Sappanone A Aggrandises Ionising Radiation–induced Damage in Oral Epithelial Cells

Xue Feng ZHAO[1#], Zhi Yong WANG[1#], Xue Mei QIU[1], Hang ZHAO[1], Rui LIU[1], Qian Ming CHEN[1]

Objective: *To analyse the role played by Sappanone A, a bioactive ingredient isolated from the heartwood of Caesalpinia sappan, in the regulation of oral epithelial cell viability under radiation.*

Methods: *Cell viability of human oral keratinocytes (HOKs) and mouse salivary gland cells under ionising radiation was analysed. Expression of Ki67 was measured by immunohistochemical staining. Fragmentation of deoxyribonucleic acid (DNA) was measured by comet assay. Cell death was analysed using trypan blue exclusion assay. Cell viability was measured using a Cell Counting Kit 8 (CCK8; Abcam, Cambridge, UK) assay.*

Results: *Sappanone A decreased cell viability of HOK cells and mouse salivary gland cells under ionising radiation. In addition, Sappanone A enhanced radiation-induced genomic DNA fragmentation, accompanied by impaired homologous recombination and non-homologous end joining DNA repair. Mechanistic evaluation revealed that Sappanone A counteracted radiation-induced inosine monophosphate dehydrogenase 2 (IMPDH2) activation, and that this effect could be abolished by reconstituted expression of a Sappanone A-binding defective IMPDH2 mutant.*

Conclusion: *The present study highlights a novel role played by Sappanone A in the modulation of radiosensitivity of oral epithelial cells.*

Key words: *DNA repair, ionising radiation, oral epithelial cell, Sappanone A*
Chin J Dent Res 2022;25(4):261–267; doi: 10.3290/j.cjdr.b3628117

Ionising radiation can cause temporary or irreversible oral complications including oral mucositis, xerostomia, caries lesions, periodontal disease, taste dysfunction, sensory disorders and salivary gland dysfunction. Radiation-induced oral mucositis (RIOM) is the most common form of damage to the normal oral mucosa of patients who receive radiotherapy to treat head and neck cancer[1]. Patients may suffer from multiple symptoms, including severe pain and difficulties in speaking and swallowing[2]. The clinical manifestations of RIOM, such as hyperaemia and redness of the oral mucosa, usually arise 2 to 3 weeks after the initiation of radiotherapy and may progress further to oral ulcers with a pseudomembrane[3].

Double-strand breaks (DSBs) are the most severe type of damage to deoxyribonucleic acid (DNA) caused by ionising radiation, and the cell fate in such a context is largely dependent on the efficiency of DNA repair[4]. Non-homologous end joining (NHEJ) and homologous recombination (HR) are the two pivotal DSB repair mechanisms. The NHEJ pathway directly rejoins the two DSB ends by DNA ligase which does not require a homologous donor sequence. In contrast, the HR

pathway utilises the homologous sister chromatid or homologous chromosome as a template[5]. NHEJ is a fast process in all cell cycle phases and usually dominant in G0/G1 and G2 phases, but is often considered imprecise due to frequent nucleotide deletions and insertions at repaired sites. HR is considered more accurate and is confined to the S and G2 phases for the use of a homologous chromatid[6]. Though the cellular factors involved in DNA repair have been studied extensively, the environmental factors that affect DNA repair efficiency are still relatively unknown.

Caesalpinia sappan, also known as Brazil or Sappan wood, is a species of plant belonging to the Leguminosae family that is mainly distributed in Southeast Asia. It is considered an important source that yields multiple herbal medicinal products, which are used in a variety of beverage and food formulations[7]. Several bioactive phenolic compounds have been extracted from *Caesalpinia sappan* so far, including xanthone, coumarin, chalcones, flavones, homoisoflavonoids and brazilin[7]. Among them, Sappanone A, belonging to the class of homoisoflavanones, is a bioactive ingredient isolated from the heartwood of *Caesalpinia sappan*. Sappanone A has been used as a treatment agent of allergic asthma, rheumatoid arthritis and inflammation-induced bone loss[8,9]. However, whether it affects cell radiosensitivity is relatively unclear.

In this study, we demonstrate that Sappanone A enhances ionising radiation–induced damage in oral epithelial and salivary gland cells. Furthermore, it disrupts DNA repair after ionising radiation, most likely by inhibiting guanosine nucleotide metabolism.

Materials and methods

DNA constructs and mutagenesis

The human IMPDH2 gene was amplified by polymerase chain reaction (PCR) and subcloned into the indicated vectors. A QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to prepare mutant constructs. The following shRNAs were used: scramble shRNA, GCT TCT AAC ACC GGA GGT T, human IMPDH2 shRNA and TCC AGC ACA CCT CCT CGG T (recognising noncoding sequences).

Cell culture

Human oral keratinocytes (HOKs) isolated from human mucosa were purchased from ScienCell (Carlsbad, CA, USA) and maintained in Oral Keratinocyte Medium (ScienCell). For subculture, cells in a 10-cm dish were washed with phosphate-buffered saline (PBS) twice and incubated with 2 ml 0.05% T/E solution for about 5 minutes. Once the cells became completely round, 10 ml medium was added to the dish and the cells were then centrifuged at 1000 rpm for 3 minutes. The cells were resuspended with fresh culture medium and cultured in a new dish at a density of 5000 cells/cm^2. To determine the effects of Sappanone A on ionising radiation–induced damage, HOKs were incubated with 50 μM Sappanone A for 2 hours prior to 10 Gy irradiation.

Mice irradiation

An 8-Gy whole-body irradiation of 6-week-old C57BL/6 mice was performed with a ^{137}Cs gamma-ray source. A single dose of radiation was given at 1 Gy per minute. Salivary gland tissues were obtained 72 hours after irradiation. The animal maintenance and treatments were conducted according to the relevant institutional and national guidelines and regulations. The use of animals was approved by the institutional review board of West China Hospital of Stomatology.

Cell viability assay

A cell viability assay was performed using Cell Counting Kit 8 (WST-8/CCK-8) (Abcam, Cambridge, UK) and measurements were performed following the manufacturer's protocol.

Comet assay

A comet assay was performed using a single cell gel electrophoresis assay/comet assay (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol.

Analyses of DNA repair efficiency

The efficiency of NHEJ and HR repair was analysed using an I-SceI- and DR-GFP-based system, which have been studied previously[10,11]. In brief, HOKs were transfected with the DR-GFP plasmid, which was unable to express DR-GFP because of the I-SceI site. After I-SceI induced DSB in the DR-GFP locus, NHEJ repair resulted in a 0.65-kb PCR band, which is resistant to double digestion with I-SceI and BcgI, while HR repair restores the expression of GFP. Primers targeting the genomic sequence of the ACTB gene were used to measure the I-SceI cutting efficiency. To measure NHEJ repair, HOK cells were treated with lovastatin to be synchronised during the G1 phase prior to ionising radiation.
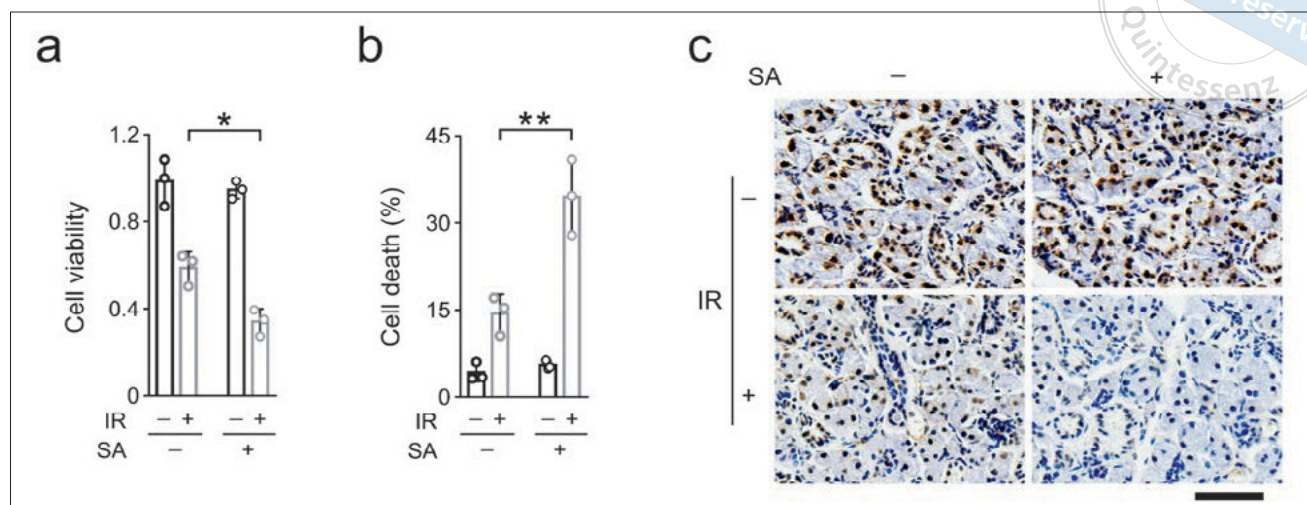
Fig 1 Sappanone A impairs the viability of oral epithelial cells under ionising radiation. **(a)** HOK cells were treated with 50 μM Sappanone A for 2 hours and challenged by 10 Gy ionising radiation. Cell viability was measured 48 hours after irradiation. The data represent the mean ± SD from three independent experiments. *$P < 0.05$. SA, Sappanone A; IR, ionising radiation. **(b)** HOK cells were incubated with 50 μM Sappanone A for 2 hours and challenged by 10 Gy ionising radiation. Cell death was measured 48 hours after irradiation. Data represents the mean ± SD from 3 independent experiments. **$P < 0.01$. **(c)** Mice (n = 7) were treated with 40 mg/kg Sappanone A. After 6 hours, the mice were exposed to 8 Gy whole-body ionising radiation. The salivary gland tissues were obtained and examined by immunohistochemical staining using an anti-Ki67 antibody. Scale bar 80 μm.

Cell death assay

Cell death was measured by trypan blue exclusion assay. Briefly, 0.05 ml of the 0.4% trypan blue solution (Thermo Fisher Scientific, Waltham, MA, USA) was mixed with 0.45 ml cell suspension. After 3 minutes, the stained cells were loaded onto a hemacytometer and the numbers of cells with or without trypan blue signal were counted.

IMPDH activity assay

IMPDH activity in the cell precipitates was measured using an IMPDH Inhibitor Screening Assay Kit (BioVision, Milpitas, CA, USA), following the manufacturer's instructions. IMPDH activity in cell lysates was measured following a previous study[12].

Statistical analysis

All data were analysed statistically using a two-tailed unpaired Student t test and presented as mean ± SD from three independent experiments/samples. The level of significance was set at $P < 0.05$.

Results

Sappanone A impairs the viability of oral epithelial cells under ionising radiation

As expected, exposure to ionising radiation largely reduced cell viability and increased cell death in HOK cells, and pre-treatment with Sappanone A further dampened the cell viability in the irradiated HOK cells (Figs 1a and b). In contrast, Sappanone A caused only minor effects in the unirradiated cells (Figs 1a and b). Further, in the mouse model, immunohistochemical staining showed that ionising radiation markedly reduced the expression of Ki67, a proliferating cell marker, in the salivary glands; this effect was largely amplified by Sappanone A treatment (Fig 1c). However, treatment with Sappanone A alone did not cause an obvious change to Ki67 expression in the salivary glands derived from unirradiated mice (Fig 1c). These results suggest that Sappanone A impairs the viability of oral epithelial cells under ionising radiation.

Sappanone A enhances ionising radiation–induced DNA damage in oral epithelial cells

Effective DNA repair is important to maintain cell viability upon ionising radiation–mediated DNA damage[13]. To determine the impact of Sappanone A on HR and
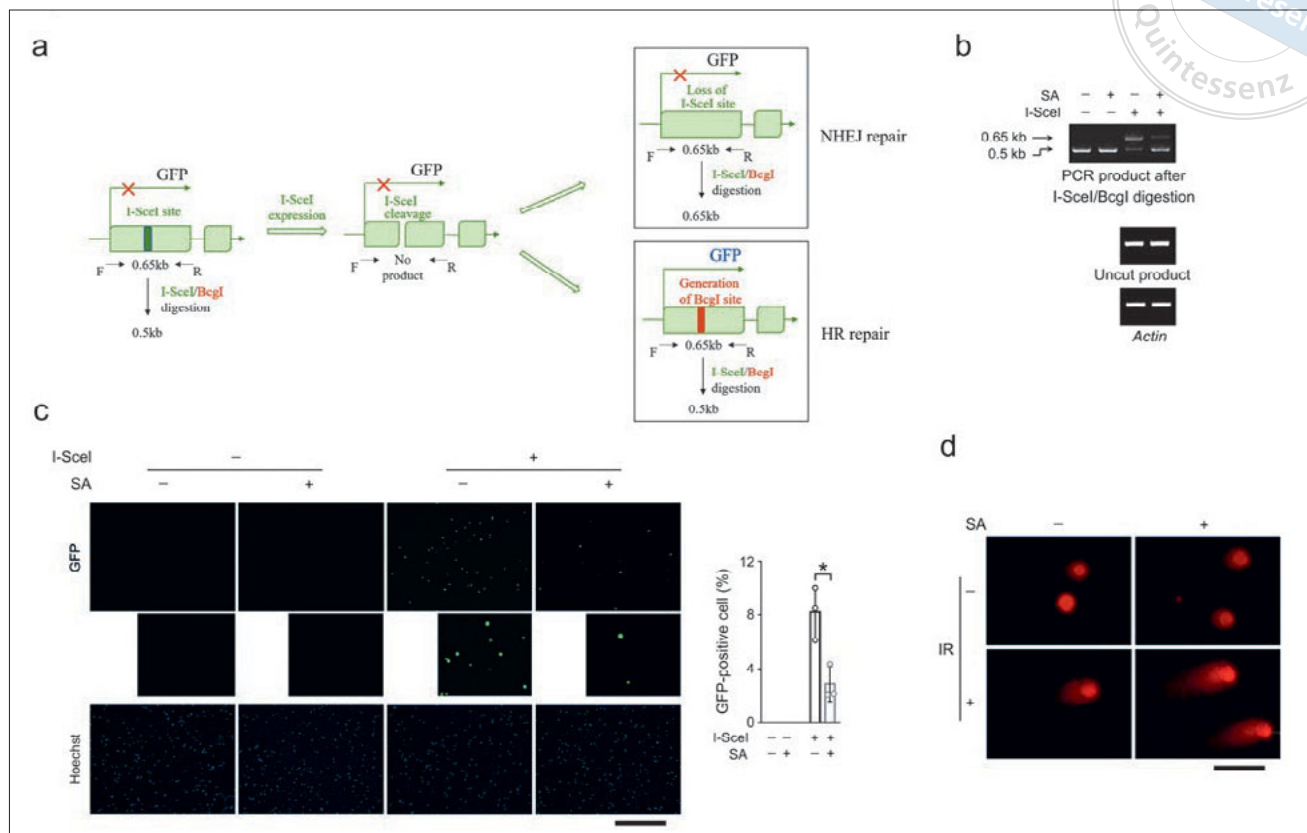
Fig 2 Sappanone A impedes DNA repair in oral epithelial cells. **(a)** A schematic for the measurement of NHEJ and HR repair. **(b and c)** HOK cells were transfected with I-SceI-expressing vector and treated with or without Sappanone A (50 µM) for 2 hours. NHEJ repair was examined 1 hour after irradiation **(b)**. HR repair was tested 72 hours after irradiation **(c)**. The data represent the mean ± SD from three independent experiments. *$P < 0.05$. SA, Sappanone A. Scale bar 300 µm for original images and 100 µm for enlarged images. **(d)** HOK cells were incubated with 50 µM Sappanone A for 2 hours and challenged by 10 Gy ionising radiation. DNA fragmentation was measured 1 hour after irradiation. Scale bar 20 µm. IR, ionising radiation.

NHEJ repair, endonuclease I-SceI-mediated DSBs in an exogenously introduced DR-GFP locus was used to measure the efficiency of HR and NHEJ repair (Fig 2a)[11]. We found that treatment with Sappanone A markedly reduced the amounts of 0.65-kb PCR product, suggesting that Sappanone A impeded NHEJ repair (Fig 2b). Further, I-SceI expression induced GFP expression in about 8% of HOK cells, which could be palpably eliminated upon Sappanone A treatment (Fig 2c), hinting at an impaired HR repair. In line with this, treatment with Sappanone A aggravated the irradiation-induced DNA fragments in the HOK cells, shown by the prolonged tail-like smear in the comet assay (Fig 2d). These results suggest that Sappanone A accentuates ionising radiation–induced DNA damage in oral epithelial cells.

Sappanone A accentuates ionising radiation–induced DNA damage by targeting IMPDH2

Enhanced synthesis of nucleotide upon ionising radiation facilitates DNA repair[11]. Inosine-5′-monophosphate dehydrogenase (IMPDH)1 and IMPDH2 produce xanthosine monophosphate (XMP) from inosine monophosphate (IMP), which is the rate-limiting step for guanine nucleotide synthesis (Fig 3a)[14]. Since Sappanone A was reported as an IMPDH2-specific inhibitor[15], which was of particular interest to us, we investigated the cellular IMPDH activity in HOK cells upon radiation. As expected, cellular IMPDH activity was sharply increased 30 minutes after ionising radiation (Fig 3b), hinting at an accelerated guanine nucleotide synthesis. Intriguingly, only the enzymatic activity of IMPDH2, not IMPDH1, was elevated manyfold (Fig 3c), suggesting that an ion-
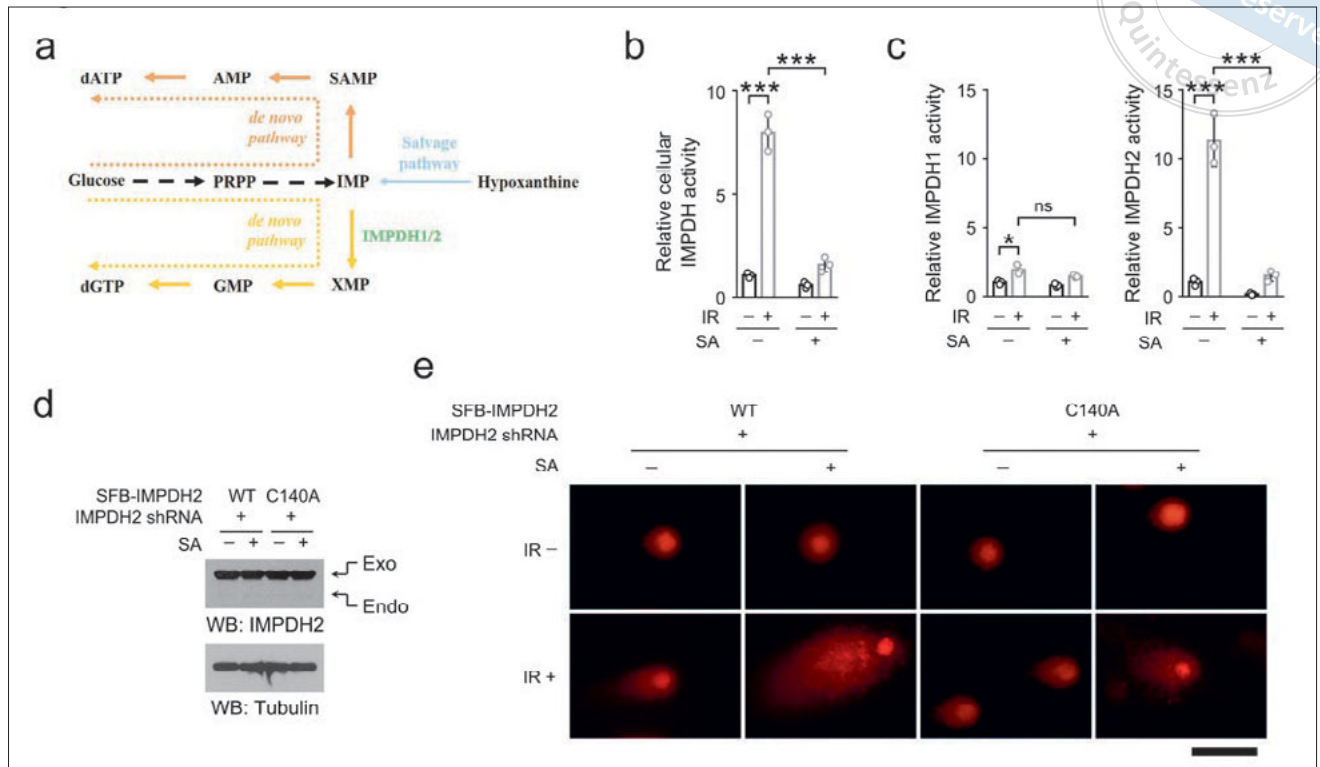
Fig 3 Sappanone A aggravates ionising radiation–induced DNA damage by binding with IMPDH2. **(a)** A schematic of purine nucleotide synthesis. **(b)** HOK cells were incubated with 50 μM Sappanone A for 2 hours and challenged by 10 Gy ionising radiation. The IMPDH activity was measured 30 minutes after irradiation. The data represent the mean ± SD from three independent experiments. ***$P < 0.001$. SA, Sappanone A; IR, ionising radiation. **(c)** HOK cells were incubated with 50 μM Sappanone A for 2 hours and then challenged by 10 Gy ionising radiation. The IMPDH1 and IMPDH2 proteins were pulled down 30 minutes after irradiation, and the activity was measured. The data represent the mean ± SD from three independent experiments. *$P < 0.05$; ***$P < 0.001$; ns, not significant. **(d)** HOK cells with expression of IMPDH2 shRNA and SFB-IMPDH2 were incubated with 50 μM Sappanone A for 2 hours. Expression of endogenous and exogenous IMPDH2 was examined by immunoblot. **(e)** HOK cells with expression of IMPDH2 shRNA and SFB-IMPDH2 were incubated with 50 μM Sappanone A for 2 hours and challenged by 10 Gy ionising radiation. DNA fragmentation was measured 1 hour after irradiation. Scale bar 30 μm.

ising radiation–induced increase in cellular IMPDH activity is mainly due to the activation of IMPDH2.

Treatment with Sappanone A prevented ionising radiation–induced IMPDH activity in HOK cell lysate markedly (Fig 3b). The increased IMPDH activity in IMPDH2 protein immunoprecipitates derived from irradiated HOK cells was consistently largely reduced in the presence of Sappanone A, whereas only minor changes were detected for IMPDH1 protein immunoprecipitates (Fig 3c). These results suggest that Sappanone A inhibits ionising radiation–induced cellular IMPDH activity in oral epithelial cells.

To determine whether IMPDH2 is the major protein target responsible for Sappanone A-regulated cell radiosensitivity, we knocked down the endogenous IMPDH2 and exogenously expressed an S protein-FLAG-Streptavidin binding peptide (SFB)-tagged wildtype IMPDH2 or IMPDH2 C140A mutant, which was defective in binding with Sappanone A (Fig 3d)[15]. Comet assay revealed that disruption of Sappanone A/IMPDH2 interaction substantially shortened the ionising radiation–induced tail-like smear after irradiation (Fig 3e). Together, these results suggest that Sappanone A aggravates ionising radiation–induced DNA damage by binding with IMPDH2.

Discussion

The oral mucosa is a vulnerable target for ionising radiation–induced damage. RIOM occurs in approximately 80% of patients receiving head and neck radiotherapy, and its incidence increases to as high a level as 100% in patients who undergo altered fractionation radiotherapy[16]. RIOM may decrease patients' quality of life sig-

nificantly and create a substantial health and economic burden. Ionising radiation can cause lethal DNA damage, resulting in apoptosis and necrosis of oral epithelial cells, which may contribute to the development of difficulties in speaking and swallowing[2].

Besides being known as a substance in traditional Chinese medicine, there is growing evidence to suggest the anti-inflammatory function of Sappanone A. Sappanone A has been proven to have inhibitory effects on nitric oxide and prostaglandin E2 in lipopolysaccharide-challenged RAW264.7 cells[17]. In a rat myocardial ischemia reperfusion injury model, Sappanone A reduced the size of myocardial infarction significantly and improved systolic and diastolic function[18]. Further, Sappanone A exhibits antioxidant activity by inducing heme oxygenase-1 protein through the nuclear factor-E2-related factor 2 (Nrf2), promoting the expression of Nrf2 downstream genes, such as NAD(P)H:quinone oxido-reductase 1 (NQO1). On the other hand, Sappanone A enhanced the phosphorylation of Nfr2 by regulating PKC and PI3K pathways[18]. The present study demonstrates that Sappanone A sensitised oral epithelial cells to ionising radiation. Treatment with Sappanone A further declined the viability in both the irradiated HOK cells and the mouse salivary gland cells. This study increases knowledge of the function of Sappanone A in regulating cellular responses in stressful conditions.

IMPDHs, which catalyse the conversion of IMP to XMP, are a rate-limiting enzyme in both the de novo and salvage biosynthesis of purine nucleotides[19]. Two IMPDHs have been discovered in humans, IMPDH1 and IMPDH2, which share 84% sequence homology but are coded by distinct genes. IMPDH1 is constitutively expressed, while IMPDH2 is often highly expressed in rapidly proliferating cell populations. Both IMPDH isoforms comprise a catalytic domain that contains cysteine residues, and a Bateman domain[15]. It has been reported that Sappanone A can selectively target Cys140 within the Bateman domain of IMPDH2, thereby reducing the enzymatic activity of IMPDH2[15]. In contrast, the corresponding Cys site does not exist in IMPDH1, which renders IMPDH1 with a low binding affinity with Sappanone A[15]. The present study has shown that ionising radiation induces a rapid elevation in cellular IMPDH activity, which is mainly caused by the activation of IMPDH2 and could be abolished upon Sappanone A treatment. Further, disruption of the association between Sappanone A/IMPDH2 by reconstituted expression of an IMPDH2 C140A mutant attenuated ionising radiation–induced DNA damage in HOK cells. Further work should be conducted to profile the altera-

tions in purine metabolism mediated by Sappanone A in the context of irradiation.

Conclusion

In conclusion, the present study demonstrates a novel role of Sappanone A in the regulation of oral epithelial cell radiosensitivity. Sappanone A interacts with and counteracts IMPDH2 to impede DNA repair after ionising radiation, thereby further repressing the viability of the irradiated oral epithelial cells.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Drs Xue Feng ZHAO, Zhi Yong WANG and Xue Mei QIU performed the experiments; Drs Hang ZHAO, Qian Ming CHEN and Rui LIU conceived and designed the study; Drs Qian Ming CHEN and Rui LIU wrote the paper, with comments provided by all authors.

References

1. Lacas B, Bourhis J, Overgaard J, et al. MARCH Collaborative Group. Role of radiotherapy fractionation in head and neck cancers (MARCH): An updated meta-analysis. Lancet Oncol 2017;18:1221–1237.

2. Elting LS, Cooksley CD, Chambers MS, Garden AS. Risk, outcomes, and costs of radiation-induced oral mucositis among patients with head-and-neck malignancies. Int J Radiat Oncol Biol Phys 2007;68:1110–1120.

3. Sroussi HY, Epstein JB, Bensadoun RJ, et al. Common oral complications of head and neck cancer radiation therapy: Mucositis, infections, saliva change, fibrosis, sensory dysfunctions, dental caries, periodontal disease, and osteoradionecrosis. Cancer Med 2017;6:2918–2931.

4. Bee L, Nasca A, Zanolini A, et al. A nonsense mutation of human XRCC4 is associated with adult-onset progressive encephalocardiomyopathy. EMBO Mol Med 2015;7:918–929.

5. van Leeuwen LA, Hoozemans JJ. Physiological and pathophysiological functions of cell cycle proteins in post-mitotic neurons: Implications for Alzheimer's disease. Acta Neuropathol 2015;129:511–525.

6. Ceccaldi R, Rondinelli B, D'Andrea AD. Repair pathway choices and consequences at the double-strand break. Trends Cell Biol 2016;26:52–64.

7. Nirmal NP, Rajput MS, Prasad RG, Ahmad M. Brazilin from Caesalpinia sappan heartwood and its pharmacological activities: A review. Asian Pac J Trop Med 2015;8:421–430.

8. Chang TS, Chao SY, Ding HY. Melanogenesis inhibition by homoisoflavavone sappanone A from Caesalpinia sappan. Int J Mol Sci 2012;13:10359-10367.

9. Choo YY, Tran PT, Min BS, et al. Sappanone A inhibits RANKL-induced osteoclastogenesis in BMMs and prevents inflammation-mediated bone loss. Int Immunopharmacol 2017;52:230–237.

10. Jiang Y, Qian X, Shen J, et al. Local generation of fumarate promotes DNA repair through inhibition of histone H3 demethylation. Nat Cell Biol 2015;17:1158–1168.

11. Liu R, Li J, Shao J, et al. Innate immune response orchestrates phosphoribosyl pyrophosphate synthetases to support DNA repair. Cell Metab 2021;33:2076–2089.e9.

12. Ji Y, Gu J, Makhov AM, Griffith JD, Mitchell BS. Regulation of the interaction of inosine monophosphate dehydrogenase with mycophenolic acid by GTP. J Biol Chem 2006;281:206–212.

13. Morgan MA, Lawrence TS. Molecular pathways: Overcoming radiation resistance by targeting DNA damage response pathways. Clin Cancer Res 2015;21:2898–2904.

14. Jain J, Almquist SJ, Ford PJ, et al. Regulation of inosine monophosphate dehydrogenase type I and type II isoforms in human lymphocytes. Biochem Pharmacol 2004;67:767–776.

15. Liao LX, Song XM, Wang LC, et al. Highly selective inhibition of IMPDH2 provides the basis of antineuroinflammation therapy. Proc Natl Acad Sci U S A 2017;114:E5986–E5994.

16. Maria OM, Eliopoulos N, Muanza T. Radiation-induced oral mucositis. Front Oncol 2017;7:89.

17. Lee S, Choi SY, Choo YY, et al. Sappanone A exhibits anti-inflammatory effects via modulation of Nrf2 and NF-κB. Int Immunopharmacol 2015;28:328–336.

18. Shi X, Tao G, Ji L, Tian G. Sappanone A protects against myocardial ischemia reperfusion injury by modulation of Nrf2. Drug Des Devel Ther 2020;14:61–71.

19. Zhang Q, Zhou X, Wu R, et al. The role of IMP dehydrogenase 2 in Inauhzin-induced ribosomal stress. Elife 2014;3:e03077.