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N^6-Furfuryladenine, Kinetin, Protects against Fenton Reaction-Mediated Oxidative Damage to DNA

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N^6-Furfuryladenine (kinetin) has been shown to have anti-ageing effects on several different systems including plants, human cells in culture, and fruitflies. Since most of the experimental data point toward kinetin acting as an antioxidant both *in vitro* and *in vivo,* and since much evidence supporting a causal role of oxidative damage in ageing is accumulating, we tested the antioxidant properties of kinetin directly. Using 8-oxo-2′deoxyguanosine (8-oxo-dG) in calf thymus DNA as a marker for oxidative damage, we demonstrate that kinetin significantly ($P < 0.005$) protects the DNA against oxidative damage mediated by the Fenton reaction. Kinetin inhibited 8-oxo-dG formation in a dose-dependent manner with a maximum of 50% protection observed at 100 μM kinetin. © 1999 Academic Press

N^6-Furfuryladenine (kinetin) belongs to the family of N^6-substituted adenine derivatives known as cytokinins. In addition to its several biological effects such as stimulation of transcription (1), cell cycle control (2), and calcium flux (3) kinetin has been shown to have anti-ageing effects on several different systems including plants (4), human fibroblasts (5), and the fruitfly *Zaprionus paravittiger* (6, 7). Although the mechanisms of action of kinetin are yet to be revealed, various lines of evidence indicate that kinetin may act directly as an antioxidant or indirectly as a regulator of antioxidants. For example, kinetin has been shown to: (i) have a direct effect on superoxide dismutase (SOD) activity in plants (8), (ii) increase the specific activity of catalase in fruitflies both during developmental stages and in adults undergoing ageing (7), (iii) prevent oxidation of unsaturated fatty acids in plant membranes (9), and (iv) slow down the accumulation of autofluorescent material (such as lipofuscin) in human cells during ageing in culture (5). However, no published data are available showing the effects of kinetin on reactive oxygen species (ROS)-mediated damage to macromolecules, especially the DNA.

It has recently become clear that extensive hits by ROS on DNA is a major cause of oxidative damage which may lead to mutations and consequently to pathology, including cancer and ageing (10). Particularly the base modification 8-oxo-dG has been a focus of much interest and is often used as a marker of oxidative damage in DNA (11). Several systems have been described which induce the formation of 8-oxo-dG, including Fenton-type reactions, singlet oxygen, and UV- or ionising-radiation (12–14). We have tested the antioxidant effect of kinetin using the Fenton reaction system which generates primarily hydroxyl radicals (•OH). Here we report that kinetin significantly inhibits the Fenton reaction-mediated formation of 8-oxo-dG in the DNA, which is a novel observation.

MATERIALS AND METHODS

Preparation of kinetin solutions. Kinetin (N^6-furfuryladenine) was purchased from either Serva (Germany) or Carmé (USA). Since kinetin is practically insoluble in water (<1.5 mg/L) all concentrated kinetin solutions were made by first dissolving 33 mg kinetin in 1 ml of 1 M HCl and then diluting to a total volume of 20 ml with H_2O. The final concentration of kinetin (approx. 8 mM) was determined using spectroscopy ($\epsilon_{Kinetin} = 18{,}650$ at 268 nm). In order to rule out any effect from the acid, adenine (Serva) solutions were also prepared the same way as kinetin solutions.

Formation of 8-oxo-dG in vitro by the Fenton reaction system. Calf thymus DNA (300 μg; Sigma-Aldrich Denmark) was dissolved in a total volume of 250 μl NaOAc, pH 5.0 (16 mM final concentration), followed by the addition of Fe_2SO_4 (25 μM final concentration). Kinetin, adenine, or acid control solution was then added and the tubes mixed thoroughly by vortexing before H_2O_2 was added. The reaction was stopped by precipitating the DNA in cold ethanol for 30 min at −20°C. The DNA was dissolved in 20 mM NaOAc, pH 5.0 at a final concentration of 6 μg/μl.

Enzymic cleavage of DNA to nucleosides. Enzymic hydrolysis of DNA was carried out as described previously (11). The DNA was digested to nucleosides by nuclease P1 (1 U/100 μg DNA; Boehringer-Mannheim) for 2 h at 37°C in 20 mM NaOAc, pH 5.0. Tris/HCl was added to a final concentration of 50 mM and pH 7.4 and the DNA was digested by bacterial alkaline phosphatase (0.2 U/100 μg DNA)

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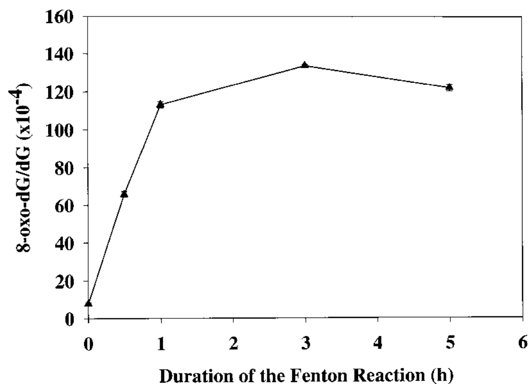

FIG. 1. Formation of 8-oxo-dG by the Fenton reaction. H_2O_2 = 250 mM, Fe^{2+} = 25 μM. Each point represents the mean ± SD of three independent experiments.

for 1 h at 37°C. After the enzymic cleavage the samples were kept at 4°C until HPLC-ECD analysis was performed.

Detection of 8-oxo-dG. The analysis of 8-oxo-dG content of DNA was carried out on a Gilson HPLC/UV system connected to an electrochemical detector (CoulArray 5600, ESA Inc. USA). Nucleosides were separated on a Vydac C18 reversed-phase column (4.6 × 250 mm) equipped with a precolumn. An isocratic mobile phase composed of 10 mM sodium phosphate, pH 5.0, and 2–5% methanol was used. The methanol content was changed to compensate for the change in retention time caused by a decrease in column performance. The potentials used for detection of 8-oxo-dG were set to 150 mV, 260 mV, and 400 mV, respectively. In order to ensure that no other compounds were co-eluting with 8-oxo-dG the ratio of the current measured at 260 mV and 400 mV was calculated. This ratio can be considered as a fingerprint for 8-oxo-dG and in most cases we have observed a ratio accuracy higher than 90%. Quantification of deoxyguanosine (dG) was done using UV absorption at 256 nm. The ESA CoulArray for Windows software was used to analyse the data. Before each analysis standard curves were generated for both 8-oxo-dG and dG to determine the concentration of these species in the samples. Data are expressed as oxidised nucleosides per unmodified nucleosides (e.g., 8-oxo-dG/dG).

Statistical analysis. The F-test was used to test the null hypothesis that any two sets of measurements had the same variances ($H_0\sigma^2$: $\sigma_a^2 = \sigma_b^2$) and in all cases the null hypothesis was accepted. The t-test was then used to test the hypothesis that two sets of measurements had equal means ($H_0\mu$: $\mu_a = \mu_b$).

RESULTS

Initially, we checked that kinetin itself did not catalyse the decomposition of H_2O_2 by monitoring the concentration of H_2O_2 spectrophotometrically at 240 nm. There was no effect on the H_2O_2 concentration after addition of kinetin (results not shown). Exposure of DNA to the Fenton reaction was found to increase the ratio of 8-oxo-dG/dG more than 15 fold from $7.86 \times 10^{-4} \pm 0.64 \times 10^{-4}$ to $1.21 \times 10^{-2} \pm 0.02 \times 10^{-2}$ within 1 h after which a plateau was reached (Fig. 1).

A series of experiments was then performed to investigate the effect of kinetin on the formation of 8-oxo-dG under different parameters of the Fenton reaction mixture, namely H_2O_2 concentration (Fig. 2A), Fe^{2+} concentration (Fig. 2B), and the duration of the stress (Fig. 2C). We found that kinetin (100 μM) significantly ($P < 0.02$) inhibited the formation of 8-oxo-dG induced by the Fenton reaction and that this protective effect of kinetin was observed at all H_2O_2 doses used (Fig. 2A). After the DNA was exposed to 250 mM H_2O_2 and 25 μM [Fe^{2+}] for 1 h the ratio of 8-oxo-dG/dG in samples containing kinetin was only $3.61 \times 10^{-3} \pm 0.23 \times 10^{-3}$ compared to the control samples where a ratio of 8-oxo-dG/dG of 8.67 ×

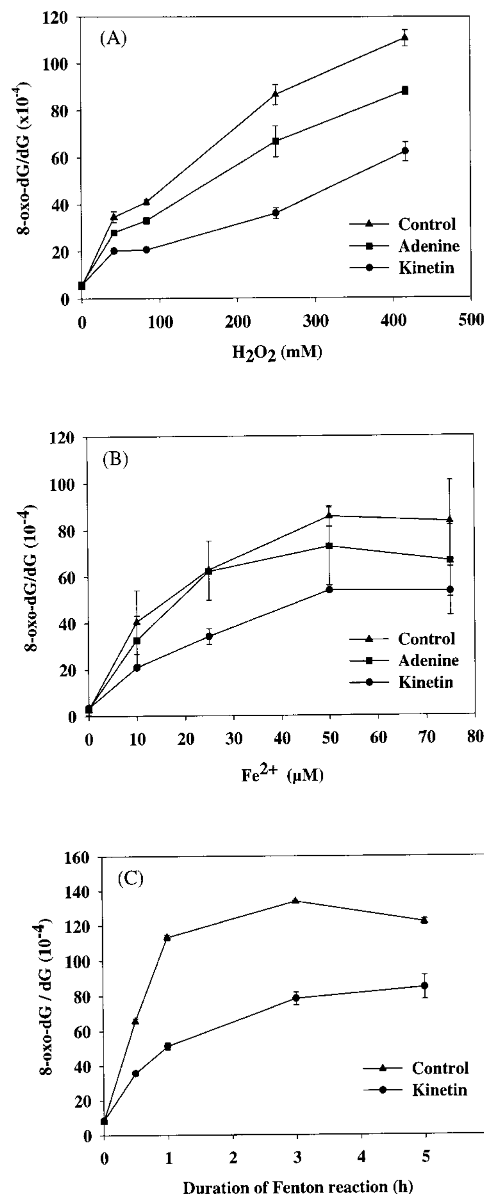

FIG. 2. Optimisation of the Fenton reaction. (A) Increasing H_2O_2 concentration in the Fenton reaction, and the effect of 100 μM kinetin or 100 μM adenine. The duration of the Fenton reaction was 1 h and 25 μM Fe^{2+}. (B) Increasing Fe^{2+} concentration in the Fenton reaction, and the effect of 100 μM kinetin or 100 μM adenine. The duration of the Fenton reaction was 1 h and 250 mM H_2O_2. (C) Extended exposure to H_2O_2, and the effect of 100 μM kinetin, 250 mM H_2O_2 and 25 μM Fe^{2+}. Each point represents the mean ± SD of values obtained from three independent experiments.

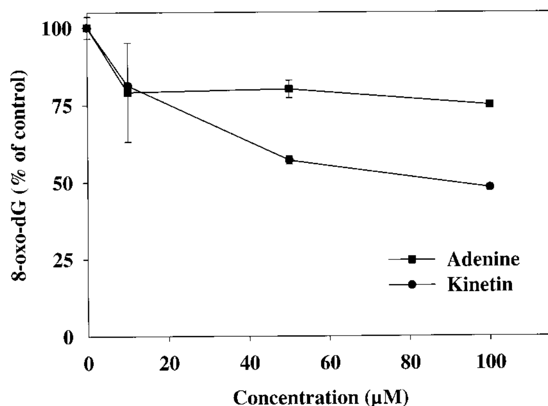

FIG. 3. Effect of increasing concentration of kinetin or adenine on 8-oxo-dG formation by the Fenton reaction system. $[H_2O_2] = 250$ mM and $[Fe^{2+}] = 25$ μM. Each point represents the mean ± SD of three independent experiments.

$10^{-3} \pm 0.43 \times 10^{-3}$ was observed. In order to evaluate the influence of the N^6-furfuryl substitution of kinetin the effect of adenine on the Fenton reaction system was also tested. Adenine (100 μM) also had some protective effect ($P < 0.03$) but the inhibition of DNA damage was less than that observed for kinetin. After the DNA was exposed to 250 mM H_2O_2 and 25 μM $[Fe^{2+}]$ for 1 h the ratio of 8-oxo-dG/dG in samples containing adenine was $6.77 \times 10^{-3} \pm 0.66 \times 10^{-3}$ (Fig. 2A).

Since production of $^{\bullet}OH$ by Fenton type reactions depends on the concentration of Fe^{2+} we investigated the effect of kinetin on 8-oxo-dG formation during increasing Fe^{2+} concentration keeping the H_2O_2 concentration constant. The formation of 8-oxo-dG increased with increasing Fe^{2+} concentration and reached a plateau after 25 μM. At all Fe^{2+} concentrations tested kinetin significantly ($P < 0.01$) inhibited the formation of 8-oxo-dG (Fig. 2B). After the DNA was exposed to 250 mM H_2O_2 and 50 μM $[Fe^{2+}]$ for 1 h the ratio of 8-oxo-dG/dG in samples containing kinetin was only $5.39 \times 10^{-3} \pm 0.01 \times 10^{-3}$ compared to the control samples where a ratio of 8-oxo-dG/dG of $8.59 \times 10^{-3} \pm 0.45 \times 10^{-3}$ was observed. Adenine also inhibited 8-oxo-dG formation though not as efficiently as kinetin. After the DNA was exposed to 250 mM H_2O_2 and 50 μM $[Fe^{2+}]$ for 1 h the ratio of 8-oxo-dG/dG in samples containing adenine was $7.27 \times 10^{-3} \pm 0.17 \times 10^{-3}$ (Fig. 2B). The effect of kinetin on 8-oxo-dG formation during increasing exposure time to H_2O_2 was then investigated. The property of kinetin for protecting DNA from oxidative damage could be observed rapidly and significantly ($P < 0.01$) within the first hour followed by further protection throughout the period of reaction for up to 5 h (Fig. 2C).

Having established that kinetin protected against DNA oxidation under all the different Fenton reaction conditions tested we investigated if the inhibition of 8-oxo-dG formation by kinetin occurred in a dose-dependent manner. For this experiment we used the optimised Fenton reaction mixture (H_2O_2 = 250 mM, Fe^{2+} = 25 μM, exposure time = 1 h). All kinetin concentrations tested resulted in significantly ($P < 0.005$) lower 8-oxo-dG levels in the DNA compared to the controls in a dose dependent manner (Fig. 3). More than 50% reduction in the amounts of 8-oxo-dG was observed in the presence of 100 μM kinetin as compared with the controls. A dose dependent reduction in the levels of 8-oxo-dG was also observed for adenine, however, the effect was less than that observed for kinetin at all concentrations tested. For example, at a concentration of 100 μM adenine 8-oxo-dG formation was inhibited only by 25% as compared to the controls (Fig. 3).

DISCUSSION

We have observed that the presence of N^6-furfuryladenine (kinetin) in the Fenton reaction mixture significantly inhibits the formation of 8-oxo-dG in DNA. Kinetin-mediated protection was consistently observed

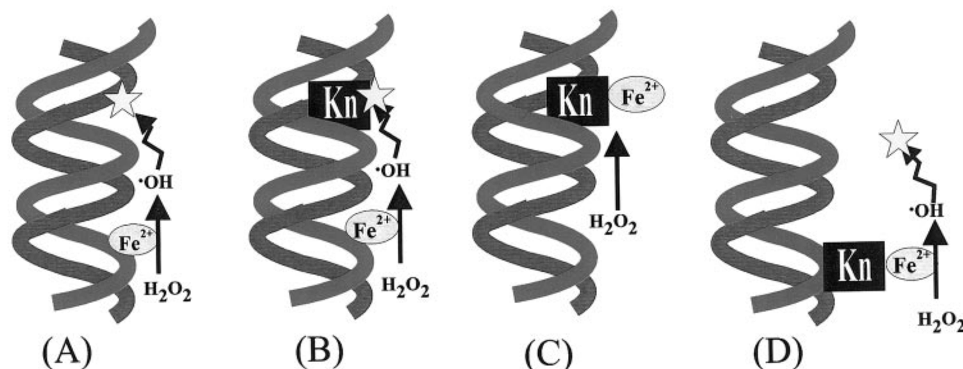

FIG. 4. Possible mechanisms of action of kinetin. (A) DNA damage by the Fenton reaction. (B) Kinetin scavenges $^{\bullet}OH$ and protects the DNA. (C) Kinetin binds Fe^{2+} and prevents $^{\bullet}OH$ formation. (D) Kinetin binds Fe^{2+} in such way that the $^{\bullet}OH$ is formed too far away from the DNA to cause any damage.

within a broad concentration range of the individual components of the Fenton reaction system. Furthermore, the inhibition of damage occurred in a dose dependent manner with more than 50% protection observed at kinetin concentrations of 100 μM. This concentration of kinetin is within the range (80–200 μM) observed to have anti-ageing effects on human skin fibroblasts and fruitflies (5–7). Furthermore, within this concentration range kinetin was significantly more effective than adenine in protecting the DNA from oxidative damage.

Although the exact mechanism for the DNA protective effects of kinetin against oxidative damage is not known, there are at least two ways by which these effects may be achieved. One, kinetin may prevent the formation of hydroxyl radical, and second, kinetin may scavenge $^{\bullet}OH$ before it reacts with the DNA. Kinetin could prevent the $^{\bullet}OH$-mediated 8-oxo-dG formation by binding iron either in such a way that it is no longer a Fenton reductant or in a way that prevents iron from associating with the DNA. It has recently been shown that only iron bound tight to the DNA results in 8-oxo-dG formation (15). Dps, a ferritin homologue, is an example of a molecule that has been proposed to protect DNA by preventing ROS formation because Dps when bound to the DNA also binds iron ions (16, 17). Therefore, it is possible that kinetin also prevents the formation of $^{\bullet}OH$ by this route. Further studies are required to test this possibility.

In another scenario kinetin can act as a radical scavenger in two different ways since oxygen radicals can: (i) directly abstract hydrogen from the carbon atom of the amine bond of kinetin (18), or (ii) undergo faster dismutation due to an SOD-like activity of metal-kinetin complexes (18). Kinetin has been shown to form a complex with Cu(II) that has some SOD-like activity (19). In biological systems O_2^- may play a double role in radical formation by the Fenton reaction since it is dismutased into H_2O_2 but also because it acts as a reductant for free iron or protein bound iron providing Fe^{2+} for Fenton type reactions (20). The SOD-like activity of a kinetin-Cu(II) complex would therefore remove O_2^- and consequently less Fe^{2+} would be available for Fenton type reactions. However, removal of O_2^- from the Fenton reactions mixture used in this study will not have any effect since Fe^{2+} was added from outside in high concentration. Furthermore, there was no increase in protection of DNA from $^{\bullet}OH$-mediated damage after addition of Cu(II) to the Fenton reaction mixture (results not shown). The fact that kinetin can bind to metal ions and has some SOD-like activity (19), indicates that in our *in vitro* system too, kinetin could prevent iron from participating in Fenton type reactions which generate 8-oxo-dG in the DNA. A schematic representation of various possible modes of action of kinetin, whereby it can prevent oxidative damage to the DNA, is given in Fig. 4.

In order to investigate if kinetin was unique in its antioxidant properties because of the N^6-furfuryl substituent, the ability of adenine to protect against DNA damage was also tested. We found that although adenine could inhibit 8-oxo-dG formation to some extent, it was much less effective than kinetin. Thus, the N^6-furfuryl substitution appears to be important for the protective function of kinetin. Since the electrochemical activity of kinetin is due to the N^6-furfuryl substitution, which makes it more easily oxidised than adenine (21), it would be interesting to test other N^6-modified purines, such as N^6-(4-hydroxy-3-methyl-trans-2-butenyl)adenine (zeatin), isopentyl adenine, and benzyladenine, for their abilities to inhibit 8-oxo-dG formation, and to establish if any correlation exists between their DNA protective capacity and their electrochemical activity.

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REFERENCES

1. Gaudino, R. J., and Pikaard, C. S. (1997) *J. Biol. Chem.* **272,** 6799–6804.
2. Zhang, K., Letham, D. S., and John, P. C. (1996) *Planta* **200,** 2–12.
3. Schumaker, K. S., and Gizinski, M. J. (1995) *J. Biol. Chem.* **270,** 23461–23467.
4. Gan, S., and Amasino, R. M. (1995) *Science* **270,** 1986–1988.
5. Rattan, S. I. S., and Clark, B. F. C. (1994) *Biochem. Biophys. Res. Commun.* **201,** 665–672.
6. Sharma, S. P., Kaur, P., and Rattan, S. I. S. (1995) *Biochem. Biophys. Res. Commun.* **216,** 1067–1071.
7. Sharma, S. P., Kaur, J., and Rattan, S. I. S. (1997) *Biochem. Mol. Biol. Int.* **41,** 869–875.
8. Kurepa, J., Herouart, D., Van Montagu, M., and Inze, D. (1997) *Plant Cell Physiol.* **38,** 463–470.
9. Leshem, Y. Y. (1988) *Free Radical Biol. Med.* **5,** 39–49.
10. Collins, A. R. (1999) *BioEssays* **21,** 238–246.
11. Barciszewski, J., Barciszewska, M. Z., Rattan, S. I. S., and Clark, B. F. C. (1995) *Pol. J. Chem.* **69,** 841–851.
12. Wei, H., Ca, Q., Rahn, R., Zhang, X., Wang, Y., and Lebwohl, M. (1998) *Biochemistry* **37,** 6485–6490.
13. Boiteux, S., Gajewski, E., Laval, J., and Dizdaroglu, M. (1992) *Biochemistry* **31,** 106–110.
14. Taffe, B. G., Larminat, F., Laval, J., Croteau, D. L., Anson, R. M., and Bohr, V. A. (1996) *Mutat. Res.* **364,** 183–192.
15. Lloyd, D. R., and Phillips, D. H. (1999) *Mutat. Res.* **424,** 23–36.
16. Martinez, A., and Kolter, R. (1997) *J. Bacteriol.* **179,** 5188–5194.
17. Grant, R. A., Filman, D. J., Finkel, S. E., Kolter, R., and Hogle, J. M. (1998) *Nat. Struct. Biol.* **5,** 294–303.
18. Barciszewski, J., Rattan, S. I. S., Siboska, G. E., and Clark, B. F. C. (1999) *Plant Science,* in press.
19. Goldstein, S., and Czapski, G. (1991) *Free Radical Res. Commun.* **12–13,** 173–177.
20. Keyer, K., Gort, A. S., and Imlay, J. A. (1995) *J. Bacteriol.* **177,** 6782–6790.
21. Barciszewski, J., Siboska, G. E., Pedersen, B. O., Clark, B. F. C., and Rattan, S. I. S. (1996) *FEBS Lett.* **393,** 197–200.