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Original Research

The Efficacy of Apigenin in the Treatment of High-Grade Hepatocellular Carcinoma: An Invitro Experiment

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Abstract

Objectives: Apigenin, a flavonoid with reported antineoplastic and anti-inflammatory properties, is being investigated for its potential in treating hepatocellular carcinoma (HCC). This study evaluated apigenin's effects on proliferation, invasion, and viability of the SNU-449 HCC cell line.

Methods: To evaluate apigenin's antiproliferative and antimetastatic effects in HCC, we performed MTT assays at 24, 48, and 72 hours, using six apigenin concentrations (2.5–100 µM). Following the determination of the minimum effective concentration at 48 hours, SRB, colony formation, and wound healing assays were performed at that dose. All results are expressed as median (interquartile range).

Results: The MTT assay identified 5 µM apigenin at 72 hours as the minimum effective dose. Absorbance at 5 µM apigenin and in the untreated control was 0.581 (IQR: 0.26) and 0.67 (IQR: 0.049), respectively (p>0.05). The SRB assay showed no significant difference between the apigenin-treated and control groups (0.54 [IQR: 0.07] vs. 0.381 [IQR: 0.365]; p>0.05). The colony formation assay revealed a modest reduction in survival fraction in the apigenin-treated group (74% relative to control). Wound areas at the end of the wound healing assay were 528,366 (IQR: 691,200) µm^2 in the apigenin-treated group and 528,861 (IQR: 523,150) µm^2 in the control group (p>0.05). Wound closure rates were similar between the apigenin-treated and control groups (59.5 [IQR: 36.9]% vs. 59.75 [IQR: 15.4]%; p>0.05).

Conclusion: The results of this study suggest that apigenin's direct antiproliferative and antimetastatic effects on HCC cells may be limited. Further research focusing on the modulation of the tumor microenvironment and the induction of antitumor immune responses could provide valuable insights.

Keywords: Apigenin, Antimetastatic treatment, Antiproliferative effects, Hepatocellular cancer, SNU-449 cell line

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Hepatocellular carcinoma (HCC) is the most frequently observed major liver neoplasm type and is predominantly accountable for liver cancer. It is becoming increasingly widespread worldwide and it displays poor prognosis.[1] Throughout the spectrum of health problems, liver cancer is the second major source of cancer-associated death.[1] Even though advanced technology and therapeutic forms have resulted in a substantial abridgment of the death rate caused by main cancer kinds, advancement in therapies for liver cancer has stayed mostly stagnant. Therefore, the development of better liver cancer therapies is deeply needed.[2]

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Apigenin is a naturally occurring polyphenol of the flavonoid family, abundant in everyday food items such as vegetables, fruits, and tea. There are emerging reports indicating that apigenin applies anti-inflammatory, anti-tumor, and pro-apoptotic properties.[3] Recent research indicates that apigenin notably reduced the development, multiplication, and expansion of liver cancer cells in treated rats.[3,4] Petroleum ether-extracted propolis elements highly enriched in apigenin significantly repressed the advancement of liver cancer in mice; this could transpire via adjusting cell cycle and prompting cell apoptosis.[3,4] Redox alteration is a main component in the tumorigenesis of hepatocellular carcinoma (HCC) in individuals and animal models. Studies have shown that an apigenin-rich diet substantially promptly ameliorated oxidative stress and decreased HCC expansion in rats.[5,6] Additionally, a variety of research reported that apigenin suppressed liver cancer advancement by prompting a rise in oxidative stress in numerous cell lines and animal models. Bioinformatical and experimental analyses have demonstrated that upregulated miR-520e prompted by apigenin holds back coding mRNA by binding to the 3'-UTR, prompting a rise in ROS, ultimately resulting in tumor cell apoptosis.[5-8]

Apigenin (4',5,7-trihydroxyflavone) is a natural compound that can be found in high levels in parsley, onions, oranges, tea, chamomile, cereal, fruit, and some herbs.[9] As a natural source of food and ingredient in traditional Chinese medicine, there are some reports pointing to low toxicity and high efficacy in the prevention and treatment of cancers.[10-12] Recent research demonstrated that apigenin could significantly regulate the PI3K/Akt signaling pathway and ultimately suppress the proliferation of hepatocellular treatment by halting the cell cycle and causing apoptosis, thus showing the potential of apigenin to act as a novel drug in the treatment of HCC.[9] In another recent vivo experiment, the results indicated that apigenin potentially inhibits the focal damage of liver tissue. Chronic CCl4-exposed rats supplemented with apigenin diminished lipid peroxidation, restored the defenses of superoxide dismutase and glutathione peroxidase, boosted the level of catalase, and reduced alanine transaminase, aspartate aminotransferase, and γ-glutamyl transpeptidase in serum. Furthermore, apigenin enhances the apoptosis of liver cells and curbs the chemical changes of the liver microenvironment induced by CCl4, therefore exerting a therapeutic effect on hepatocirrhosis.[13-15] More notably, accumulating evidence demonstrates the superior bioavailability of apigenin among flavonoids. These findings collectively underscore apigenin's potential as a safe and practical drug. Due to the pleiotropic properties of apigenin in viruses, for example, anti-proliferate, anti-angiogenic, antioxidative, and anti-proinflammatory actions, as well as its inhibitory effects on mitochondrial biogenesis, there is an urgent need for relevant trials of exploration targeting apigenin supplementation to develop drug therapy for liver disorders and HCC.[15-17]

SNU449 cell line (ATCC® CRL-2234™) is an HCC cell line with grade II/III differentiation that developed on the background of hepatitis B virus (HBV) related cirrhosis.[18] The cell line is positive for HBV deoxyribonucleic acid (DNA). It can be used in experiments that require biological characteristics and behavior of HCC.[18] We designed the study aiming to evaluate the invitro biological effects of apigenin on the proliferation and migration of SNU449 HCC cell lines.

Methods

Cell Culture

Human hepatocellular carcinoma SNU-449 cells (ATCC, CRL-2234) were maintained in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma) and 1% penicillin-streptomycin-neomycin (Sigma) under standard cell culture conditions (37 °C, 5% CO_2, humidified atmosphere).

Cell Viability Assay

SNU-449 cells were resuspended in RPMI-1640 medium and seeded at a density of 10,000 cells per well in 96-well plates. Cells were treated with apigenin (Sigma) at concentrations of 2.5, 5, 10, 20, 50, and 100 µM in RPMI-1640 for 24, 48, and 72 hours. Following the designated incubation period, MTT solution (5 mg/mL in PBS) was added to each well and incubated for four hours. Subsequently, 100 µL of dimethyl sulfoxide (DMSO, Merck) was added to dissolve the formed formazan crystals. Absorbance was measured at 570 nm using a microplate reader (Biotek, Synergy H1m), and the IC50 value was determined.

Sulforhodamine B (SRB) Assay

SNU-449 cells were seeded at 10,000 cells/well in 96-well plates containing RPMI-1640 medium and treated with apigenin (5, 10, and 20 µM) for 48 hours. Following fixation with ice-cold 10% trichloroacetic acid (TCA) and staining with 0.04% sulforhodamine B (SRB, Sigma), unbound dye was removed by rinsing with TCA. Bound SRB was then solubilized with 10 mM Tris-base, and after adding 100 µL of dimethyl sulfoxide (DMSO, Merck), absorbance was measured at 510 nm using a microplate reader.

Wound Healing Assay

SNU449 cells were seeded in a 60 mm cell culture petri dish and cultured until they reached 80% confluence. A scratch

was made in the cell monolayer using a sterile 100 µL pipette tip, and the cells were washed twice with PBS to remove debris. The cells were then treated with 5 µM Apigenin for 48 hours, and images of the scratch were captured at specified time points using a cell imager (Leica, Paula). The wound healing area was quantified using ImageJ, and the wound closure rate was calculated.

Colony Formation Assay

SNU449 cells were seeded in 6-well plates at a density of 1,000 cells per well, incubated overnight, and treated with 5 µM Apigenin for 48 hours. The medium was then replaced with fresh RPMI-1640, and it was changed every 2-3 days. The cultures were washed with PBS, fixed with methanol: acetic acid (3:1) for 5 minutes, stained with 0.5% crystal violet in methanol for 15 minutes, and washed with water. Colonies were counted, and the plating efficiency (PE) and surviving fraction (SF) were calculated using the following formulas:

PE = (number of colonies formed/number of cells seeded)*100

SF = (PE of Apigenin-treated cells/PE of control cells)*100

Statistical Analysis

The normal distribution of the variables was evaluated using the Shapiro-Wilk Test due to the small sample size. Continuous variables were expressed as median and interquartile range (IQR). The evaluation of the colony formation assay was explained previously. The Continuous variables were compared among the control and apigenin-treated groups using the Mann-Whitney U test. Comparison of the responses to different doses of apigenin in cell survival and SRB assays was performed using the Kruskal-Wallis Test for multiple group comparisons. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using the Statistical Package for the Social Sciences software version 27 (SPSS v.27) (IBM, Armonk, NY, USA).

Results

Cell Viability Assay

The IC50 of apigenin was determined using the MTT assay. Results indicated that 5 µM at 72 hours was the minimum effective dose. The absorbance values for the 5 µM apigenin-treated and control groups were 0.581 (IQR: 0.26) and 0.67 (IQR: 0.049), respectively (p>0.05) (Fig. 1A), demonstrating a lack of statistically significant difference.

SRB Assay

We confirmed apigenin's effects on SNU449 cell survival using the SRB assay. We tested null (untreated), 5 µM, 10 µM, and 20 µM doses. The SRB assay demonstrated no statistically significant difference in cell viability between cells treated with various apigenin doses and the untreated control group (0.54 [IQR: 0.07] vs. 0.381 [IQR: 0.365]; p>0.05) (Fig. 1B).

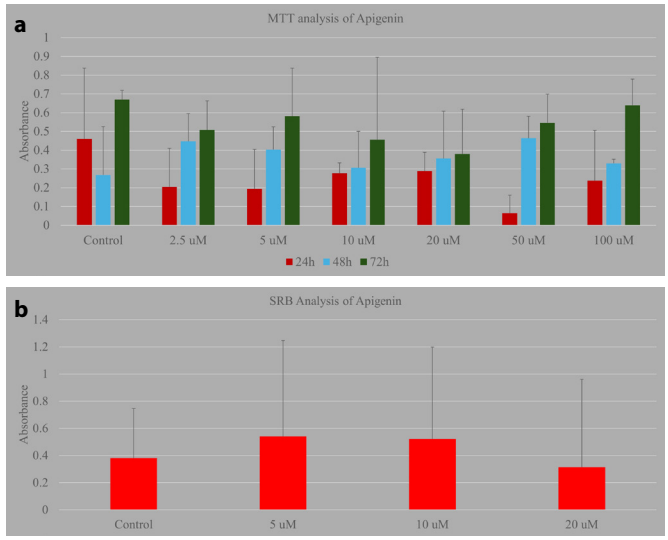

Figure 1. Summary of the results of the cell survival and SRB assay. **(a)** Dose-dependent changes in cell survival are seen. **(b)** SRB assay showing the dose-dependent changes in proliferation in SNU449 cell lines. Abbreviations: SRB: Sulforhodamine B.

Wound Healing Assay

The wound healing assay showed no significant difference in wound closure between the apigenin-treated and control groups. Wound areas at 96 hours were 528,366 (IQR: 691,200) µm^2 and 528,861 (IQR: 523,150) µm^2, respectively (p>0.05) (Fig. 2A). Wound closure rates were also similar (59.5 [IQR: 36.9] % vs. 59.75 [IQR: 15.4]%; p > 0.05) (Fig. 2B). Although the wound area appeared larger in the apigenin-treated group at 96 hours (Fig. 2C), this difference was not statistically significant.

Colony Formation Assay

The colony formation assay revealed a modest difference between apigenin-treated and untreated cells. The plating efficiency (PE) was 4.65 for control cells and 3.45 for apigenin-treated cells, resulting in a surviving fraction of 74.15% in the treated group compared to the control. These results are shown in Figure 3A-D.

Discussion

We have evaluated the effects of apigenin on the biological behavior of HCC. We have found that although it reduces the proliferative and survival capabilities of HCC cell-line SNU-449, this did not reach statistical significance. This can be interpreted as a lack of efficacy of

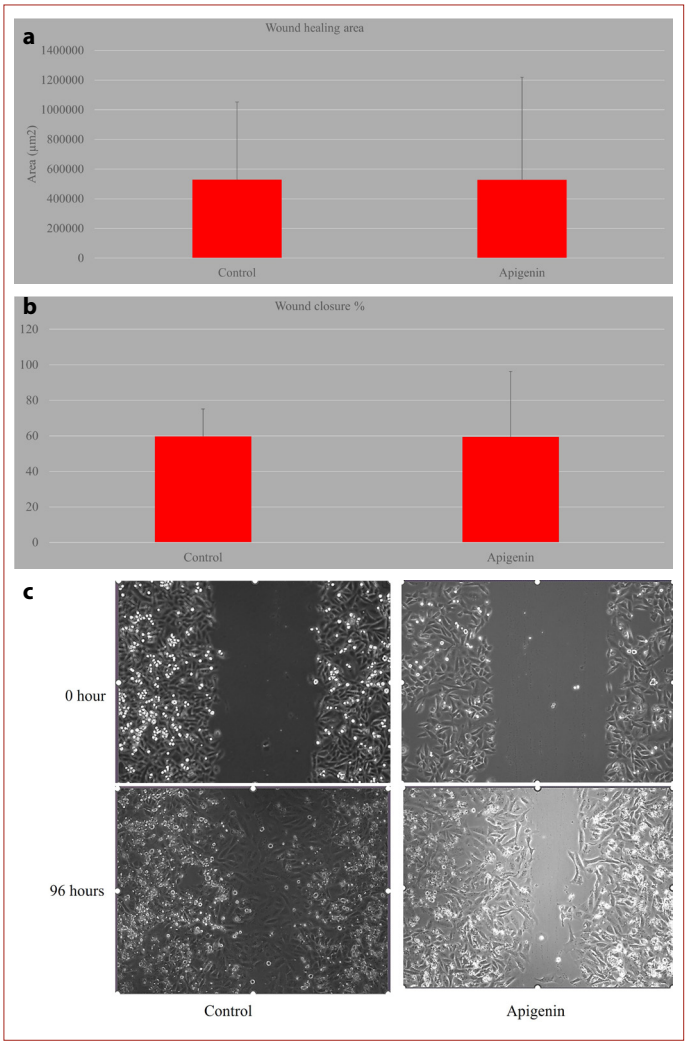

Figure 2. The results of the wound healing assay are summarized. **(a)** Wound surface area is shown. **(b)** Wound closure rates are summarized. **(c)** The figure shows the changes in the streak width of the study groups.

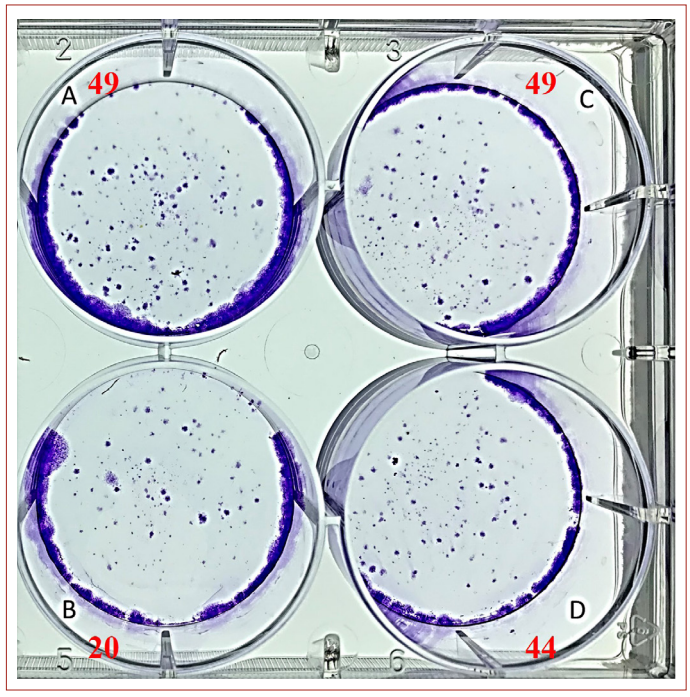

Figure 3. The results of the colony formation assay included the control and the apigenin-treated group. **(a)** and **(b)** Apigenin treated group at the end of the observation. **(c)** and **(d)** Control group at the end of the observation.

apigenin in the HCC treatment. On the other hand, it may show its effects by interacting with components of the tumor microenvironment.

Apigenin is a flavonoid that is mostly present in fruits and vegetables like parsley, celery, celeriac, and others. Since ancient times, apigenin has been used to appraise many ailments like tremors, sleep disorders, and anxiety.[14] Preclinical studies illustrate the usefulness of apigenin in the prevention as well as treatment of peri-operative stress challenges. Pre-clinical in-vitro as well as in-vivo investigations confirm the anti-tumor potentiality of apigenin in various cancers. Several studies note that apigenin can curb growth, as well as metastasis of hepatocellular carcinoma cells.[15] HCC is the fundamental kind of liver malignancy with an overall grim ending. Apigenin exhibits anticancer capability through incentives of apoptosis and autophagy in HepG2 and considerable growth suppression of HCC in HepG2 and Huh-7. In addition, apigenin can recover the anti-cancer effect of chemotherapeutic agents such as doxorubicin and Sorafenib in a mice model as well as in HepG2 and Huh-7 cells.[16] Moreover, apigenin and its unique analogs are competent to cut down the activity of multiple drug resistance mediated CYP3A4, which is impressive in improvement of the oncological treatment effect of doxorubicin.[9] Cellular alarm and demise or programmed cell death routes are accountable for the development as well as replacement tissues and suppression of cells and carcinogens bacteria. Therefore, apoptotic cell demise is vigorous against growth-propagating impact as compared to mortal natures. In diseased cells, apoptosis refuses to work correctly and transmutes into tumor progression. Necrosis is unprogrammed with disastrous results and deteriorates debris contents are discharged in the surroundings and starting inflammation.[14-16] As a result, vicious cell endeavors are developed. In any case, ATP-deficient surroundings necroptosis or programmed necrosis can be activated. In the direction of the specific genetic condition of HCC type and grade of "tumor-stage-dependent," specialists are not perpetually capable of disposing of efficacy and prevention. The bioactive plant constituent, apigenin, is located in a diversity of green provisional, cereals, and therapeutic herbs.[19] Api is a nontoxic cure with originating health

profits. The treatment of Hep3B cells with Apigenin significantly concentrated the caused demise. Hence, findings present that apigenin was prosperous in fostering the API-enhanced API-induced cell committal as well as necroptosis in Hep3B cells. On this account, Apigenin exhibited the API-induced necroptosis toxic aftermath as well as emerging it as an ally with chemotherapeutic drugs in liver cancer cure.[14-16] We have not found such effects of apigenin on SNU449 cell lines. However, what we could confirm was a modest reduction of proliferation.

There is much interest on dietary interventions with polyphenols as a new strategy in the prevention of cancer.[20] Bioactive compounds of plant origin, such as flavonoids, reduce the incidence of cancer. Epidemiological evidence suggests that a diet rich in flavonoids from fruits and vegetables may be associated with a reduced risk of lung cancer and potentially HCC.[19,20] HCC cell lines representing different genetic backgrounds were exposed to physiologically relevant apigenin levels to assess effects on cell proliferation, cell cycle, and superoxide radical formation.[21] Mouse and zebrafish HCC models were established and exposed to apigenin to evaluate effects on HCC progression, oxidative stress, and immunoediting.[22] The combinatorial effect of apigenin with current chemical HCC therapies was analyzed in HCC cell lines and zebrafish.[22] Our results suggest that the biological effects of apigenin should be together with the tumor microenvironment of HCC.

Chemotherapy is one of the most common cancer treatments that employ chemical compounds that can kill, or limit the growth of, cancer cells. Currently, chemotherapy is the treatment of choice for many types of cancers.[19-22] However, potent antineoplastic chemicals harm healthy tissues, especially tissues with high cell turnover rates, such as the gastrointestinal tract, hair follicles, and bone marrow. Moreover, long-term chemotherapies harshly affect the liver, kidneys, and heart, which are responsible for detoxifying the body from potentially toxic agents.[19-22] Many chemotherapeutic agents have a very narrow therapeutic window due to their low bioavailability, rapid metabolic clearance, and considerable toxicity. Inhibition of certain metabolic enzymes by some phytochemicals has previously been reported.[23] Since many clinically used drugs, including chemotherapeutic agents, are substrates for these enzymes, chemopreventive phytochemicals that inhibit metabolic enzymes may enhance the action of these drugs by altering their pharmacokinetics. So, the use of specific phytochemicals in combination therapy with specific chemotherapeutic drugs can result in a significant increase in drug efficacy and, at the same time, it may lead to a reduction in side effects.[24] Among the co-administered phytochemicals, flavonoids have an advantageous effect on the pharmacokinetic parameters of drugs. Hepatocellular carcinoma (HCC) is the sixth most common cancer and the fourth leading cause of cancer-related deaths globally. Surgical resection and liver transplantation offer curative treatment for early-stage HCC.[23,24] Chemotherapy is considered a palliative way to prolong the patient's life by 2–3 months. In many hepatocellular carcinoma patients, their impaired liver function eliminates the option of surgical intervention.[23,24] The overall 5-year survival rate is about 5%. Chemotherapeutics with plant extracts, including certain compounds, may be used to inhibit the growth of cancer cells. Certain compounds potently inhibit parasite hepatic cell growth by blockading the cell cycle and inducing apoptosis.[23,24] These compounds produce a concentration-dependent decrease in parasitic hepatic cell survival, with elevated values from 20 μM to 80 μM. In combination with certain inhibitors, these compounds synergistically inhibit the hepatic cell growth of parasites. These compounds suppress hepatocellular carcinoma cell growth by targeting specific genes. By targeting these genes, they abate hepatocellular carcinoma cells' migrating and invading abilities. The repurposing of these compounds, together with the current treatment protocol, might improve the effectiveness.[23,24] Apigenin radiosensitizes HCC by promoting radiation-induced apoptosis and G2/M phase cell cycle arrest, and inhibiting AP-1 and HIF-1α.[25,26] However, the potential of apigenin to enhance the radiosensitivity of tumor cells has yet to be sufficiently studied.[25,26] We have concentrated on the antiproliferative effects of apigenin and we could not prove our hypothesis. We plan to use apigenin in combination with various chemotherapeutics to search the synergistic effects.

Our study is an in vitro study evaluating the effects of apigenin on the proliferation of HCC cell line SNU449. This is the most important limitation of the present study. We did not consider the component of the tumor microenvironment. Therefore, in the future, we intend to use apigenin alone and in combination with antitumor agents in animal models to evaluate the mechanism of action of this flavonoid.

Conclusion

There is a rapidly emerging body of in vivo and ex vivo data from animal-based studies that supports the contention that apigenin has real promise as an anti-cancer agent. Essentially, these studies are now broad enough in scope to suggest possible mechanistic themes for future investigations and to justify progression to human applications. However, to provide an effective foundation for undertaking randomized controlled trials based on these observations, it is important to establish parameters for dosing frequency, total dosage, bioavailability, effective

serum concentration, routes of administration, and efficacy in preventing, reversing, or modifying dysplasia and neoplastic disorders. Efficient translation from the bench to the clinic necessitates comprehensive efficacy studies with well-characterized standardized products and dosing regimens. Randomized controlled trials must be based on validated marker metrics showing a significant reduction in tumor burden or tumor progression. Highly relevant to the digestive tract, such trials also require validated data on efficacy in specific cell types and a comprehensive understanding of interaction with key signaling pathways that ultimately determine enzyme activation and cell progression. Experimental design must also involve the interrogation of the therapeutic window (i.e., the interval between optimal dose for effective treatment and the maximum dose that can be taken before adverse effects occur). Comprehensive understanding of apigenin metabolism and a wider series of well-conducted preclinical studies that are directly translatable into clinical settings are then the preclinical requirements. At the very least, all of these factors should be established in the literature before embarking on phase I clinical trials, the primary goal of which would be validation of the safety of apigenin exposure at the anticipated doses. Currently, we could only prove a modest antiproliferative effect of this flavonoid.

Disclosures

Ethics Committee Approval: None.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

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