ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
29 (2002) 361–369

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Short communication

Spectroelectrochemical study of the interaction between antitumor drug daunomycin and DNA in the presence of antioxidants

Guifang Cheng, Haiyun Qu, Dongmei Zhang, Jidong Zhang, Pinggang He, Yuzhi Fang *

Department of Chemistry, East China Normal University, ZhongShan Road North 3663, Shanghai 200062, People's Republic of China

Received 22 October 2001; received in revised form 21 January 2002; accepted 16 February 2002

Abstract

Anthracycline drug daunomycin (DNR) is a widely used clinical drug. But its side effects, especially cardiotoxicity, have greatly restrained its application. The side effects were due to free radical formation in the metabolic process of DNR. The purpose of this study is to diminish the side effects by using antioxidants. Two kinds of free radical scavengers have been investigated, that is, vitamins: vitamin C (V_C), rutin (V_P) and vitamin B6 (V_{B6}); amino acids: cysteine (CysH) and methionine (Met). Free radical scavenging efficiency (E°_{eff}) of these antioxodants had been calculated. Under the experimental condition, the values of E°_{eff} of V_C, V_P, V_{B6}, CysH and Met were 23.8, 15.3, 6.4, 48.2 and -7.7%, respectively. The relationship between the free radical scavenging activities and its chemical structure has also been discussed.

Keywords: DNA damage; Spectroelectrochemistry; Free radical; Antioxidant; Scavenger

1. Introduction

The antitumor antibiotic daunomycin (DNR) is in wide clinical use for the treatment of various neoplastic diseases [1,2]. The metabolism and action mode of DNR has been investigated extensively [3–5]. Some of its biological activities have been thought to be an intimate correlation with its redox characteristic. The usefulness of this drug is, however, limited by its significant toxicity. Recent studies demonstrated that the main toxicity of this drug is due to the metabolic products of DNR in vivo. Lown et al detected there was metabolic intermediate—semiquinone existence in the reduction process of DNR under anaerobic condition [6]. Many evidences showed that DNR is able to form an electrostatic complex with cardiolipid, a phospholipid of the mitochondria in membrane, to restrain the activity of respiratory system enzyme [7] and to cause lipid peroxidation on cell membranes [8]. Studies on cultured cardiac

* Corresponding author. Tel.: +86-21-622-32627; fax: +86-21-624-51921.

0731-7085/02/$ - see front matter
PII: S0731-7085(02)00074-2

cells showed that DNR also affects both ATP and phosphocreatine [9]. Some people believe that the cardiotoxicity is owing to co-operation of free radical and Fe(III)–DNR complex [10,11]. Peroxidation and harmful secondary product such as hydroxyl radical ($^{\bullet}OH$) and superoxide anion radical ($^{\bullet}O_2^-$) etc. produced in metabolic process of DNR were toxic to neighbor molecules in vivo.

We have ever studied the redox behavior of DNR under physiological condition and found that DNR could be reduced to form two kinds of free radicals, that is, semidaunomycinone and 7-deoxysemidaunomycinone [12]. Like other free radicals in vivo, semidaunomycinone and 7-deoxysemidaunomycinone preferentially react to mitochondrial DNA in the cytoplasm where much NADH reductase exists and can reduce DNR to semiquinone free radicals. Heart, where is plenty of mitochondrial DNA and active oxygen, short of catalase and glutathione peroxidase becomes a special target of anthracycline drugs. Therefore, cardiotoxicity is one of the main causes for their application restriction [13].

Many research works have been done to reduce the toxicity of DNR by remodeling the structure of DNR [14,15]; forming metal ion–DNR complex to protect quinone structure from being reduced [16–19]. But these methods are usually too complicated. As we known, most of antioxidants are nurtures and beneficial to improve human immune system and would not bring about other side effects so that using antioxidants in scavenging free radicals is widely applied [20–23], but its application in decreasing side effects during chemotherapy is limited.

Spectroelectrochemistry is a new sphere of analytical science technology, not only can it get the information about current and potential on the electrode, but the information about spectrophotometric characteristics of the substances on the working electrode as well. It becomes a very fruitful method used in the study of mechanism of the electrode reaction, especially in dynamic mode.

In this paper, in situ spectroelectrochemistry has been used as a tool to imitate and control the metabolic process of DNR in vivo and to observe the change of DNA by UV spectra measurement. The effect of antioxidants in decreasing the side effects of DNR has been investigated.

The arm of this paper is to diminish side effects of DNR conveniently by means of clearing out free radicals with antioxidants. It is of significance in clinic and pharmacy.

2. Experimental

2.1. Materials

DNR hydrochloride, obtained from Shanghai Institute for Drug Control, was used without further purification. Its aqueous stock solution was prepared by dissolving the drug in a mixed phosphate and Tris–HCl solution (pH 7.0). Fish sperm DNA was purchased from Sangon Bioengineering Company Ltd., (Shanghai), dissolved in Tris–HCl-EDTA (TE) solution (pH 7.0). Both of them were kept below 4 °C. The concentrations of these stored standard solutions were determined spectrophotometrically by using the molar absorption coefficient at the wavelength of their respective maximum absorbency.

Vitamin C (V_C), vitamin B6 (V_{B6}), rutin (V_P), cysteine (CysH) and methionine (Met) were of analytical reagents grade and were dissolved in phosphate buffer (pH 7.0).

All other chemicals were of analytical reagents grade. Doubly distilled water was used throughout the experiments.

2.2. Apparatus

The spectroelectrochemical cell being used was homemade. A three-electrode system was used with an unglazed porcelain tube to connect the catholyte and anolyte [12]. A spectroscopical pure graphite plate was used as the working electrode ($1.0 \times 0.6 \times 0.3$ cm), which was fixed on the plexiglass plate connected with a Cu wire. A thin layer of epoxy resin was covered on the graphite plate except the electrode surface. The counter electrode was a coiled platinum wire. Ag/AgCl (saturated KCl) electrode was used as the reference electrode.

Pretreatment of working electrode: after being ultrasonic cleaned, the surface of the graphite electrode was polished with emery paper, washed with 1:1 HNO_3, acetone and distilled water successively, then oxidized at 0.90 V (vs. Ag/AgCl, saturated KCl) for 5 min in 1 mol/l NaOH solution.

Spectroelectrochemical measurements were made with a Cary 50 Probe UV–Vis Spectrophotometer (Varian, USA), with a ZF-3 potentiostat (Shanghai Second Component Factory, China). All spectra were collected in 1 cm optical-path cells.

2.3. Procedures

All mixed solutions were bubbled with N_2 for about 15 min to remove dissolved oxygen completely before measurements and put into the thin-layer spectroelectrochemical cell. A positive nitrogen atmosphere was maintained during the experiment. In order to ensure light passing through the thin layer on the surface of the working electrode, the optimum position of the working electrode was selected and fixed according to [24]. Two steps of potential transition (0 to -0.55 to -0.80 V) were applied and the UV spectra of DNA were measured in each period. As DNR and antioxidants applied in our experiments absorb UV light and interfere with the spectrum of DNA, the solution of DNR or DNR with antioxidant was used as blank in respective experiment.

All experiments were repeated for three times and the data were the average of three measurements.

3. Results and discussion

3.1. Spectroelectrochemical study of the interaction between DNR and fish sperm DNA

Followed the experimental procedure in Section 2.3, the UV spectra of DNA in DNA–DNR adduct were measured in situ. When the first potential step -0.50 V was applied on, the adduct was just adsorbed on the surface of the electrode and there were no other electrochemical reactions taking place [12]. The absorption of DNA at 260 nm increased after a potential -0.8 V was applied (see Fig. 1 and Table 1). The base hyperchromic effect happened and it meant DNA was injured. The hyperchromic effect (H) could be expressed as:

$$H = \frac{(A_{260} - A^{\circ}_{260})}{A^{\circ}_{260}} \quad (1)$$

A°_{260} is the absorption at 260 nm of DNA in the adduct at open circuit and A_{260} is that measured after being electrolyzed. The hyperchromic effect increased with the concentration ratio $C_{\mathrm{DNAp}}/C_{\mathrm{DNR}}$ in adduct which was changed from 6:1 to 1:1.

According to [25], If the base hyperchromicity is caused only by disassembling double helix DNA into denatured single helix DNA, it is lower than 40%. But, in the experiment, the hyperchromic effect rose to 713.7% (when C_{DNAp}:$C_{\mathrm{DNR}} = 1$:1). Here, it is guessed that DNA was damaged not only to be denatured but to be fragmentized as well. The deduction was confirmed by atomic force microscope (AFM) experiments. Before being electrolyzed, pure DNA molecules with millions base pairs are hundreds of micrometers long and only a small part of which can be observed by AFM, (see Fig. 2a). After being electrolyzed, DNA broke into fragments of different length and some of them even could not be made out their linear structure (shown in Fig. 2b). The influence of active oxygen and other free radicals produced depending on oxygen could be excluded, for electrolysis was done under anaerobic condition. It is more reasonable to believe that free radicals, semidaunomycinone and 7-deoxysemidaunomycin, induced free radical chain reactions around DNA helixes, then lead DNA damage.

3.2. Effect of antioxidants

As discussed above, the hyperchromic effect can effectively reflect the degree of DNA damage. To keep DNA from damage by free radical, two kinds of antioxidants, vitamins and amino acids, were investigated.

3.2.1. Effect of vitamins

UV spectra of DNA in DNA–DNR-vitamin were measured followed the procedure in Section 2.3. The results are shown in Fig. 3 and Table 2.

3.2.2. Effect of amino acids

Amino acids are nurture and beneficial to health. Some of them have the action to eliminate free radicals. In the experiment, Met and CysH were used as scavengers and their efficiencies were measured according to Section 2.3 (see Fig. 4 and Table 3).

Since the amount of antioxidant added in each solution was different, H_{max} can not be used as a standard to compare the efficiency of scavenging free radical of different antioxidant.

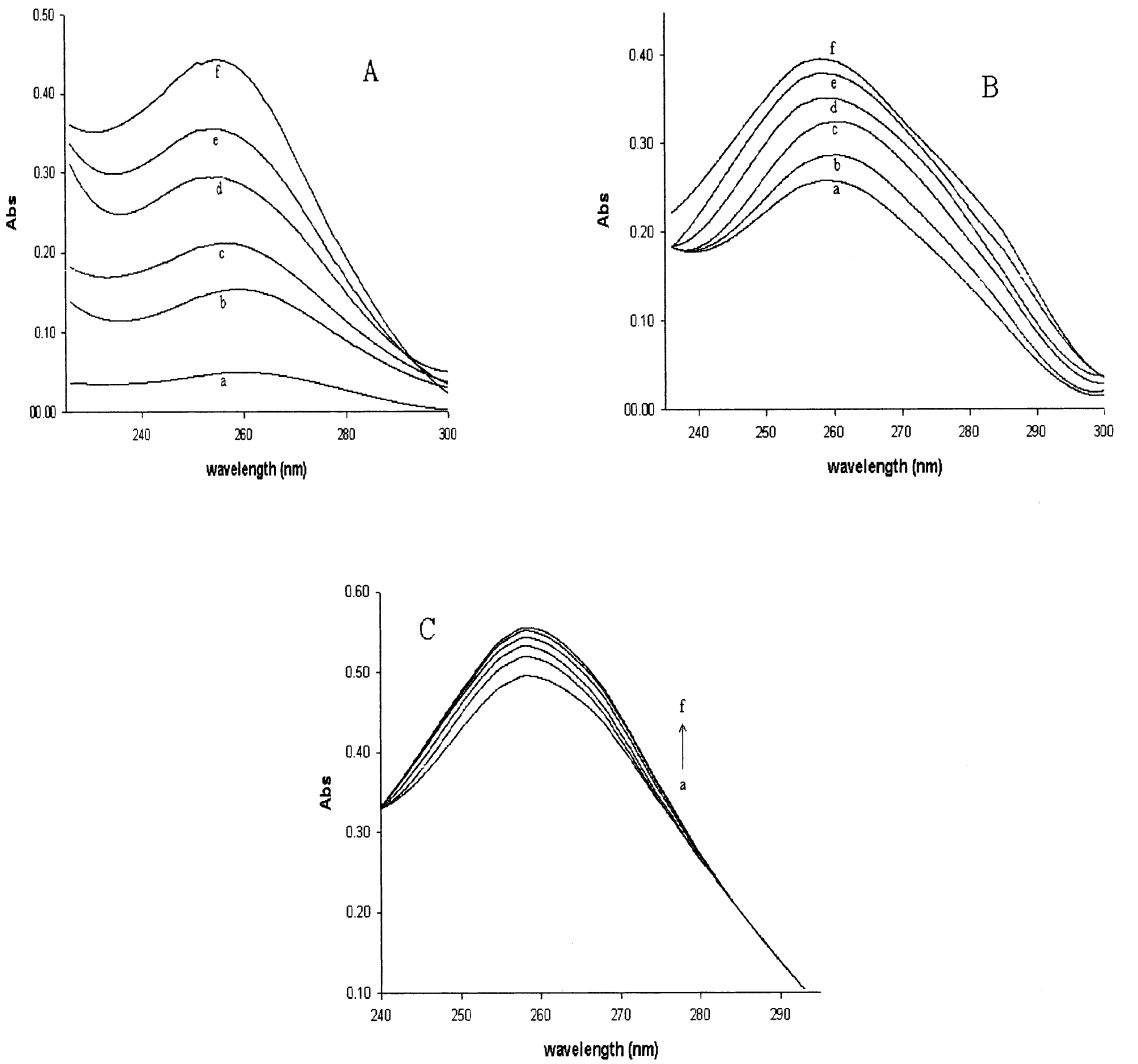


Fig. 1. Absorption spectra of DNA–DNR adducts, $C_{DNR} = 1.0 \times 10^{-5}$ mol/l. (A) C_{DNAp}:C_{DNR} = 1:1; (B) C_{DNAp}:C_{DNR} = 3:1; (C) C_{DNAp}:C_{DNR} = 6:1, (a) open circuit, Applied potential stepped to -0.8 V, for (b) 0.2 min, (c) 0.5 min, (d) 0.8 min, (e) 1 min and (f) 1.2 min.

Table 1
H_{max} of different DNA-DNR adducts

C_{DNAp}/C_{DNR}	$H_{max}(\%)$[a]
6:1	12.2
3:1	53.0
1:1	713.7

[a] H_{max} is the maximum base hyperchromic effect under the experimental condition.

According to [26], the equation of scavenging free radical efficiency (E_{eff}) can be expressed as following:

$$E_{eff} = \frac{(H^{o}_{max} - H^{x}_{max})}{H^{o}_{max}} \quad (2)$$

where H^{o}_{max} is the hyperchromic effect in DNA–DNR adduct and H^{x}_{max} is that in the system of DNA–DNR-scavenger. To conveniently compare scavenging efficiency, equimolar scavenging effect (E°_{eff}) is applied as a standard:

$$E^{\circ}_{eff} = \frac{E_{eff}}{n} = \frac{(H^{o}_{max} - H^{x}_{max})}{nH^{o}_{max}} \quad (3)$$

where n is the ratio of the mass of antioxidant to DNR. E°_{eff} of three vitamin antioxidants and two amino acid antioxidants were calculated by Eq. (3).

The results are shown in Table 4.

As shown in Table 4, E°_{eff} of V_C is the best, V_P the second and V_{B6} the worst in three vitamins. Compared with two of amino acids, it is very obviously that CysH is a very high efficient scavenger. But in the presence of Met, DNA damaged heavily.

3.3. Discussion of the antioxidative ability of scavengers

The mechanism of antioxidants to restrain side effects of DNR is on the basis of antioxidants scavenging radicals produced in the metabolic process of DNR. There may be, we think, two ways for antioxidants to scavenge free radicals. One is, antioxidants react with active free radicals such as $^{\bullet}OH$ and $^{\bullet}O_2^-$, to form new free radicals with less activity and then the radical chain reactions could be terminated. The other is that antioxidants lose electrons to have single electron in free radicals paired.

The antioxidant ability of V_C, V_P and V_{B6} is related to their reductive abilities representing in losing hydrogen atom in hydroxyl group existing in their structure and stability of the oxidized products. Structure difference is one of the main reasons for different scavenging radical ability

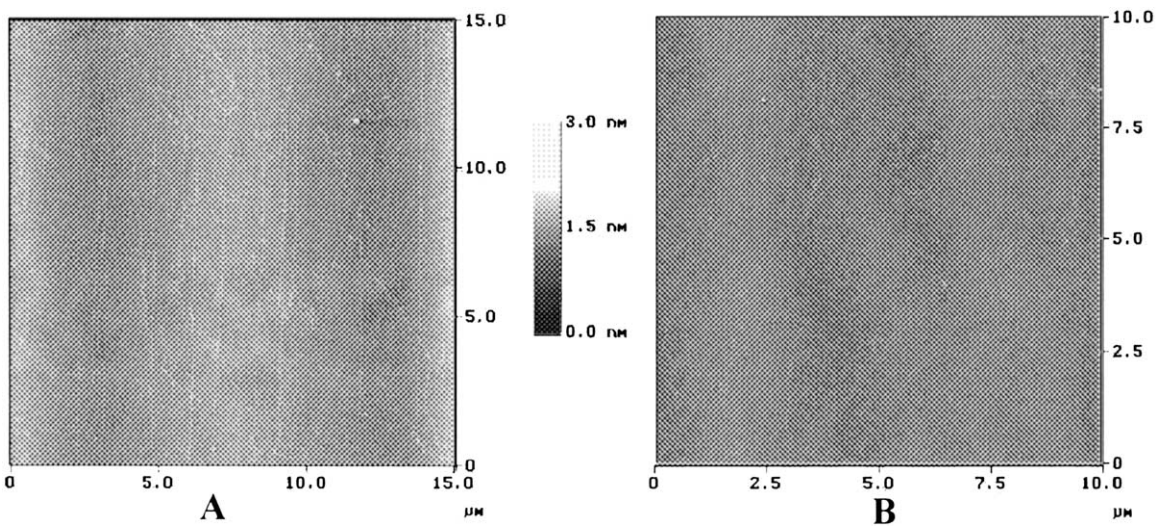


Fig. 2. Graph of AFM of DNA (A) DNA adduct without electrolysis and (B) after electrolysis.

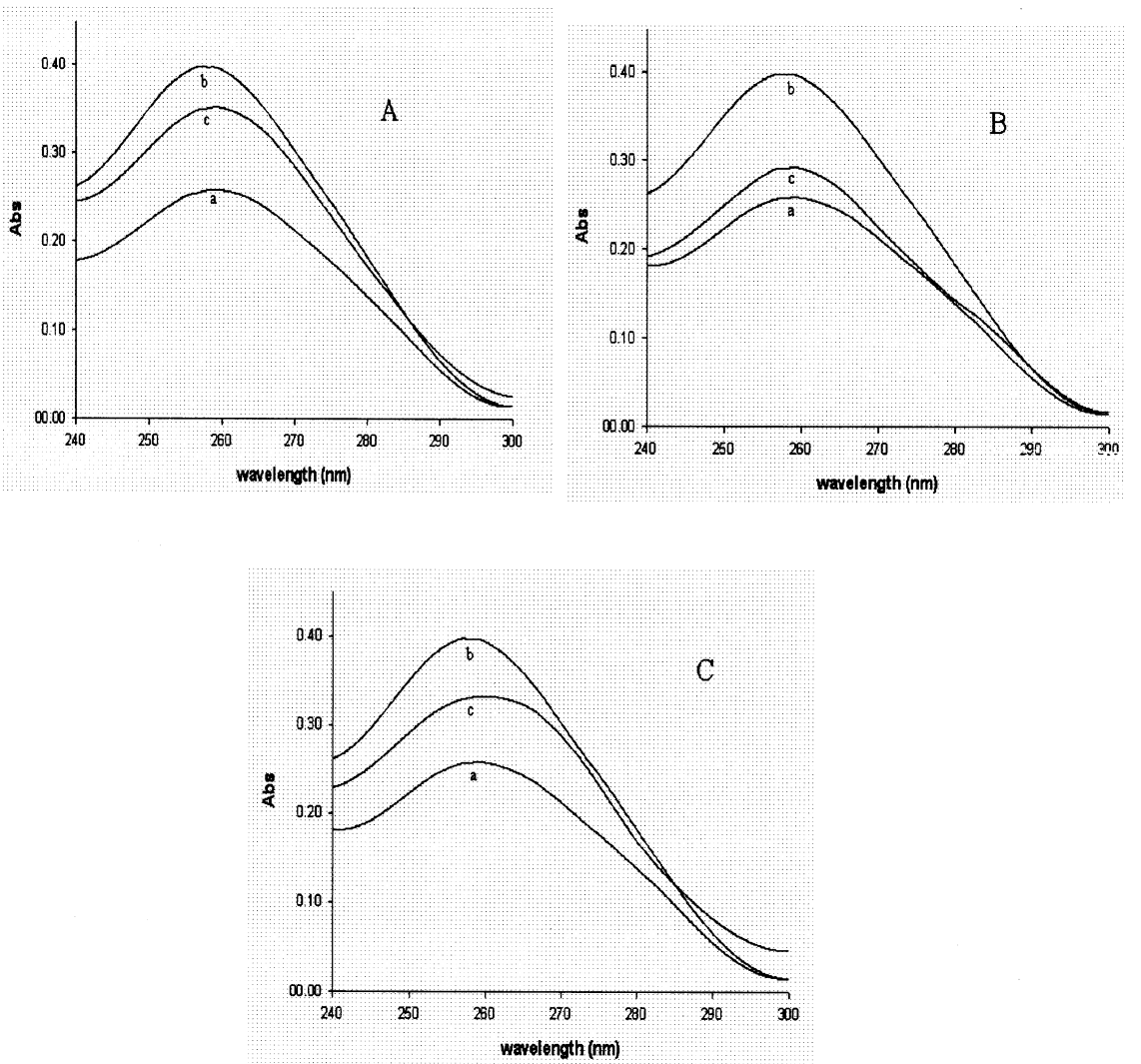
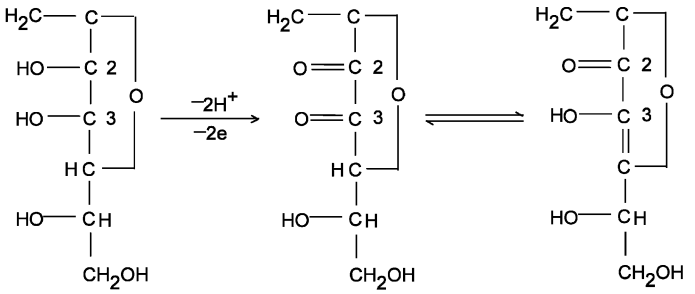


Fig. 3. The effect of vitamins, $C_{DNAp} = 3.92 \times 10^{-5}$ mol/l at pH 7.0. (A) the effect of V_{B6} (C_{DNAp}:C_{DRN}:C_{VB6} = 3:1:5); (B) the effect of V_C (C_{DNAp}:C_{DRN}:C_{VB6} = 3:1:3); (C) the effect of V_P (C_{DNAp}:C_{DRN}:C_{VB6} = 1:1:3). (a) Adduct with antioxidant free measured at open circuit; (b) the adduct with antioxidant free and (c) presence, measured at the applied potential was -0.80 V and when the absorption of DNA at 260 nm achieved highest.

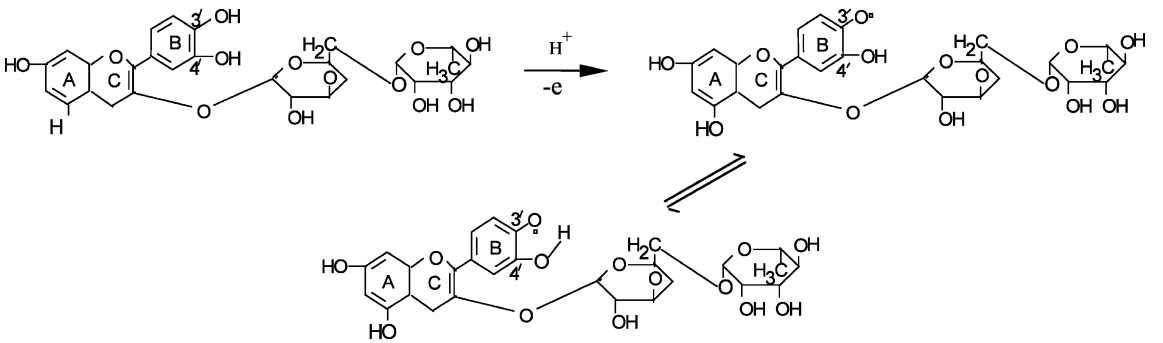
(see Scheme 1). Hydroxyls on C2 and C3 in the structure of V_C can lose two electrons and two hydrions easily so that V_C has the high reductive ability in neutral, especially in alkaline medium. The antioxidative group of V_P is phenol group. Hydroxyls on C3′ and C4′ of ring B also easily lose a hydrogen atom and form phenoxy radical. Through intramolecular hydrogen bond, *o*-xophenic radical can resonate and tautomerize to

Table 2
The degree of DNA damage in different experiment

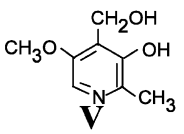
Components	Proportion of the component	H_{max} (%)
DNA–DNR–V_C	3:1:3	15.1
DNA–DNR–V_{B6}	3:1:5	36.0
DNA–DNR–V_p	3:1:3	28.7



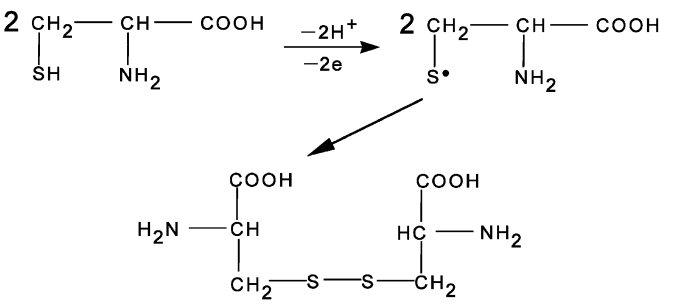
V_C



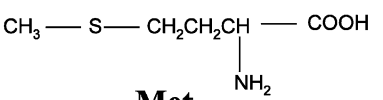
V_P



B6



CysH



Met

Scheme 1.

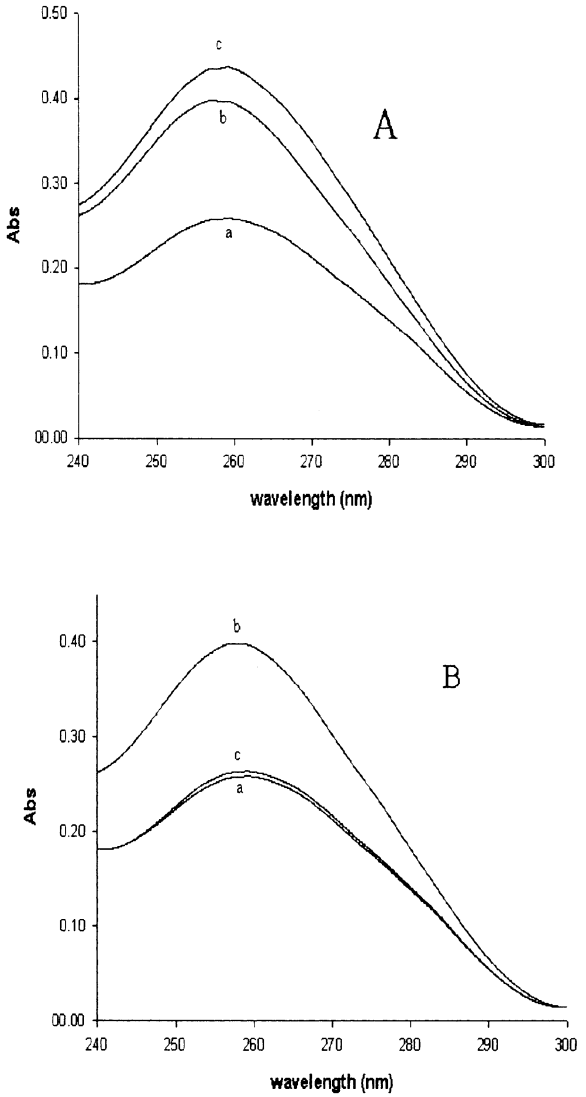


Fig. 4. The effect of amino acids, $C_{DNAp} = 3.92 \times 10^{-5}$ mol/l at pH 7.0. (A) the effect of Met (C_{DNAp}:C_{DRN}:$C_{Met} = 3:1:5$); (B) the effect of CysH(C_{DNAp}:C_{DRN}:$C_{CysH} = 3:1:2$); (a) adduct with antioxidant free measured at open circuit; (b) the adduct with antioxidant free and (c) presence, measured at the applied potential was -0.80 V and when the absorption of DNA at 260 nm achieved highest.

stable *o*-hydroxybenzoione, so that V_P is a good scavenger following V_C. In the structure of V_{B6}, although there is a hydroyl connecting with insatiable pyrrole ring, the oxidized product is unstable because there are electronic repulsive groups CH_3– and –CH_2OH on *ortho*-position decreasing the stability of the oxidized product and directly diminishing the ability of radical scavenger. There is a mercapto in the structure of both CysH and Met. After drawing-out hydrogen, –SH in CysH becomes –S$^{\bullet}$ radical and two of them connect to form stable –S–S– structure. Mercapto in Met is –S–, which is stable so that it is hard for Met to be oxidized [27]. Met has no scavenging radical ability.

4. Conclusion

Antioxidants such as V_C, V_P and CysH, basic substance and harmless in vivo, can efficiently scavenge semiquinone radicals and do not cut down the action of DNR. Furthermore, V_C, V_P and CysH are nurtures and much helpful.

Normally, free radicals such as hydroxyl ($^{\bullet}OH$) and superoxide anion ($^{\bullet}O_2^-$) formed in physiological action or biochemical reactions can be cleared out by many kinds of special radical scavenger enzymes in human body, such as superoxide dismutase (SOD), catalase and glutathione peroxidase, also including some nonenzyme small molecules, i.e. V_C, V_E and β-carotene etc.. If there are much more drug entering the body, it will

Table 3
H_{max} of DNA-DNR adduct in the presence of different amino acid antioxidants

Components	Proportion of the component	H_{max} (%)
DNA–DNR–CysH	3:1:2	1.98
DNA–DNR–Met	3:1:5	73.4

Table 4
Equimolar scavenging effect (E°_{eff}) of scavengers

Scavenger	E°_{eff} (%)
V_{B6}	6.4
V_C	23.8
V_P	15.3
Met	−7.7
CsyH	48.2

generate semiquinone radicals and initiate free radical chain reactions. The concentration of free radical in vivo may be too high to be cleared out by body defensive system. Supplying some antioxidant CysH, V_C and V_P would conveniently and efficiently decrease the side effect of the DNR in chemiotherapy.

The result obtained from our experiment, we think, will show a realistic way to decrease the toxicity of anthracycline drugs.

Acknowledgements

This work was carried out with the support of the foundation of national natural science of China. Supported by National Science Foundation of China (Grant number 29875005).

References

[1] G.P. Murphy, W. Lawrence Jr, R.E. Lenhard, American Society Textbook of Clinical Oncology, second ed., American Society, Atlanta, GA, 1995.
[2] P.H. Wiernik, J.P. Dutcher, Clinical importance of anthracyclines in the treatment of acute myeloid leukemia, Leukemia 6 (Suppl l) (1992) 67.
[3] X. Chu, G.-L. Shen, J.-H. Jiang, T.-f. Kang, B. Xiong, R.-Q. Yu, Anal. Chim. Acta 373 (1998) 29.
[4] D.L. Kleyer, T.H. Koch, J. Am. Chem. Soc. 105 (1983) 2504.
[5] S. Mazzini, R. Mondell, E. Ragg, J. Chem. Soc., Perkin Trans. 9 (1998) 1983
[6] J.W. Lown, H.H. Chen, Can. J. Chem. 59 (1981) 3212.
[7] E. Goormaghtight, P. Huart, et al., Biophy. Acta 861 (1986) 83.
[8] C.E. Myers, W.P. Mcguire, R.H. Liss, et al., Science 197 (1997) 165.
[9] B.K. Sinha, R.H. Sik, Biochem. Pharmacol. 30 (1981) 2026.
[10] O. Marcillat, Y. Zhang, K.J.A. Davies, Biochem. J. 259 (1989) 181.
[11] M.M.L. Fiallo, A. Garinee-Suillerot, B. Matzanke, H. Kozlowski, J. Inorg. Biochem. 75 (1999) 105.
[12] H. Qu, G. Cheng, P. He, Y. Fang, Chem. J. Chinese Univ. 12 (2001) 2000 Chinese.
[13] J. Fisher, K. Ramakrishnan, J.E. Becvar, Biochemistry 22 (1983) 1347.
[14] T. Masahiko, T.-K. Shozo, I. Masamoto, J. Coord. Chem. 18 (1988) 77.
[15] S. Akihiro, O. Takashi, S. Masakatsu, Tetrachdron 58 (2002) 75.
[16] S.I. Dikalov, G.V. Rumyanteeeeseva, A.V. Piskunov, L.M. Weiner, Biochemistry 31 (1992) 8947.
[17] M. Spinelli, J.C. Dabrowiak, Biochemistry 21 (1882) 5862.
[18] S. Mazzini, R. Mondelli, E. Ragg, J. Chem. Soc. Perkin Trans. 2 (1998) 1983.
[19] B.B. Hasinoff, K.T. Tran, J. Inorg. Biochem. 77 (1999) 257.
[20] S.T. Palayoor, E.A. Dump, K. Malaker, Int. J. Radiat. Oncol. Biol. Phys. 29 (1994) 289.
[21] H. Zhang, D. Chen, Acta Biophysica. Sinica. (Chinese), 16 (2000) 1.
[22] W. Ma, E. Cao, J. Zhang, J. Qin, Acta Biophysica. Sinica. 14 (1998) 156 Chinese.
[23] N. Salh, N.J. Miller, G. Paganga, Arch. Biochem. Biophys. 322 (1993) 339.
[24] Y.Z. Fang, D.J. Long, J.N. Ye, Anal. Chim. Acta 342 (1997) 13.
[25] J.D. Rawn, Biochemistry, Haper & Raw Publishers, New York, 1983.
[26] H. Zhang, F. Wang, M. Li, H. Zhang, M. Li, J. Radiat. Res. Radiat. Proc. 15 (4) (1997) 224 Chinese.