Bioorganic & Medicinal Chemistry 20 (2012) 340–345

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Reactive metabolites of desipramine and clomipramine: The kinetics of formation and reactivity with DNA

Ekaterina A. Korobkova *, John Nemeth, Mikeisha Cadougan, Abhishek Venkatratnam, Mohanram Bassit, Nikolay Azar

Science Department, John Jay College of Criminal Justice, CUNY, 445 W 59th St., New York, NY 10019, USA

ARTICLE INFO

Article history:
Received 4 August 2011
Revised 21 October 2011
Accepted 27 October 2011
Available online 3 November 2011

Keywords:
Tricyclic antidepressants
One-electron oxidation
Michaelis–Menten kinetics
Rate constants
DNA damage

ABSTRACT

Tricyclic antidepressants (TCAs), along with phenothyazines and some industrial chemicals, are shown to react with enzymes that exhibit peroxidase activity. These reactions result in the formation of reactive intermediates having unpaired electrons. The peroxidase oxidation and reactivity of two TCAs, desipramine and clomipramine, were investigated. As a model of peroxidase, horseradish peroxidase (HRP) was employed. The products of the peroxidase catalyzed oxidation of desipramine and clomipramine were identified as N-dealkylated compounds iminodibenzyl and 3-chloroiminodibenzyl using the GC/MS technique. Both drugs formed broad UV/vis absorption spectra in the presence of HRP and H_2O_2, indicating the formation of a radical cations—reactive intermediate of the oxidation reaction. The dynamics of the formation of the desipramine intermediate was studied using UV/vis spectroscopy. The extinction coefficient was measured for the reactive intermediate, $7.80 \times 10^3\ M^{-1}\ cm^{-1}$, as well as the apparent Michaelis–Menten and catalytic constants, 4.4 mM and $2.3\ s^{-1}$, respectively. Both desipramine and clomipramine degraded DNA in the presence of HRP/H_2O_2, as was revealed by agarose gel electrophoresis and PCI extraction. Manipulating the kinetic parameters of drug's radical formation and determining the extent of degradation to biomolecules could be potentially used for designing effective agents exhibiting specific reactivity.

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1. Introduction

The discovery of tricyclic antidepressant's (TCAs) radicals traces back to the 1960s. During this time, an EPR spectrum of an imipramine radical was detected in the presence of one-electron oxidation systems such as ceric ion and horseradish peroxidase (HRP)/H_2O_2 and in 1983, pentavalent vanadium ion.[1,2] Consequently, a number of studies have demonstrated the ability of TCAs to promote the formation of free radicals and oxidative stress in vivo. Thus, clomipramine, imipramine and nortriptyline increase the level of hydroxyl radicals in rats.[3–5] Also, imipramine and amitriptyline lead to oxidative stress in astrocyte cell lines.[6] It is even proposed that the ability of amitriptyline to increase the level of reactive oxygen species (ROS) in cancerous cells could be utilized in antitumor chemotherapy.[7] Clomipramine and desipramine are shown to inhibit the activity of mitochondrial complexes of cancerous cells.[8–11] On the other hand, TCAs are proven to be capable of performing protective tasks against the formation of lipid peroxide and to enhance antioxidant defensive mechanisms in cells.[12,13] Even though there is sufficient evidence of the radical nature of TCA's reactivity with biomolecules, the mechanisms and dynamics of the formation of these reactive species have not been studied. The dynamics of the radical formation, however, has been investigated for molecules having structural motifs similar to those of TCAs such as phenothiazines[14] and aminopyrine.[15] The focus of the present work was desipramine and clomipramine (Fig. 1). Both drugs are used in the treatment of

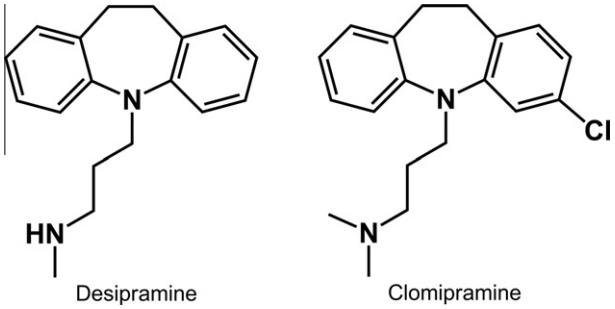

Figure 1. The molecular structures of the experimental TCAs.

Abbrevations: HRP, horseradish peroxidase; TCAs, tricyclic antidepressants; EPR, electron paramagnetic resonance; ROS, reactive oxygen species; ALS-GC/MS, automatic liquid sampler-gas chromatography/mass spectrometry; PCI, Phenol–Chloroform–Isoamyl alcohol.

* Corresponding author. Tel.: +1 212 237 8064; fax: +1 212 237 8318.
E-mail address: ekorobkova@jjay.cuny.edu (E.A. Korobkova).

0968-0896/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.bmc.2011.10.075

obsessive-compulsive disorder.[16] Specifically, desipramine is found to be effective in impeding cocaine dependence[17,18] and relieving postherpetic neuralgia.[19] Clomipramine appears to be more successful than other TCAs in the treatment of panic disorder[20] and trichotillomania.[21] We performed structural and kinetic studies on the formation of reactive intermediates of the drugs under the peroxidase oxidation. Reactivity with DNA was examined for the drugs under the conditions of one-electron oxidation.

2. Material and methods

2.1. Materials

Desipramine hydrochloride, purity ⩾98% (Saint Louis, MO); clomipramine hydrochloride, purity ⩾99% (Saint Louis, MO); peroxidase from horseradish type VI, 250–330 units/mg (Allentown, PA); and deoxyribonucleic acid type I from calf thymus (Allentown, PA) were purchased from Sigma Aldrich. Hydrogen peroxide, 35% (New Jersey, USA) was purchased from Acros Organics.

2.2. Als-gc/ms

An Agilent Automatic Liquid Sample injector (ALS-G4513A) was employed with a syringe size of 10 μL. Two microliters of sample were injected at a speed of 6000 μl/min and set at a fast plunger speed. The injector was washed with acetone and methanol seven times before and after every injection, utilizing three sample washes, five sample pumps and a standard L1 air gap injection type for all sample injections.

An Agilent 7890A GC was utilized. The inlet parameters were as follows: heater, 250 °C isocratic; pressure, 10.523 psi; total He flow, 27 ml/min; septum purge flow, 1 ml/min; septum purge flow mode, standard; injection mode, split; split ratio, 25:1; split flow, 25 ml/min. The column parameters were 30 m × 250 μm × 0.25 μm, column out—to vacuum. The carrier gas (He, 5.0 UHP) flow rate was 1 ml/min with post run flow of 1 ml/min. The initial oven temperature was 100 °C, ramped at 5 °C/min for 40 min, to the final temperature of 300 °C that was held for 2 min, with a post run temperature of 100 °C. The transfer line was kept at 300 °C for the entire 42 min run.

An Agilent 5975C Mass Selective Detector (MSD) was used with a GC sample inlet, a solvent delay of 6 min and an EMV mode gain factor of 1.00 = 1129 V. The MSD was run in 'scan' acquisition mode at normal scan speed with a scan mass range of 50.00–800.00 amu. The MSD's threshold was set at 150 amu with 1.99 scans/s and a sampling rate (2^n) of 2. The MS source temperature was set at 230 °C and MS quadrupole temperature was set at 150 °C.

An Agilent MSD ChemStation, Version E.02.00.493, was used for data acquisition and analysis with the NIST 98 library.

2.3. UV/vis spectroscopy

UV/vis spectra were recorded with a Shimazu UV–visible Spectrophotometer UV-1700 in 1000 μl quartz cuvettes at 25 °C. The solutions were prepared in 500 μl of 133 mM Sorenson buffer (pH 7.0) containing 133 mM dibasic sodium phosphate and 133 mM monobasic potassium phosphate. The spectra were recorded at the wavelength interval of 200–800 nm right after mixing desipramine or clomipramine with HRP and H_2O_2. The concentration of HRP was calculated using the extinction coefficients at 404 nm of 102,000 M^{-1} cm^{-1}.[22] The extinction coefficient of the drugs were determined to be 7.61×10^3 M^{-1} cm^{-1} at $\lambda = 251$ nm for desipramine and 7.32×10^3 M^{-1} cm^{-1} at $\lambda = 252$ nm for clomipramine. The extinction coefficients were determined by mass measurements combined with UV/vis spectroscopy.

2.4. Kinetics of the reaction of catalytic oxidation

Kinetic studies were performed with a Shimazu UV–visible Spectrophotometer, UV-1700. 1000 μl quartz cuvettes were used. The temperature in the cuvette chamber was thermostated at 25 °C. The reactions were carried out in a 500 μl solution of 133 mM Sorenson buffer (pH 7.0). An aliquot of desipramine was added to a solution of HRP and H_2O_2. The solution was thoroughly mixed via pipetting immediately after the addition of the drug. The absorption intensity was recorded over the time period of 1–20 min at $\lambda = 523$ nm.

2.5. Determination of the extinction coefficient of the product of one-electron oxidation of desipramine

The absorbance was monitored over two minutes at 251 and 523 nm for the desipramine reaction after the drug was mixed with HRP/H_2O_2. At 251 nm, the absorbance decreased with time indicating the disappearance of the drug in the oxidation reaction. At 523 nm, the absorbance increased indicating the formation of the one-electron oxidation product of the drug. The reactions were performed at various concentrations of HRP and a fixed concentration of desipramine. The concentration of the drug was chosen such that the initial absorbance did not exceed 1. The initial slope of the drug's decay kinetics was plotted against the initial slopes of the kinetics of radical appearance. The linear fit of the data provided the resulted slope, which was equal to the ratio of the extinction coefficient of the drug and the extinction coefficient of the radical.

2.6. Analysis of the kinetic data, the determination of rate constants

The absorbance versus time curves were obtained at different concentrations of desipramine and analyzed in the following way. The linear fits were performed at the onsets of the kinetics curves. The values of the rate of absorbance growth were divided by the extinction coefficient of the desipramine radical and normalized by the concentration of HRP. The values of the normalized rates of product formation were plotted against the drugs concentration. The error bars were determined using the formula of the standard deviation of a ratio of two independent variables: absorbance growth rate and the extinction coefficient of the radical. The apparent catalytic and Michaelis–Menten constants were determined by fitting the data with the equation derived by Wang et al.[23]

2.7. Agarose gel electrophoresis

1% agarose gels were prepared in TAE buffer. Electrophoresis was performed at 80 V for 120 min. The loading samples contained 20 μl of the reaction mixture and 2.2 μl of the 10× loading buffer. The 10× loading buffer consisted of 50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanole FF in TAE buffer. The gel was stained in ethidium bromide (0.5 μg/ml) for 20 min and then distained in distilled water for 20 min. The gel images were captured with the PerkinElmer Geliance 200 Imaging system.

3. Results and discussion

3.1. UV/vis spectra of desipramine oxidation intermediate

The incubation of desipramine (Fig. 1) in the presence of HRP and H_2O_2 led to the initial formation of a pink color followed by an increasing brown color over time, which produced the corresponding

broad UV/vis spectra: Figure 2. Figure 2A shows the spectra recorded as the reaction of desipramine with HRP and H_2O_2 proceeded for 24 min. The negative control reactions containing HRP/H_2O_2, HRP/desipramine or desipramine/H_2O_2 did not result in the appearance of new spectra. In the mixture containing HRP, H_2O_2 and clomipramine, no color was observed in the buffer due to the drug's lack of solubility with the buffer used. A spectrum with the absorption maximum at 520 nm (Fig. 2B) was observed in water with a clomipramine concentration 10 times greater than that of desipramine (Fig. 2A). Broad absorption spectra were observed earlier under the one-electron oxidation of aminopyrine,[15] phenothyazines,[14] imipramine[24] and some industrial arylamines.[25,26] The spectra were associated with the formation of the products of one-electron oxidation, radical cations. The assignment of the spectra to radical cations is also supported by the stoichiometry studies of the redox transitions between the oxidized forms of HRP. In this experiment, the 'one-electron-gain' conversions—HRP-I to HRP-II and HRP-II to HRP[27]—were observed upon the addition of one HRP equivalent of desipramine or clomipramine (data not shown). A radical formed is potentially very stable. This stability originates from the delocalization of the unpaired electron over the benzene rings of desipramine and clomipramine. This conjugation phenomenon is known to stabilize nitrogen-centered radicals.[28,29]

3.2. GC/MS studies of the oxidation products of desipramine and clomipramine

The products of the oxidation of the drugs were studied with GC/MS. In the case of desipramine, a precipitant formed after the incubation of the drug with HRP and H_2O_2 in the Sorenson buffer. The precipitant was dissolved in acetonitrile and injected into a GC column. A single peak was observed at 20.5 min. The mass spectrum of the peak matched that of iminoibenzyl, an N-dealkylated product from desipramine (m/z: 195.1).

In the case of clomipramine, the enzymatic oxidation reaction could only be performed in water due to the insolubility of clomipramine in the buffer. No precipitant was observed after the drug was incubated with HRP and H_2O_2. A reaction mixture was loaded on the GC column, which led to the detection of two major peaks at 24.9 and 29.4 min. The mass spectrum of the first peak matched that of 3-chloroiminodibenzyl (systematic name—5H-dibenz[*b*,*f*]azepine,3-chloro-10,11-dihydro-) as an N-dealkylated product from clomipramine (m/z: 229.1), and the second peak was identified as clomipramine (m/z: 314.1). The two molecules, iminodibenzyl and 3-chloroiminodibenzyl, were purchased and were analyzed with GC/MS. The retention times and the mass spectra of the commercial compounds matched those of the reaction products. The N-dealkylating products were previously observed in the reactions of one-electron oxidation of imipramine,[24] crystal violet[30] and aminopyrine.[15] In these instances, radical species were detected as reaction intermediates.

Based on the experimental observations presented, we proposed the kinetic schemes shown in Figure 3.

3.3. Determination of the extinction coefficient of desipramine radical cation

The extinction coefficient of desipramine radical cation was determined by measuring the rates of the absorbance decrease for the drug (251 nm for desipramine and 252 nm for clomipramine) and applying the equation:

$$\frac{d[\text{Drug radical}]}{dt} = -\frac{d[\text{Drug}]}{dt} \quad (1)$$

The equation holds true for the initial times after the reagents are mixed (when the contribution of the N-dealkylation process is negligible). Using Beer's law:

$$\frac{dA_{\text{radical}}}{dt} \times \varepsilon_{\text{drug}} = -\frac{dA_{\text{drug}}}{dt} \times \varepsilon_{\text{radical}} \quad (2)$$

where A_{drug} is the absorbance at 251 nm, while A_{radical} is the absorbance value at 523 nm, and $\varepsilon_{\text{radical}}$ and $\varepsilon_{\text{drug}}$ are the extinction coefficients for drug radical and drug, respectively. The initial slopes of

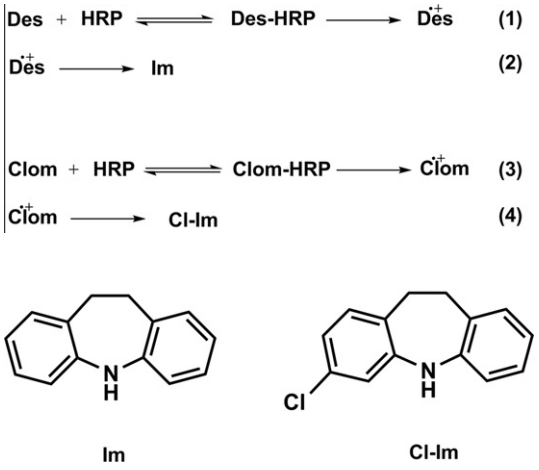

Figure 3. Reaction scheme of the drug's oxidation in the presence of HRP and H_2O_2 and the structures of the products. Des—desipramine, Im—iminodibenzyl, Clom—clomipramine and Cl-Im—3-chloriminodibenzyl.

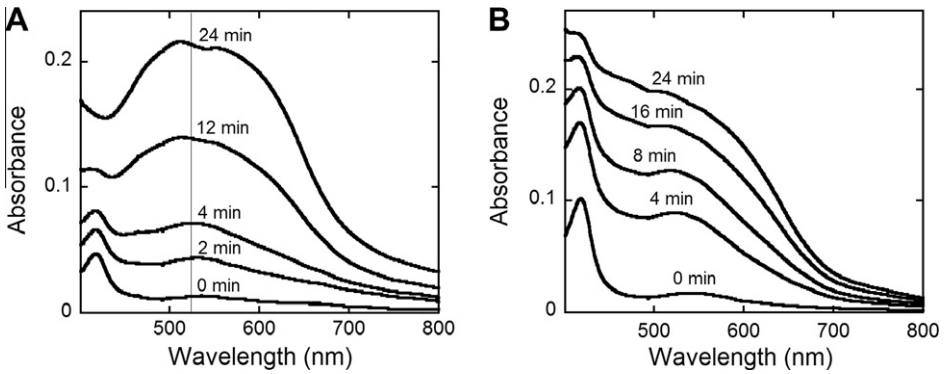

Figure 2. UV/vis absorption spectra of the drug's reactive intermediate formed during the oxidation by the HRP/H_2O_2 system. (A) Reaction with desipramine performed in 133 mM Sorenson buffer pH 7.0. [HRP] = 0.6 μM; [H_2O_2] = 10 mM; [Desipramine] = 92 μM. (B) Reaction with clomipramine performed in water. [HRP] = 1 μM; [H_2O_2] = 10 mM; [Clomipramine] = 1 mM. The indicated times were when data were taken after the reagents were mixed.

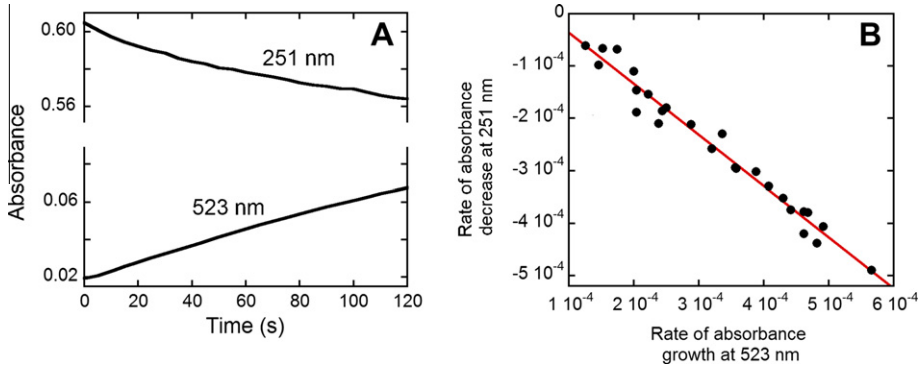

Figure 4. Determination of the extinction coefficients of desipramine radical cation. (A) Kinetic curves recorded at 251 nm and 523 nm; [HRP] = 2.48 μM, [H_2O_2] = 10 mM, [desipramine] = 37 μM. (B) Initial slopes of the kinetic curves recorded at 251 nm versus initial slopes of the kinetic curves recorded at 523 nm. The concentration of desipramine was 37 μM, and the concentration of H_2O_2 was 10 mM. The concentration of HRP varied from 0.44 to 3.5 μM.

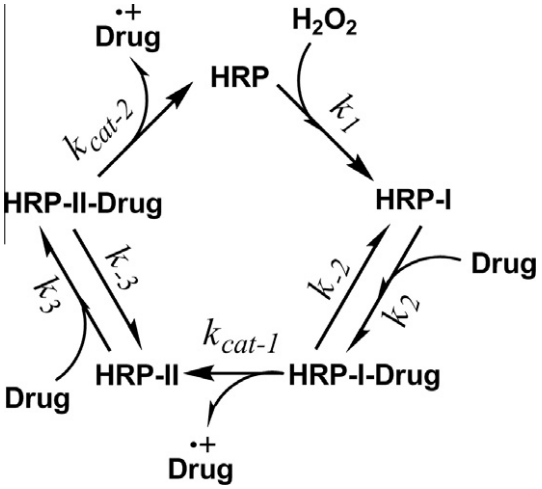

Figure 5. Kinetic scheme of the reactions of a drug's oxidation by H_2O_2 catalyzed by HRP.

the 'absorbance versus time' curves were measured at different concentrations of enzyme.

Figure 4A shows kinetic curves recorded at 251 and 523 nm, respectively. The values of (dA_{drug}/dt) were plotted against ($dA_{radical}/dt$). The slope was found to be -0.976 ± 0.034, and $\varepsilon_{radical}$ was determined to be $(7.80 \pm 0.29) \times 10^3\ M^{-1}\ cm^{-1}$ (Fig. 4B). The $\varepsilon_{radical}$ value is consistent with the extinction coefficients found for phenothiazines that range between 6507 $M^{-1}\ cm^{-1}$ for promazine (λ_{max} = 518 nm) and 12,500 $M^{-1}\ cm^{-1}$ for chlorpromazine (λ_{max} = 530 nm).[14]

3.4. Kinetic studies of desipramine peroxidase oxidation

Figure 5 shows the mechanisms underlying reactions (1) and (3) from Figure 3. These mechanisms involve the formation of the oxidation forms of HRP, HRP-I and HRP-II. In a reduced form of peroxidase, an iron atom sitting on the porphyrine ring is in its ferric state. In HRP-I, the iron atom is complexed with oxygen and is in its ferryl state. The heme group of HRP-I has an unpaired electron and a unit positive charge. In an HRP-II state, the ferryl iron atom forms a covalent complex with oxygen.[27,31] Transitions HRP-I→HRP-II and HRP-II→HRP are associated with the transmission of one electron from the substrate (drug) and the peroxidase porphyrine ring.[27] The formation of complexes between the peroxidase active site and the substrate is necessary for the electron transfer (HRP-I-Drug and HRP-II-Drug, Fig. 5). Ling et al. (2008) applied spectroscopic measurements and computational docking simulations to describe the structure of the complex intermediates formed between HRP oxidized forms and *N*-(indole-3-ethyl) cyclic amines. They found that the substrates bind to the active site of the enzyme via their benzene rings.[32] The benzene rings of both desipramine and clomipramine represent the most electronegative part of the molecule. Also, due to the electron overlap between the nitrogen atom electron pair and the π orbitals of the benzene rings, the tricyclic motif of the molecules possesses high reduction

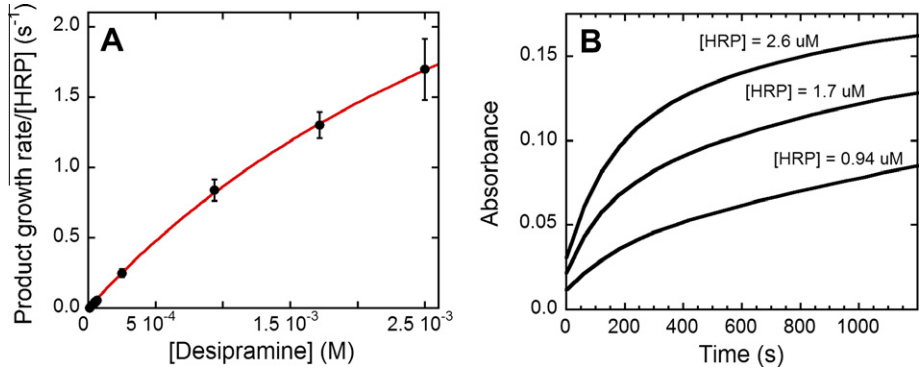

Figure 6. The kinetics of the formation of the desipramine radical monitored by UV/vis spectroscopy at 523 nm. (A) The inicial slope (d*Abs*/d*t*) normalized by the extinction coefficient of the radical and the concentration of HRP, 0.498 μM, plotted against the concentration of desipramine. The error bars are standard deviations determined based on the 6–8 measurements of the absorbance growth rate and combined with the standard deviation of the extinction coefficient of desipramine radical. The red line represents the best fit curve obtained with Eq. (3). (B) The kinetics of the oxidation reactions of desipramine in the presence of HRP and H_2O_2 recorded for 20 min. [Desipramine] = 47 μM, [H_2O_2] = 10 mM. The concentration of HRP for each curve is shown.

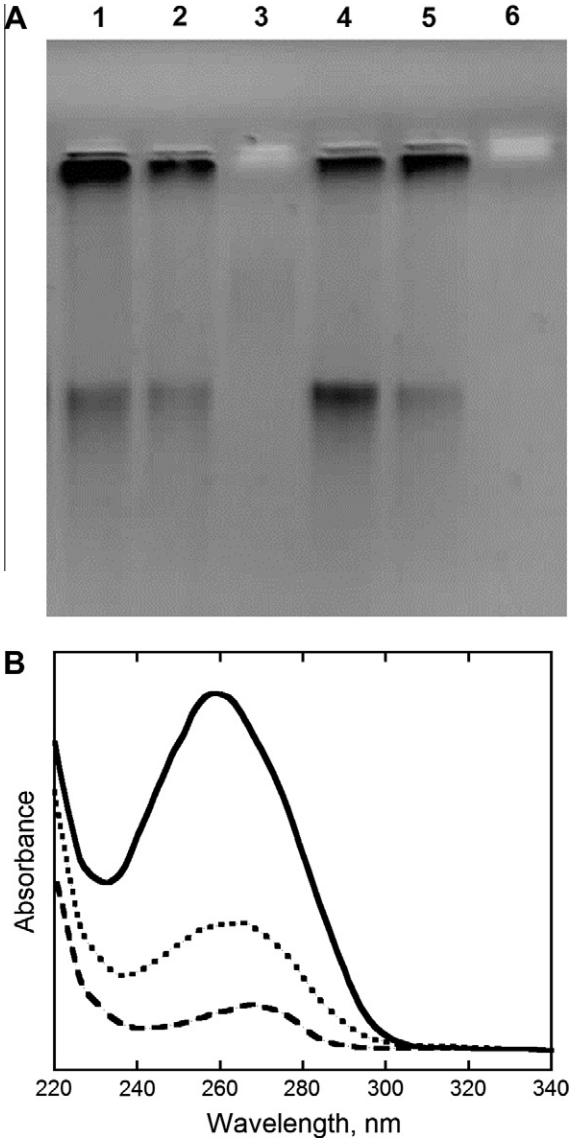

Figure 7. Agarose gel electrophoresis of reactions with calf thymus DNA and PCI extraction. For gel electrophoresis studies, all reactions mixtures were incubated for 1 hour at 37 °C in water. The concentrations of desipramine and clomipramine were 0.5 mM, concentrations of HRP and H_2O_2 were 2 and 10 mM, respectively. The concentration of calf thymus DNA was 6 μM per bp in all reaction mixtures. (A) Reactions with desipramine. Lane 1, DNA with clomipramine; lane 2, DNA with clomipramine and H_2O_2; lane 3, DNA with clomipramine and HRP/H_2O_2; lane 4, DNA with desipramine; lane 5, DNA with desipramine and H_2O_2; lane 6, DNA with desipramine and HRP/H_2O_2. (B) PCI extraction of DNA reacted with drugs in the presence of HRP/H_2O_2. Solid spectrum: 1 mM of DNA only; dashed spectrum: [DNA] = 1 mM, [H_2O_2] = 10 mM, [Desipramine] = 2 mM, and [HRP] = 1 μM; dotted spectrum: [DNA] = 1 mM, [H_2O_2] = 10 mM, [Clomipramine] = 2 mM, and [HRP] = 1 μM. The reaction mixtures were incubated at room temperature for 2 h after which the extraction was performed. The spectra were recorded with the NanoDrop 2000C Spectrophotometer.

(electron donating) potential, which makes it highly likely that the drug binds to the enzyme heme pocket with its benzene ring.

We studied the kinetics of the oxidation of desipramine in the presence of HRP and H_2O_2 by monitoring the absorbance at 523 nm. The absorption at λ_{max} was recorded for a period of 1–20 min at various concentrations of desipramine and fixed concentrations of HRP and H_2O_2. The initial slopes were normalized by the extinction coefficient of the radical and the concentration of HRP. The normalized product formation rates plotted against the concentration of the drug (Fig. 6), and the data were fitted with the following equation:[23]

$$\frac{v_0}{[\mathrm{HRP}]_0} = 2 \times \frac{\kappa_{\text{cat-app}} \times \kappa_1 \times [\mathrm{H_2O_2}]}{\kappa_{\text{cat-app}} + \kappa_1 \times [\mathrm{H_2O_2}]} \times \frac{[\mathrm{Drug}]}{\kappa_{\text{M-app}} \times \frac{\kappa_1 \times [\mathrm{H_2O_2}]}{\kappa_{\text{cat-app}} + \kappa_1 \times [\mathrm{H_2O_2}]} + [\mathrm{Drug}]} \quad (3)$$

where v_0 is the initial rate of the formation of the product, and $k_{\text{cat-app}}$ and $K_{\text{M-app}}$ are the apparent catalytic and Michaelis–Menten constants, respectively. These constants represent functions of the kinetics constants indicated in Figure 5.

The k_1 constant (Fig. 5) was taken to be $1.8 \times 10^7\ M^{-1}\ s^{-1}$ as was determined by Wang and co-workers who studied HRP-catalyzed oxidation of di-(*N*-acetyl-L-tyrpsin) and dipeptide Gly–Tyr.[23] The apparent Michaelis constant, $K_{\text{M-app}}$, of desipramine oxidation was found to be 4.4 ± 0.3 mM (Fig. 6A). The apparent catalytic constant for desipramine reaction was determined to be 2.3 ± 0.1 s^{-1} (Fig. 6A). Figure 6B shows the kinetic curves monitored at different concentrations of HRP. The initial slope of the curves proved to be a linear function of HRP concentration (data not shown).

3.5. Studies of DNA damage cased by desipramine and clomipramine in the presence of HRP/H_2O_2

Some TCAs are known to cause DNA degradation when present with one-electron oxidation systems.[24] Potential DNA damage was examined for desipramine and clomipramine using calf thymus DNA and agarose gel electrophoresis. Reaction mixtures containing a drug, HRP and H_2O_2 were incubated at 37 °C for 1 h in a water bath and gel-electrophoresed along with the control reactions (Fig. 7). While DNA bands appeared in all control bands (lanes 1, 2, 4 and 5), no DNA was visible in the gel when incubated with one of the drugs in the presence of HRP/H_2O_2 (lanes 3 and 6), suggesting that reactive intermediates of both desipramine and clomipramine degrade DNA. The DNA degradation was confirmed by PCI extractions followed by UV/vis spectra (Fig. 7B). The solid line represents DNA extracted from a control sample with no other components added to the solution. The other two spectra were measured from DNA extracted from the reaction mixture containing HRP/H_2O_2 and desipramine (dashed line) or clomipramine (dotted line). The absorbance at 260 nm was essentially lower than that in a control sample (solid line). PCI extractions were performed as described earlier.[24] DNA damage studies have been performed earlier with both drugs. Thus DNA fragmentation has been observed with C6 glioma cells[33] and human colon cancer cells.[34] Desipramine was also shown to induce chromosomal aberrations in mouse bone marrow cells.[35] Clomipramine was shown to be genotoxic to wing cells of *Drosophila melanogaster*.[36] DNA fragmentation has been observed under clomipramine treatment of human peripheral lymphocytes[37] and human leukemic cells.[38]

4. Conclusion

In this study, we have demonstrated that both desipramine and clomipramine undergo one-electron oxidation in the presence of HRP and H_2O_2, followed by the formation of reactive species. The kinetics of the oxidation of desipramine was studied in detail, and the apparent Michaelis and catalytic constants were found to be 4.4 mM and 2.3 s^{-1}, respectively. We hypothesize that the reactive intermediates of the oxidation of the drugs may play important roles in living species. With identifying the toxicity and dynamics behind the drugs oxidation in addition to previous research that emphasizes the importance of these drugs in affecting mitochondria of cancer cells, this mechanistic study can assist in the design of molecules that would enable control over specific activities in living systems.

Acknowledgments

This work was sponsored by the PRISM program at John Jay College and the John Jay College Research Assistance Grant. The authors cordially thank Professor Nathan Lents for technical help and Zully Santiago for help with the editing of the manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.10.075.

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