



Cytotoxic and Apoptotic Effects of the Combination of Borax (Sodium Tetraborate) and 5-Fluorouracil on DLD-1 Human Colorectal Adenocarcinoma Cell Line

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ABSTRACT

Objectives: Colorectal cancer (CRC) remains a crucial health problem due to the toxicity of 5-Fluorouracil (5-FU) as first-line chemotherapy agent for treating CRC. The anticancer effects of boron and its compounds have been shown in various cell lines. This study aimed to examine the cytotoxic and apoptotic effects of borax (sodium tetraborate) alone or along with 5-FU on human CRC cells, DLD-1.

Materials and Methods: Cytotoxicity and apoptosis were determined by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay, 4',6-diamidino-2-phenylindole and annexin V/propidium iodide staining.

Results: The results showed that combined treatment revealed a significant time- and concentration-dependent cytotoxic effect on DLD-1 cells compared with borax or 5-FU treatment alone. The combination of borax and 5-FU induced a clear increase in the early apoptotic cell percentage, compared to the cells treated with monotherapies. Additionally, a significant increase in condensed and fragmented nuclei was detected in DLD-1 cells treated with the combination treatment compared with borax or 5-FU alone.

Conclusion: Our current findings suggest that the combination of borax with 5-FU has a strong cytotoxic and apoptotic effect on the human CRC DLD-1 cells.

Key words: Apoptosis, borax, colorectal adenocarcinoma, DLD-1, 5-fluorouracil

INTRODUCTION

Colorectal cancer (CRC) is the second most commonly identified cancer and is the third leading occur in cancer-related deaths in the worldwide.¹ 5-Fluorouracil (5-FU) has been widely used intravenously as the first-line drug for treating both advanced and early stage CRC.² However, the patients' low response rates to therapy, development of chemoresistance and serious adverse reactions severely limit the clinical application of 5-FU in advanced CRC.³⁻⁶ Recently, many aggressive adjuvant therapies combined with 5-FU have been developed to

overcome clinical resistance.^{7,8} The combination of 5-FU and other agents as an advanced-stage CRC treatment has shown success in prolonging patient survival,^{9,10} but leads to increased vulnerability of patients to disease relapse together with high costs and some side effects.¹⁰

Previous clinical studies have demonstrated the possible beneficial effects of high boron intake in lung and prostate cancer.^{11,12} Boron, a nutrient element, is present in food and drinking water and categorized as "probably essential" for humans by the World Health Organization.¹³ Boron is found

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abundantly in nature as boric acid (a soluble form of boron) and inorganic salts called borates.^{14,15} Sodium tetraborate known as borax is a salt of boric acid.¹⁵ A study by Wei et al.¹⁵ revealed the anticarcinogenic effect of borax in hepatocellular carcinoma. Another boron compound, boric acid inhibited the proliferation of prostate cancer cell lines, DU-145 and LNCaP¹⁶ and MDA-MB-231 human breast cancer cells¹⁷ and inhibited cell growth, apoptosis, and morphological alterations of DU-145 cells.¹⁸ Currently, bortezomib, which is made from boric acid polymers, is used as an anticancer chemotherapeutic agent for treating multiple myeloma cells.¹⁹ Additionally, boron compounds have been used in neutron capture therapy for different types of cancer.^{20,21}

The use of natural products along with a conventional chemotherapeutic agent, 5-FU enhanced efficacy in anti-CRC treatment.^{2,22,23} Accordingly, the primary aim of this study was to evaluate the effects of borax (sodium tetraborate) combined with 5-FU on the viability and apoptosis of DLD-1 CRC cells.

MATERIALS AND METHODS

Cell culture and chemical treatments

Human colorectal adenocarcinoma DLD-1 cells (CCL-221) were bought from American Tissue Culture Collection (ATCC, Manassas, VA). The cells were cultured in RPMI-1640 medium (Capricorn Scientific, Germany) supplemented with 10% fetal bovine serum (Gibco, USA) in a humidified incubator (Sanyo MCO-20AIC, California, USA) containing 5% CO₂ at 37°C.

Borax was obtained from Sigma-Aldrich (St. Louis, MO, USA), and prepared as 0.25 M stock solution in RPMI-1640 medium (Capricorn Scientific, Germany). 5-FU was diluted with physiological saline solution to obtain 50 mg/mL stock solution. Both aliquots were stored at -20°C until further experiments.

Cell viability assay

Cell viability was determined by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously.²⁴ The effect of borax, either alone or with 5-FU, on CRC cell survival was evaluated by cell viability assay using MTT (Sigma-Aldrich, St. Louis, MO, USA). DLD-1 cells were cultured in 96 well plates (2 x 10³ cells/well) with 100 µL of complete medium. At the end of 24 h incubation, cells were treated with various concentrations of borax (50 to 1000 µM) or 5-FU (5 to 100 µg/mL). In combination treatment, increasing concentrations of borax ranging from 50 to 500 µM were mixed with IC₅₀ value of 5-FU (50 µg/mL) and applied to the wells. Upon 24 and 48 h treatment, 20 µL of MTT reagent (5 mg/mL in RPMI) was added to each well. After 4 h incubation at 37°C, culture medium containing MTT was removed, formazan crystals were dissolved in 100 µL isopropanol. The absorbance of the wells was measured at 590 nm in a micro-plate reader (ThermoScientific, USA).

Quantification of apoptotic cells by flow cytometry

Quantitative assessment of the cell apoptosis rate was determined by the Annexin V-FITC Apoptosis Detection

Kit (Thermo Scientific, Waltham, MA, USA) in line with manufacturer's protocol. In the experiments, we selected the IC₅₀ concentration of borax (500 µM) and 5-FU (50 µg/mL) for 48 h according to the MTT assay. Firstly, DLD-1 cells were cultured in 25 cm² flasks with a density of 1 x 10⁶/mL and incubated for 24 h. After treating DLD-1 cells with borax or 5-FU alone or a combination of these reagents for 48 h, cells were harvested by trypsinization and washed twice with cold phosphate buffered saline (PBS) *via* centrifugation at 1000 rpm for 3 min. The cell pellets then were resuspended in 100 µL of 1X Annexin binding buffer and 5 µL of FITC-conjugated Annexin V.²⁵ After the addition of 20 µL of propidium iodide (PI), samples were vortexed gently and 30 min incubation process was initiated in the dark, then 400 µL of 1X Annexin binding buffer was added into each tube. Finally, the number of viable, necrotic, and apoptotic cells quantified by a flow cytometer (BD Bioscience, USA) with CellQuest software for data analysis.

4',6-Diamidino-2-phenylindole (DAPI) staining

DAPI staining was performed on DLD-1 cells treated with borax, 5-FU, and a combination of both to investigate nuclear morphological changes. After 24 and 48 h incubation in 6-well plates at a density of 2.4 x 10⁴ (cells/cm²), the cells were harvested and centrifuged at 1000 rpm for 10 min. Then, cell pellets were rinsed with PBS and fixed using 100 µL of 4% formaldehyde for 10 min at room temperature. The fixed cells were centrifuged at 3000 rpm for 2 min and cell pellets were washed with sterile PBS.²⁶ Finally, cells were stained with 20 µL DAPI (Thermo Fisher, USA) at room temperature for 20 min in a dark place. After the incubation, the supernatant was discarded by centrifugation at 3000 rpm for 2 min, and 20 µL of sterile PBS was added to the cell pellet. One µL of the final mixture was placed on slides.²⁶ Morphological changes in cell nuclei were visualized under the Thermo Fisher EVOS M5000 imaging system equipped with a DAPI filter.

Statistical analysis

Statistical analysis was carried out using SPSS 19.0 for Windows (SPSS, Chicago, IL, USA). The numerical parameters were reported as mean ± standard deviation. Differences between the control and treatment groups were examined by one-way ANOVA test for triplicate experimental data. Test results ($p < 0.05$) were considered statistically significant.

RESULTS

Cytotoxicity of borax, 5-FU, and their combination in DLD-1 cells

Based on the results of MTT assay, borax (150-1000 µM) or 5-FU (20-100 µg/mL) treatment for 24 and 48 h suppressed DLD-1 cell growth dose and time-dependently ($p < 0.05$), with an IC₅₀ value of 500 µM and 50 µg/mL for 48 h, respectively (Figure 1). Additionally, the combination of four different concentrations of borax (150, 200, 250, and 500 µM) with IC₅₀ concentration of 5-FU (50 µg/mL) for 24 and 48 h displayed strong growth-inhibitory activity in DLD-1 cells compared with control as shown in Figure 2 ($p < 0.05$).

The percentage of viable cell amount was 81.5 ± 4.28 at $150 \mu\text{M}$ borax and 53.7 ± 3.19 at $50 \mu\text{g/mL}$ 5-FU in DLD-1 cells for 48 h, while it was decreased to 38.59 ± 2.28 in DLD-1 cells treated with a combination of $150 \mu\text{M}$ borax with $50 \mu\text{g/mL}$ FU for 48 h (Figure 2).

Analysis of Annexin V-FITC/PI staining

To quantitatively analyze the apoptosis- and necrosis-related cell death, DLD-1 cells were treated with borax or 5-FU alone or in combination for 48 h and stained with Annexin V-PI. As shown in Figure 3, DLD-1 cells treated with borax or 5-FU alone and borax + 5-FU combination demonstrated significantly increased early (Annexin V+/PI-) and late (Annexin V+/PI+) apoptotic cell percentages compared with untreated control cells. Besides,

more apoptotic cell death was observed in combined treatment (66.3%) compared with borax (46.8%) or 5-FU (32.2%) alone in DLD-1 cell lines. Based on the flow cytometry results, the percentage of early apoptotic cells in DLD-1 cells treated by borax ($25.5 \pm 2.1\%$) was similar to 5-FU ($21.8 \pm 1.8\%$) as a common approved anticancer drug. A significantly greater percentage of early apoptotic cells was found in combination treatment ($43.9 \pm 3.2\%$) compared to borax or 5-FU alone. These findings demonstrate that borax and 5-FU could mediate DLD-1 cell growth inhibition through the induction of apoptosis.

Analysis of DAPI staining

To clarify whether combination treatment with borax and 5-FU for 48 h induced apoptosis of DLD-1 cells, DAPI staining was

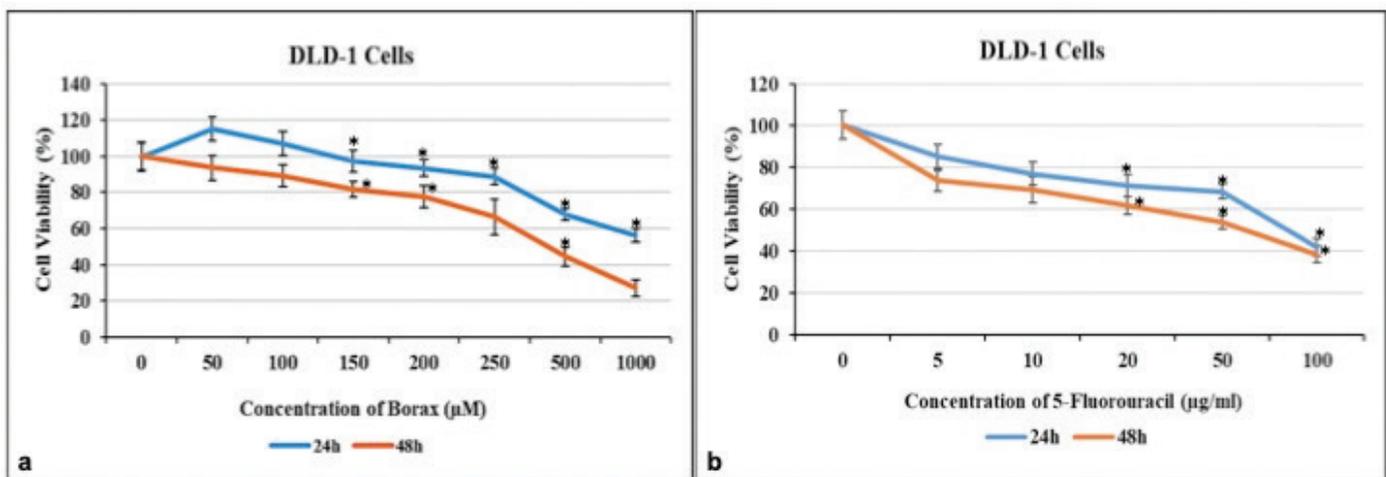


Figure 1. Cytotoxicity of borax (50-1000 μM) and 5-FU (5-100 $\mu\text{g/mL}$) against DLD-1 cells after 24 and 48 h of treatment. The IC_{50} of borax was 500 μM (a) and the IC_{50} of 5-FU was 50 $\mu\text{g/mL}$ (b) for 48 h. Error bars represent mean \pm SD of the three independent experiments

* $p < 0.05$ demonstrates the statistical significance between treatment groups and control cells, SD: Standard deviation (n: 3), 5-FU: 5-Fluorouracil

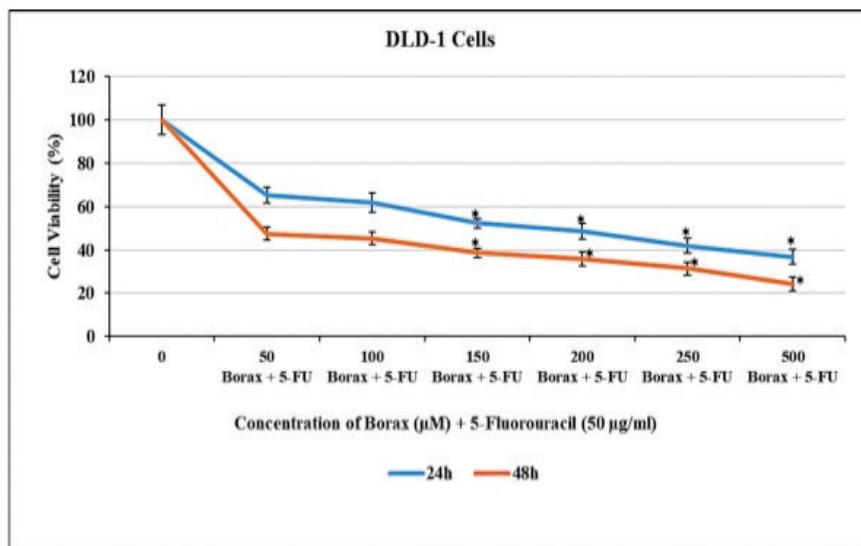


Figure 2. Effects of 5-FU at IC_{50} concentration on the viability of DLD-1 cells after incubation with borax (50, 100, 150, 200, 250 and 500 μM) for 24 and 48 h. Error bars represent mean \pm SD of the three independent experiments

* $p < 0.05$ demonstrates the statistical significance between treatment groups and control cells, SD: Standard deviation (n: 3), 5-FU: 5-Fluorouracil

conducted. As shown in Figure 4, DLD-1 cells treated with either borax or 5-FU showed fragmented nuclei and cellular disintegration into apoptotic bodies compared with untreated control cells. However, much stronger morphological and apoptotic changes that involve condensed and fragmented nuclei were observed in combination treatment compared with borax or 5-FU alone.

DISCUSSION

No article considers the evaluation of borax, which is a salt of boric acid, on DLD-1 cells with it is cytotoxic and apoptotic effects. In this study, the cytotoxic and apoptotic effects of borax combined with or without 5-FU were investigated on DLD-1 cells. Combined treatment exhibited a more significant reduction in DLD-1 cell viability compared to borax or 5-FU alone in a time and dose-dependent manner. As revealed using different methods (Annexin V-FITC/PI and DAPI staining), it appeared that the anti-proliferative effect of boric acid or 5-FU treatment alone, and their combination on DLD-1 cells is mediated by induction of apoptosis.

In the current study, the concentration of borax between 150 and 1000 μM and 5-FU between 20 and 100 $\mu\text{g}/\text{mL}$ reduced the cell proliferation in DLD-1 cells compared with control. Additionally, combination of 150 μM and higher concentrations of borax with

5-FU at 50 $\mu\text{g}/\text{mL}$ concentration exerted cytotoxic effects on DLD-1 cells for 24 and 48 h. Similarly, borax treatment inhibited cell proliferation in human hepatocellular carcinoma cell line HepG2.¹⁵ Canturk et al.²⁷ demonstrated cytotoxic effect of boric

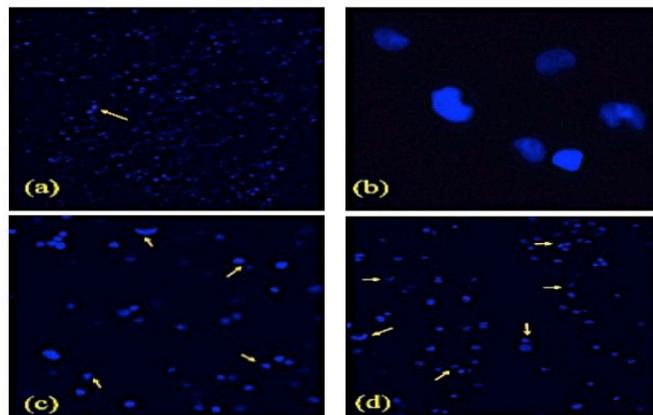


Figure 4. Analysis of DAPI staining. Control group cells (a), DLD-1 cells treated with IC_{50} concentration of borax (500 μM) (b), DLD-1 cells treated with IC_{50} concentration of 5-FU (50 $\mu\text{g}/\text{mL}$) (c), DLD-1 cells treated with borax (500 μM) + 5-FU (50 $\mu\text{g}/\text{mL}$) (d). This was photographed using a confocal inverted microscope with the DAPI filter at magnification of 100X
5-FU: 5-Fluorouracil, DAPI: 4',6-Diamidino-2-phenylindole

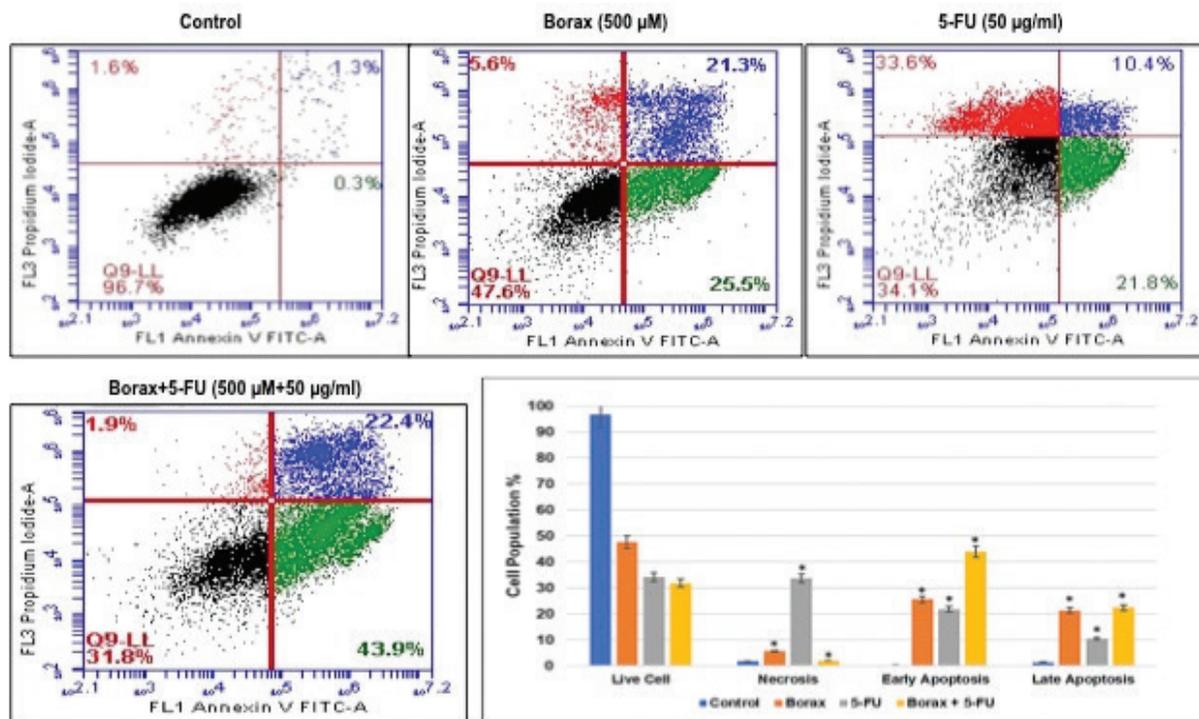


Figure 3. Flow cytometry analysis of DLD-1 cells treated with IC_{50} concentration of borax and 5-FU for 48 h *via* staining with Annexin V-FITC/PI. The lower left quadrant represents viable cells, the lower and upper right quadrant represent early and late apoptotic cells, and the upper left quadrant represents non-viable necrotic cells. Control group cells (a), DLD-1 cells treated with IC_{50} concentration of borax (500 μM) (b), DLD-1 cells treated with IC_{50} concentration of 5-FU (50 $\mu\text{g}/\text{mL}$) (c), DLD-1 cells treated with borax (500 μM) + 5-FU (50 $\mu\text{g}/\text{mL}$) (d). The analysis of viable, necrotic, and early and late apoptotic cells of control DLD-1 cells and those which have been treated with IC_{50} concentration of borax, 5-FU, and both (e). The data present the mean \pm SD of three independent experiments

* $p < 0.05$ demonstrates the statistical significance between control cells and treatment groups, SD: Standard deviation (n: 3), 5-FU: 5-Fluorouracil, PI: Propidium iodide

acid and borax on HL-60 human acute leukemia cell line using MTT. Exposure to boric acid reduced viability of MDA-MB-231 breast cancer cells and DU-145 human prostate cancer cells in a dose-dependent manner.^{16,17} Murmu et al.²⁸ reported that boron compounds inhibited cell growth of myeloid leukemia cell lines (HL-60 and U-937).

According to Annexin V/PI double-staining assay of DLD-1 cells treated with either borax alone (500 µM), 5-FU alone (50 µg/mL) or a combination of the two drugs for 48 h, the early apoptotic rates were 25.5, 21.8, and 43.9%, respectively, compared with the control 0.3%. The percentage of total apoptotic cell amount was 46.8% in the borax-treated group at 500 µM concentration and 32.2% in the 5-FU-treated group at 50 µg/mL concentration. The combined treatment group demonstrated higher percentages of apoptotic cells (66.3%) compared to the either borax or 5-FU treatment alone at 48 h. Similarly, staining of HepG2 cells with Annexin V and PI demonstrated promotion of borax-induced apoptosis.¹⁵ Additionally, a derivative of boric acid, boron oxide, demonstrated significant apoptotic effects for both L929 fibroblast and DLD-1 CRC cell lines.²⁹ Moreover, borax (a salt of boric acid) and boric acid concentration-dependently induced apoptosis by increasing the expression levels of tumor suppressor p53 gene and decreasing anti-apoptotic Bcl-2 mRNA expression levels in HepG2 human hepatocellular carcinoma cancer cell line.³⁰ In a previous study by Scorei et al.¹⁷, calcium fructoborate induced apoptosis in MDA-MB-231 breast cancer cells.

To further investigate the apoptotic effects of borax alone or along with 5-FU, DAPI staining was performed. In this study, the induction of apoptosis accompanied by condensed and fragmented nuclei was observed with higher efficiency in DLD-1 cells treated with a combination of borax and 5-FU than monotherapies. Another study demonstrated nuclear fragmentation in boron compound-treated leukemia cells.²⁷

CONCLUSION

In conclusion, cancer- and apoptosis-inducing effects of combination of borax and 5-FU were stronger than that of an individual treatment. These results suggest that borax could be a promising adjunct therapeutic agent for CRC by eliminating adverse effects of 5-FU and increasing treatment efficiency. However, further research is needed to identify the underlying molecular mechanisms of borax-induced apoptosis and understand the anticarcinogenic effect of borax in CRC.

Ethics

Ethics Committee Approval: Not necessary.

Informed Consent: There is no requirement for informed consent in the current study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Ö.F.K., E.K.S., T.Ö., Design: Ö.F.K., E.K.S., T.Ö., Data Collection or Processing: Ö.F.K., E.K.S., S.Ö., S.G., Ö.Y., T.Ö., Analysis or Interpretation: Ö.F.K., E.K.S., S.Ö., S.G., Ö.Y., T.Ö., Literature Search: Ö.F.K., E.K.S., T.Ö., Writing: Ö.F.K., E.K.S.

Conflict of Interest: No conflict of interest was declared by the authors.

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