor volume from the day of treatment. An approximately 7-fold increase in tumor volume was observed in the untreated mice compared to those treated with Tp.

**Effect of topotecan, dimer oxime and their combination (topotecan + oxime) on tumor volume**

As shown in the Figure 3, Tumors mass grew exponentially, if left untreated. A remarkable reduction in tumor volume was observed in Tp + Ox-treated mice as compared to the untreated mice. Photomicrographs in Figure 4a and b show that mice with subcutaneous implanted tumors when treated with Tp (Group II) showed reduction in tumor size compared to those of the untreated mice (Group I). Similar observation was recorded with Ox treatment. The combination of Tp and Ox showed even further inhibition of tumor growth resulting in smaller sized tumors. At the termination of treatment regimen, the body weight was recorded, the tumor mass was then excised and weighed. The ratio of tumor weight and
body weight was calculated. Figure 5 shows that Tp-treated animals had a significant \( (P < 0.05) \) reduction in tumor body weight ratio compared to untreated mice. The Tp + Ox-treated mice showed even further reduction in tumor body weight ratio compared to untreated mice \( (P < 0.01) \). However, Ox treatment did not cause a significant reduction in tumor body weight ratio. The combination treatment (Tp + Ox) was better than Tp \( (P < 0.05) \) or Ox \( (P < 0.01) \) treatment given separately.

For anticancer activity of these treatment regimens in mice implanted with NCI-H640 cells in the thoracic cavity, thoracic/body weight ratio was determined. Tp treatment showed a significant reduction in thoracic/body weight ratio compared to untreated control \( (P < 0.05) \). However, Ox treatment did not show a significant reduction. Combination of Tp and Ox treatment showed more reduction in thoracic/body weight ratio \( (P < 0.01) \) [Figure 6].

**Effect of topotecan + oxime treatment on the mortality/survival data**

As shown in Figure 7, in the untreated mice, mortality was observed on day 9 and by day 13, 50% mice had died. The Ox-treated group showed mortality on day 11 and 33% of animals had died by day 13. However, no mortality observed in Tp-treated and Tp + Ox-treated animals.

![Figure 3: Reduction in the tumor volume in mice treated with combination of topotecan and oxime as compared with untreated mice within a week to 10 days](image)

**Effect of topotecan + oxime treatment on the daily body weight**

In the subcutaneous model, none of the groups showed a substantial change in body weights; the OX + Tp-treated mice showed a decreasing trend in their body weight after 10 days of treatment [Figure 8]. Similarly, in the thoracic model, all groups showed a decreasing trend in their body weights [Figure 9].

**DISCUSSION**

Animal models have been extensively used as the frontline in evaluating the efficacies of new chemical entities and finding toxicities of new chemical therapeutic agents before reaching the clinical practice. This has led to the development of many different animal models of malignant diseases. Athymic (nu/nu) nude mice allow relatively efficient transplantation and propagation of human tumors in mice. Established *in vitro* human cell lines can be propagated subcutaneously in these mouse strains. Moreover, human tumor tissue explants obtained from the biopsy can also be transplanted directly in this strain of mice. Innovations in scientific research resulted in the establishment of orthotopic murine lung cancer models using either human xenograft lung cancer cells or Lewis lung carcinoma cells, constructing a platform to examine novel drug entities in orthotopic animal models. Subcutaneously or orthotopic tumor xenografts in nude mice are available for many tumor types and have become the major model for preclinical *in vivo* anticancer screening and drug development. It should be borne in mind that every animal model for anticancer drug development does present its pros and cons. Having said that, comparison of drug responses in many xenografts developed from different types of tumors and individual patient has shown that xenograft model has >90% possibility in correctly predicting the clinical response. In the present study, we successfully produced subcutaneous and lung cancer models in nude mice. The subcutaneous model is relatively simple to reproduce. The lung cancer model was also established with minimal invasiveness. However, further work in this arena with specific tumor markers for monitoring tumor
Anticancer effect of dimer oxime against thoracic and subcutaneous tumors

Development would certainly provide deeper understanding of its pathobiology and enhance its relevance on preclinical anticancer drug development. Furthermore, we validated our cancer model by treating the tumor-bearing mice with topotecan – a known anti-cancer drug. Numerous recent studies have shown that Tp is a potent chemotherapeutic agent[20] that increases the survival rate and decreases the tumor growth.[27,28] Our results are also in accordance with the previous studies that reported the amelioration of tumor growth by Tp treatment.

Topotecan may cause apoptosis by forming topotecan-DNA complex (topoisomerase I inhibitor) that induces cell cycle arrest at S/G2-M leading to inhibition of mitosis.[29] Furthermore, topotecan can also induce oxidative stress by increasing the levels of reactive oxygen species (ROS) and nitrite. Elevation of ROS can cause irreversible damage and modification to proteins by inducing the formation of protein carbonyl derivatives.[30] Artemisinin compounds, on the other hand, appear to regulate multiple pathways including nuclear factor-kappa B, survivin, NOXA, hypoxia-inducible factor-1α, and BMI-1 proto-oncogene that may affect drug response, drug interactions, and drug resistance.[31] It has been suggested that semi-synthetic artemisinin derivatives possess higher anticancer activity than their monomeric compounds through mechanisms such as apoptosis, arrest of cell cycle at G0/G1, and oxidative stress.[32] These two drugs suppress cell proliferation through two different targets. Therefore, it was anticipated to have a synergistic or additive anticancer effect. However, the exact mechanism and molecular basis of these anticancer effects are not fully understood.

In the mouse models of cancer presented here, we attempted combination therapy of these implanted tumors with Ox and Tp – known anticancer drug. Our results, though very preliminary, show that in the subcutaneous-implanted tumors,
the combination therapy caused a significant reduction in tumor burden as compared to Tp or Ox treatments alone. However, it is difficult to assess if a synergistic effect occurred. In the thoracic-implanted tumors the tumor burden was not significantly reduced with Ox treatment. These results suggest that the combination treatment was more effective in reducing tumor burden in the subcutaneous tumors than in the thoracic cavity. The relative inefficient activity of Ox in reducing tumor burden in the thoracic model may be related to the dose of the drug. In a breast cancer model, dihydroartemisinin (a related compound of artemisinin dimer oxime) a dose of up to 100 mg/kg has been used, whereas in our study, only 10 mg/kg was administered. Had the dose of Ox been raised, the efficacy for thoracic tumors in this study would have shown better results. This also underscores the bioavailability factor of dihydroartemisinin in two different anatomical locations which may also play a role in its effectiveness. These factors need to be considered for designing further studies.

### Conclusions

We have successfully established a subcutaneous and a xenograft lung tumor model in nude mice with NCI-H640 (human nonsmall cell lung cancer cell line). The importance of this model lies in its short duration (within 7-day postcancer cell inoculation). Our results confirm that in the subcutaneous model, Tp treatment (2.0 mg/kg) significantly reduced tumor volume compared to that of untreated animals. However, the relative ineffectiveness of these drugs regimens in the thoracic model raises questions regarding the dose and/or the bioavailability of the drug(s) in other anatomical sites. This xenograft tumor model can be used to screen natural products for their anticancer effect in vivo. In the present study, we have attempted treatment with Ox in combination with Tp. We have found that Ox along with Tp can be used as chemotherapeutic agents. Further studies are required to determine the appropriate dose and the mechanism of action of Ox.

### Acknowledgment

We would like to thank the Vivarium staff of the University of Mississippi for their help in animal care. We also thank Harlan Laboratories Inc. (now Envigo) for providing mice for these studies at no cost.

### Financial support and sponsorship

Nil.

### Conflicts of interest

There are no conflicts of interest.

### References

25. Wong CC, Cheng KW, Rigas B. Preclinical predictors of anticancer drug efficacy: Critical assessment with emphasis on whether nanomolar potency should be required of candidate agents. J Pharmacol Exp Ther