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Kojic acid and its manganese and zinc complexes as potential radioprotective agents

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Abstract—The naturally occurring fungal metabolite kojic acid and its manganese and zinc complexes have been evaluated as potential radioprotectors in mice. Their toxicity and radioprotective activity (survival rate) have been determined and compared with that of WR-2721 (amifostine). The results of in vivo radioprotection showed that these compounds exhibited significant radioprotective effects against lethal dose of γ-irradiation in mice.

The search for an ideal radioprotector is one of the current topics of research in radiation biology and medicinal chemistry. Radioprotectors can protect normal tissue intimately associated with tumor and in the path of treatment beam, and enable the application of relatively higher doses of radiation so as to favor more killing of cancer cells than normal.[1] Therefore any chemical agent that can improve the tolerance of normal tissue to radiation is of paramount interest. These agents can also be useful to protect individuals against accidental exposure. This has particular relevance in nuclear warfare, nuclear accidents and nuclear terrorism.[2,3]

A variety of compounds with different molecular structures, original sources, therapeutic activities and metabolic functions are known to have radioprotective action. Although a great many compounds synthesized for this purpose showed good in vitro radioprotective activity, most of them failed in vivo application because of their acute toxicity to the mammalian system.[2,3] Research on various thiol radioprotectors, such as WR-2721 (**1**, amifostine) alone or in combination with prostaglandins, have demonstrated prevention of radiation-induced damage to the living cells.[4–6] However, no ideal, safe synthetic radioprotectors are available to date, so the search for alternative sources, including natural products, has been on going for several decades. Radioprotective activities of foods including vitamins, garlic extract, squalene, caffeine, *miso* (fermented soy bean paste), citrus extract and hesperidin have been reported.[7,8]

The role of reactive oxygen species in ionizing radiation injury and the potential of antioxidant to reduce these deleterious effects have been studied in animal models for more than five decades. Naturally occurring antioxidants, such as vitamin E and selenium, are less effective radioprotectors than synthetic thiols but may provide a longer window of protection against lethality and other effects of low dose, low-dose rate exposures.[9] The antioxidants of plant origin, such as chlorogenic acid, curcumin, and garlic were reported to provide protection against radiation-induced genetic damage in mice.[10,11]

In the present work, the radioprotective potential of naturally occurring fungal metabolite kojic acid **2** (Fig. 1) and its complexes with manganese and zinc (**3a** and **3b**) have been evaluated. Kojic acid [5-hydroxy-2-(hydroxymethyl)-4-pyrone] is a fungal metabolite produced by some species of *Aspergillus*, *Penicillium* and *Acetobacter*. It was discovered during investigating on the fermentation of steamed rice (koji).[12,13] Kojic acid occurs in such traditional Japanese fermented foods, including *shoyu* (soy sauce), *miso* (soybean paste) and *sake* (rice wine). Because kojic acid is often produced during the fermentation of dietary staples, it has a long

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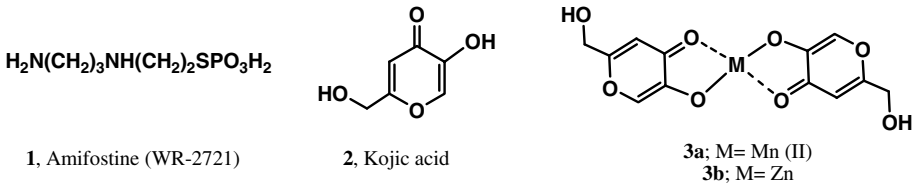

Figure 1.

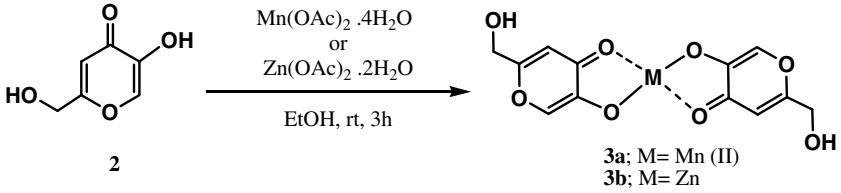

Figure 2. Preparation of complexes **3a** and **3b**.

history of consumption. Thus, due to the biochemical properties of kojic acid, they are considered to be one of the safest food products. Kojic acid is widely used as a food additive for preventing enzymatic browning of raw crabs and shrimps, and as a cosmetic agent for skin whitening, based on its excellent inhibitory on the polyphenol oxidase (tyrosinase).[14–16] It has also been shown that kojic acid enhances neutrophil phagocytosis and lymphocyte proliferation, it enhances number of leukocytes while scavenging reactive oxygen species generated in tissues or blood.[17] These findings suggest that kojic acid is very likely to have radioprotective potential. On the other hand, kojic acid is studied extensively because it forms complexes with various metal ions. These complexes have reasonable hydrolytic stability, neutral charge and significant lipophilicity.[18]

Therefore, we investigated the radioprotective potential of kojic acid **2** by the evaluation of survival rates of mice exposed to γ-irradiation and compared with its manganese and zinc complexes **3a** and **3b** (Fig. 1).

The commercially available starting material kojic acid **2** was used for biological evaluation and preparation of manganese and zinc complexes **3a** and **3b**. Compounds **3a** and **3b** were synthesized by complexation of kojic acid **2** (2 equiv) with manganese (II) acetate tetrahydrate or zinc acetate dihydrate in ethanol at room temperature in high yields (Fig. 2).[19–21]

The toxicity effects of kojic acid **2** and its complexes **3a** and **3b** were determined in vivo against male NMRI mice.[22] The LD_{50} value for kojic acid **2** was determined by the probit analysis method.[23] For complex compounds the maximum injectable dose with regard to fluidity of suspension, administrated to animals. As noted in Table 1, the LD_{50} value for kojic acid was 1400 mg/kg (9.86 mM/kg). The complex compounds did not show any mortality at the maximum injected dose, thus the LD_{50} for compounds **3a** and **3b** was found to be more than 1500 mg/kg.

For evaluation of radioprotective activity,[24] 0.5 and 1.0 mM/kg of compounds (aproximately 1/20 and 1/10 of the toxic $LD_{50}$for kojic acid) were used. These doses were 71 and 142 mg/kg for kojic acid **2**, and 169 and 339 mg/kg for **3a** and 174 and 349 mg/kg for **3b**. These compounds were administrated 24 h before 8.2 Gy irradiation. The amifostine **1** administrated at dose 214 mg/kg (1 mM/kg) to animals 1 h prior γ-irradiation. The survival results of the radioprotection studies are summarized in Table 1 and Figure 3.[25,26] Exposure of 8.2 Gy γ-irradiation-induced mortality and %100 of animals in the vehicle + 8.2 Gy irradiation group died within in 30-days.

Table 1. Toxicity and radioprotective effects of kojic acid **2** and its manganese and zinc complexes **3a** and **3b**

Compound	LD_{50} (mg/kg)	Dose injected (mg/kg)	30-days survival[a] (%)
Control	—	—	0
2, Kojic acid	1400	142 (1 mM/kg)[b]	63
		71(0.5 mM/kg)	28
3a, Kojic acid–Mn (II) complex	>1500	339 (1 mM/kg)	50
		169 (0.5 mM/kg)	47
3b, Kojic acid–Zn complex	>1500	349 (1 mM/kg)	38
		174 (0.5 mM/kg)	45
1, Amifostine[c]	—	214 (1 mM/kg)	81

[a] Male NMRI mice were injected sc with a suspension of compounds 24 h before cobalt-60 γ-radiation at dose 8.2 Gy. Thirty-days survival was used.
[b] The dose was based on 1/10 and 1/20 of the LD_{50} value for kojic acid.
[c] Amifostine was dissolved in sterile distilled water and injected 1 h prior irradiation.

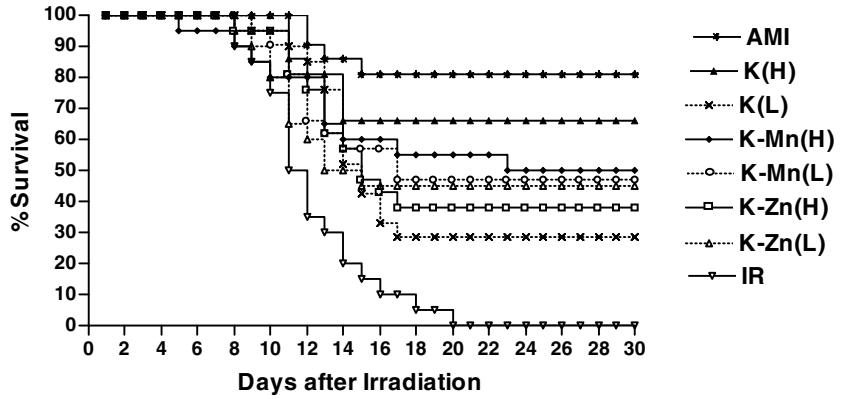

Figure 3. Effect of kojic acid **2** and its complexes **3a** and **3b** on the survival of irradiated mice. NMRI mice received these agents subcutaneously at 24 h before γ-irradiation with 8.2 Gy. $N = 20$ mice per group. AMI, amifostine **1**; K, kojic acid **2**; H, high dose (1 mM/kg); L, low dose (0.5 mM/kg); K–Mn, kojic acid–manganese complex **3a**; K–Zn, kojic acid–zinc complex **3b**. IR, irradiation.

As is evident from the data, kojic acid **2** and its complexes **3a** and **3b** significantly reduced mortality induced by radiation. The percentage of survival in each groups were as follow: vehicle, 0%, kojic acid (**2**) 63% and 28%, kojic acid–Mn complex (**3a**) 50% and 47%, kojic acid–Zn complex (**3b**) 38% and 45% for high and low doses of these compounds, respectively. Amifostine **1** exhibited a survival 81% at dose 214 mg/kg when injected 1 h before irradiation (Table 1 and Fig. 3). Survival increased significantly, in all compounds-treated groups respect to the vehicle treated group ($P < 0.0001$). The lowest mortality was observed in the animals treated with 1.0 mM/kg of kojic acid **2** and amifostine **1**. There was no significant difference between amifostine **1** (1.0 mM/kg), kojic acid **2** (1.0 mM/kg), **3a** (1.0 mM/kg) and **3a** (0.5 mM/kg) groups ($P > 0.4$). The 30-day survival for 1.0 and 0.5 mM/kg kojic acid-injected mice were 63% and 28%, respectively, this difference was statistically significant ($P < 0.01$).[25,26]

The exposure of animals to γ-rays resulted in radiation-induced sickness and mortality. The results of present study revealed that kojic acid **2** and its complexes **3** reduced mortality induced by γ-irradiation when it injected 24 h prior irradiation. Kojic acid exhibited significant action in delaying deaths as well as effective protection against γ-rays induced mortality comparable to reference drug amifostine. Although synthetic aminothiols, such as amifostine are potent radioprotective agents, they have a narrow therapeutic index and are plagued by significant side effects such as hypotension, nausea, vomiting and allergy even at low doses.[27] Thus, the use of these agents limited because of their toxic effects, and the search for more effective and less toxic radioprotectors has spurred interest in the development of nonthiol-based compounds. According to literature the LD_{50} value of amifostine is 609 mg/kg (ip).[28] However, kojic acid has a long history of consumption and to be one of the safest food products.[14] In this study we showed that kojic acid has a LD_{50} value 1400 mg/kg (sc), and complexation with manganese and zinc diminish its toxicity ($LD_{50} > 1500$ mg/kg for complexes). It may also be mentioned that the doses of kojic acid used in the present work were relatively quite low compared to its LD_{50} dose (aproximately 1/20 and 1/10 of $LD_{50}$for kojic acid). On the other hand, the usefulness of amifostine in the clinic may be affected by its short half-life in circulation. A high level of radioprotection (90%) was observed in mice injected sc with amifostine 30 min before irradiation. However, mice injected sc at 2 h before irradiation had a 0% survival.[29] We showed that administration of kojic acid afforded a significant protection when injected sc at 24 h prior γ-irradiation. It can be another advantage of kojic acid compared to amifostine.

Kojic acid, a fungal metabolite is a natural antioxidant and scavenges reactive oxygen species (ROS).[17,30] In addition, kojic acid and its manganese complex showed potent superoxide dismutase (SOD) and lipid peroxidation inhibitory activities. The manganese complex was more potent than its ligand and trolox.[19] Furthermore, Kojic acid and its complex showed protective effects against neurodegenerative diseases induced by free radicals.[19]

The radioprotective agents are known to exert their biological activity through different mechanisms, such as enhancement of repair processes, target stabilization, detoxification of radiation induced species, and scavenging of free radicals. Thus, antioxidants mainly provide protection by the removal of free radicals.[2,3] Therefore it is quite possible that kojic acid, being an antioxidant, might have scavenged the radiolytically generated free radicals and in turn provided protection against radiation-induced mortality.

Surprisingly, complexation of kojic acid **2** with manganese (II) and zinc did not enhance its radioprotectant action. The other studies showed that incorporation of manganese and zinc in ligands and complex formation increased radioprotecive activity, but we did not find the similar results.[31,32] At present it is difficult to explain these results but numerous possibilities can be considered, for example different solubility, half-lives or poor pharmacokinetics of these complexes in the animal body.

In conclusion, we investigated the radioprotective potential of the naturally occurring fungal metabolite

kojic acid **2** and its manganese and zinc complexes **3a** and **3b** in mice. Their toxicity and radioprotective activity (survival rate) have been determined and compared with that of WR-2721 (amifostine). The results of in vivo radioprotection showed that these compounds exhibited significant radioprotective effects against lethal dose of γ-irradiation in mice. Kojic acid exhibited significant action in delaying deaths as well as effective protection against γ-rays induced mortality comparable to reference drug amifostine.

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21. General procedure for the preparation of complexes **3a** and **3b**: kojic acid **2** (1420 mg, 10 mmol) was dissolved in ethanol (25 mL) and slightly heated to clear the solution. The solution of manganese (II) acetate tetrahydrate or zinc acetate dihydrate (5 mmol) in ethanol (8–10 mL) was added dropwise to the solution of kojic acid **2** and the reaction mixture was stirred at room temperature for 3 h. The precipitated complex compound was collected by filtration and washed with cold ethanol. The obtained solid was dried in vacuo over desiccant to give **3**.[19,20]
22. For toxicity studies, kojic acid **2** was dissolved in sterile distilled water and was injected subcatenus (sc) in mice. Male NMRI mice were injected with a different doses through the dose–response range for lethal toxicity. Six animals were used for each subgroup dose, and four doses were used for determining of each LD_{50}. Death observations were analyzed by Probit analysis to determine the LD_{50}.[23]The complex compounds **3a** and **3b** were suspended in sterile distilled water having 0.2% polysorbate 80 (Tween 80), and for the toxicity studies, the maximum doses were injected with regards to suspension fluidity.
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24. Whole-body irradiation was performed with a cobalt-60 γ-radiation source (Teratron 780, Canada). Mice were placed in a well-ventilated perspex box and irradiated in groups of 10 mice simultaneously. The source-to-skin distance was 80 cm with a dose rate of 1.16 Gy/min at room temperature (23 ± 2 °C). For radioprotective studies, groups of twenty mice were injected sc 24 h prior to γ-radiation for each compound. Mice were irradiated with a dose equal to the $LD_{100/30}$ (lethal dose for mice in 30 days) of control mice (8.2 Gy). The mice treated with kojic acid **2** and its manganese and zinc complexes **3a** and **3b** at doses 1 and 0.5 mM/kg 24 h prior lethal γ-irradiation. The amifostine was administrated 1 h before γ-irradiation at dose 214 mg/kg. Treated animals were kept for 30 days and lethality was recorded each day. The control group received an equal volume of sterile distilled water having 0.2% Tween 80 in the same manner. Survival was monitored on a daily basis, and the number of animals 30 days after irradiation was recorded.
25. Daily survival graph was taken using the Kaplan-Meier equation. The percentage of survival of various doses was compared using two-sample test for proportions with Fisher's exact test.[26]
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