Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 1903–1913
Bioorganic & Medicinal Chemistry

Free-radical-scavenging effect of carbazole derivatives on AAPH-induced hemolysis of human erythrocytes

You-Zhi Tang and Zai-Qun Liu*

Department of Organic Chemistry, College of Chemistry, Jilin University, Changchun 130021, China

Received 23 November 2006; revised 4 January 2007; accepted 5 January 2007
Available online 10 January 2007

Abstract—Since the research on antioxidants provides theoretical information for the medicinal development, and supplies some in vitro methods for quick-optimizing drugs, it attracts more scientific attention from bioorganic and medicinal chemists. In addition to the traditional O–H bond-type antioxidant, carbazole and its related tricyclic amines (Ar_2NHs), in which N–H bond functioned as the antioxidant, have attracted much research attention because Ar_2NHs have always been the central structure in many currently used drugs. Thus, the investigation on the structure–activity relationship (SAR) between Ar_2NHs and their free-radical-scavenging capacities in detail will benefit the development of novel radical-scavenging drugs containing Ar_2NHs as the central structure. Therefore, carbazole (CazNH) and its structural analogues including phenoxazine (PozNH), phenothiazine (PtzNH), iminostilbene (IsbNH) together with diphenylamine (DpaNH) were applied to protect human erythrocytes against 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH)-induced hemolysis in vitro. By introducing the *chemical kinetic formula* related to free radical reaction, namely, the quantitative relationship between inhibition period (t_{inh}) and the concentration of antioxidant (AH), $t_{inh} = (n/R_i)[AH]$, into AAPH-induced hemolysis, the values of stoichiometric factor (n) of Ar_2NHs indicated that the free-radical-scavenging sequence of Ar_2NHs is PozNH > DpaNH > CazNH > IsbNH > PtzNH > α-tocopherol (TocH). Another aim of this work was to investigate the antioxidative effect of Ar_2NHs used together with other antioxidants including Trolox (TroH), VC, L-ascorbyl-6-laurate (VC-12), and TocH. The obtained data revealed that n value of PozNH when used together with all the other antioxidants decreases, whereas, n values of CazNH, DpaNH, IsbNH, and PtzNH when used in combination with TroH increase, demonstrating that two different interaction styles existed in the case of Ar_2NHs used with other antioxidants. These findings may be useful for the development of agents for various ROS-mediated diseases in vivo.
© 2007 Elsevier Ltd. All rights reserved.

1. Introduction

The hydroxyl groups attaching to aromatic ring generate a series of compounds that can scavenge radicals by trapping initiating and/or propagating radicals, thus, called 'antioxidant',[1] which attracts more scientific attention from bioorganic and medicinal chemists because the research in this field provides theoretical information for the medicinal development, and supplies some in vitro methods for quick-optimizing drugs.

In addition to the traditional O–H bond type antioxidant, the antioxidative properties of the compounds containing N–H bond attract much research attention. In particular, some tricyclic amines (Ar_2NHs), that is, carbazole and its structural analogues, have been the central structure in some neuroleptic and antihistaminic drugs, such as promazine, chlorpromazine, prometazine, carvedilol.[2–5] Nowadays, the free-radical-scavenging mechanism of Ar_2NHs has been discussed from the view of chemical kinetics.[6] These works motivate us to investigate whether the antioxidative effect of Ar_2NHs can also be available for free-radical-induced peroxidation in an in vitro biological system: 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH, R–N=N–R, R = $-C(CH_3)_2C(NH_2)=NH$) initiated hemolysis of human erythrocytes,[7–9] in which the free radical generation rate (R_g) can be controlled by the concentration of AAPH, $R_g = 1.3 \times 10^{-6}[AAPH]s^{-1}$.[10] Furthermore, the *chemical kinetic formula* related to the AAPH-induced peroxidation of linoleic acid[11] is introduced into this biological

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane hydrochloride); Ar_2NH, carbazole derivatives; CazNH, carbazole; IsbNH, iminostilbene; DpaNH, diphenylamine; PtzNH, phenothiazine; PozNH, phenoxazine; TroH, Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid); TocH, α-tocopherol; VC, L-ascorbic acid; VC-12, L-ascorbyl-6-laurate.

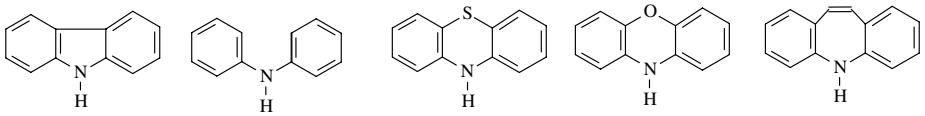
Keywords: Antioxidant; Free radical; Erythrocyte; Hemolysis; Tricyclic amines.

* Corresponding author. Tel.: +86 431 88499174; fax: +86 431 88499159; e-mail: zaiqun-liu@jlu.edu.cn

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.01.007

experimental system to explore more chemical kinetic information of the biological system.

In addition to carbazole (CazNH) as the aromatic Ar_2NH[12] and diphenylamine (DpaNH) with two *free* benzene rings as a nonaromatic amine,[13] iminostilbene (IsbNH),[6] phenothiazine (PtzNH),[14] and phenoxazine (PozNH)[15] can be regarded as the two *free* benzene rings in DpaNH connected by C=C, S, and O, respectively, to form a nonaromatic Ar_2NH.



So, presented here is the study on the antioxidative effect of the aforementioned Ar_2NHs on human erythrocytes against AAPH-induced hemolysis, in which Ar_2NHs are used individually or in combination with some traditional antioxidants including L-ascorbic acid (VC), Trolox (TroH), α-tocopherol (TocH), and L-ascorbyl-6-laurate (VC-12).

2. Results and discussion

2.1. The stoichiometric factors of Ar_2NHs

Figure 1 outlines the hemolysis process in the presence of CazNH (a), IsbNH (b), DpaNH (c), PtzNH (d), and PozNH (e) with various concentrations. All these curves were expressed by the Boltzmann equation in order to obtain the 50% hemolysis time (t_{lag}). The hemolysis is still lagged in the absence of Ar_2NH because the endogenous antioxidants in the membrane protect erythrocytes against AAPH-induced hemolysis.[7,8] In order to eliminate the influence of erythrocytes from different donors, inhibition period ($t_{inh} = t_{lag} - t_{lag0}$, t_{lag0} is the lag time in the absence of Ar_2NHs) has been designated to reveal the function from Ar_2NH, and is listed in Table 1. Then, the quantitative relationships of $t_{inh} \sim [Ar_2NH]$ are found and collected in Table 2.

Taking the n of TocH as 2,[10,16] our previous work revealed that $R_i = 0.641$ μM/min, and $R_g = 2.340$ μM/min in the case of 30 mM AAPH, thus, the$\varepsilon = 27.4\%$.[17] On the basis of the known R_i, the n of Ar_2NH can be calculated following (the slope value in $t_{inh} \sim [Ar_2NH]) \times 0.641$, and is collected in Table 2 as well. Table 2 indicates that antioxidant capacities of Ar_2NHs are higher than the traditional antioxidants, and the sequence is PozNH > DpaNH > CazNH > IsbNH > PtzNH. The n_{PozNH} is as high as 11.9, and even the lowest one, $n_{PtzNH} = 2.58$, is higher than that of PozNH and PtzNH in chemical experimental system, 5.0 and 1.8, respectively.[6] Although at present we have no reasonable interpretation of these absolute values, this sequence is in agreement with that in chemical experimental system.[6] The kinetic data, that is, k_H and k_{inh}, of PozNH and PtzNH in chemical reaction system[6] may be useful to understand our results.

$$Ar_2NH + R^{\cdot} \xrightarrow{k_H} Ar_2N^{\cdot} + RH \quad (1)$$

$$Ar_2NH + LOO^{\cdot} \xrightarrow{k_{inh}} Ar_2N^{\cdot} + LOOH \quad (2)$$

Either k_H or k_{inh} of PozNH (4.8×10^5 M^{-1} s^{-1}, 2.9×10^7 M^{-1} s^{-1}) is one magnitude higher than those of PtzNH (6.0×10^4 M^{-1} s^{-1}, 8.8×10^6 M^{-1} s^{-1}), demonstrating that the ability of PozNH either to trap initiating radical ($R^{\cdot}$) or to inhibit propagating radical ($LOO^{\cdot}$) is stronger than that of PtzNH.[6] Thus, it is reasonable to understand that antioxidant activity of PozNH in AAPH-induced hemolysis is higher than PtzNH. Moreover, the N atoms locating in middle ring in PozNH, IsbNH, and PtzNH are the *secondary* amine, leading to the observation that middle ring is not planar. Once they form radicals, the whole molecule will locate

Table 1. Inhibition periods of Ar_2NHs used individually in the AAPH-induced hemolysis of human erythrocytes

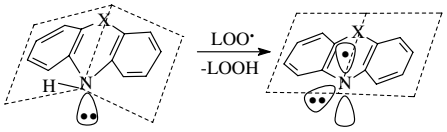
Antioxidant	Concentration (μM)	t_{lag} (min)	t_{inh} (min)
CazNH	0	197	0
	6.17	265	68
	9.26	298	101
	12.3	327	130
	15.4	364	167
	20.6	406	209
DpaNH	0	233	0
	3.00	257	24
	6.00	346	113
	9.00	400	167
	12.0	458	225
	15.0	489	256
PozNH	0	187	0
	9.27	345	158
	10.8	379	192
	12.4	425	238
	13.9	461	274
	15.5	509	322
IsbNH	0	212	0
	8.26	264	52
	12.4	297	85
	16.5	333	121
	20.7	365	153
	24.8	381	169
PtzNH	0	211	0
	20.3	275	64
	30.5	330	119
	37.3	368	157
	50.8	408	197
	61.0	451	240

Table 2. The quantitative relationships between t_{inh} and concentrations of Ar_2NHs

Antioxidant	$t_{inh} = (n/R_i)$[concentration (μM)] + B[a]	n[b]	Correlation coefficient
PtzNH	t_{inh} = 4.02[PtzNH] − 4.27	2.58	0.9934
IsbNH	t_{inh} = 7.14[IsbNH] − 1.69	4.58	0.9963
CazNH	t_{inh} = 10.2[CazNH] + 3.72	6.56	0.9985
DpaNH	t_{inh} = 18.5[DpaNH] − 7.83	11.9	0.9901
PozNH	t_{inh} = 20.3[PozNH] − 11.9	13.0	0.9911

[a] The slope in the equation means n/R_i. When TocH is assigned to be the reference antioxidant and its n is taken as 2, resulting in R_i = 0.641 μM/min,[17] the n of other antioxidants can be calculated by the slope × R_i.
[b] n_{TocH} = 2.00, n_{TroH} = 0.30, n_{VC} = 0.25, $n_{VC\text{-}12}$ = 1.11.[17]

at the same plane as Eq. 3 shows. Thus, the p orbital of N will conjugate with the whole molecule, and its lack of electron will be supplemented by the conjugation system.



(3)

As for DpaNH and CazNH, because of the lack of the kinetic data (k_H and k_{inh}), it is difficult to discuss the SAR precisely, so this will be the topic of quantum calculation in the future work.

2.2. The stoichiometric factors of Ar_2NHs used together with other antioxidants

Figures 2–5 outline the hemolysis curves of Ar_2NH with various concentrations mixed with different concentrations of TroH, TocH, VC, and VC-12 in protecting erythrocytes against AAPH-induced hemolysis, and t_{inh} in the presence of various concentrations of Ar_2NHs and other antioxidants are collected in Table 3.

Given Ar_2NH and other antioxidants protect erythrocytes simultaneously, t_{inh} should be affected by the concentrations of Ar_2NHs and other antioxidants. Consequently, the relationship between t_{inh} and concentrations of Ar_2NHs and other antioxidants should be expressed by multiple linear regressive Eq. 4,[18]

$$t_{inh} = A[Ar_2NH] + B[\text{other antioxidants}] + C \quad (4)$$

and is listed in Table 4. On the basis of R_i= 0.641 μM/min, the stoichiometric factors of Ar_2NHs and other antioxidants in this case, n_{Ar2NH} and n_{other}, are calculated by the corresponding coefficient × R_i, and listed in Table 4, in which the data in parentheses are the n when the compound is used individually (see Table 2).

As it can be found that n_{Ar2NH} and n_{other} vary remarkably though we have no reasonable explanation for so large values, that is,n_{DpaNH} = 68.3 and n_{PozNH} = −59.3 in the case of mixed usage with TroH, Table 5 indicates the increase and decrease of stoichiometric factors of Ar_2NHs used with other antioxidants.

As can be seen in Tables 4 and 5, n_{PozNH} decreases to negative value in the mixed usage with other antioxidants, revealing that the increase of the concentration of PozNH cannot contribute positively

Table 3. Inhibition periods of Ar_2NHs used in combination with TroH, TocH, VC, and VC-12 in the AAPH-induced hemolysis of human erythrocytes

Concentration of Ar_2NH (μM)	Concentration of other antioxidants (μM)	t_{lag} (min)	t_{inh} (min)
CazNH	TroH		
0	0	162	0
1.41	20.0	203	41
2.81	30.0	244	82
4.22	40.0	277	115
5.62	50.0	319	157
7.03	70.0	350	188
DpaNH	TroH		
0	0	156	0
3.00	40.0	248	92
4.00	50.0	305	149
5.00	60.0	357	201
6.00	70.0	413	257
7.00	80.0	448	292
PozNH	TroH		
0	0	168	0
5.8	40.0	238	70
6.76	50.0	298	130
7.73	60.0	369	201
8.69	70.0	425	257
9.66	80.0	489	321
CazNH	TocH		
0	0	222	0
1.4	1.93	254	32
2.8	2.89	294	72
4.2	3.86	327	105
5.6	4.82	361	139
7.0	5.79	389	167
DpaNH	TocH		
0	0	214	0
3.00	2.01	261	47
4.00	3.01	281	67
5.00	4.02	304	90
6.00	5.02	346	132
7.00	6.03	373	159
PozNH	TocH		
0	0	218	0
5.57	1.93	269	51
6.50	2.89	313	95
7.42	3.86	352	134
8.35	4.82	369	151
9.28	5.79	410	192

(*continued on next page*)

Table 3 (*continued*)

Concentration of Ar_2NH (μM)	Concentration of other antioxidants (μM)	t_{lag} (min)	t_{inh} (min)
CazNH	VC		
0	0	170	0
1.41	20.0	202	32
2.81	30.0	239	69
4.22	40.0	260	90
5.62	50.0	297	127
7.03	70.0	315	145
DpaNH	VC		
0	0	165	0
5.00	40.0	282	117
6.00	50.0	331	166
7.00	60.0	360	195
8.00	70.0	398	233
9.00	80.0	425	260
PozNH	VC		
0	0	167	0
5.89	40.0	217	50
6.88	50.0	251	84
7.86	60.0	282	115
8.84	70.0	317	150
9.82	80.0	339	172
CazNH	VC-12		
0	0	195	0
3.30	4.00	252	57
4.95	5.00	273	78
6.60	6.00	291	96
8.25	7.00	309	114
9.90	8.00	323	128
DpaNH	VC-12		
0	0	208	0
3.00	4.00	249	41
4.00	5.00	277	69
5.00	6.00	296	88
6.00	7.00	330	122
7.00	8.00	359	151
PozNH	VC-12		
0	0	195	0
6.21	4.00	238	43
7.24	5.00	311	116
8.27	6.00	348	153
9.31	7.00	403	208
10.3	8.00	439	244
IsbNH	TroH		
0	0	199	0
2.00	20.0	235	36
4.00	30.0	276	77
6.00	40.0	371	172
8.00	50.0	419	220
10.0	70.0	469	270
PtzNH	TroH		
0	0	162	0
8.94	40.0	244	82
11.9	50.0	281	119
14.9	60.0	314	152
17.9	70.0	353	191
20.9	80.0	379	217

Table 3 (*continued*)

Concentration of Ar_2NH (μM)	Concentration of other antioxidants (μM)	t_{lag} (min)	t_{inh} (min)
IsbNH	TocH		
0	0	189	0
4.38	4.9	256	67
6.57	6.54	287	98
8.76	8.17	349	160
10.9	9.81	369	180
13.1	13.1	401	212
PtzNH	TocH		
0	0	200	0
8.46	3.27	235	35
11.3	4.90	270	70
14.1	6.54	298	98
16.9	8.17	320	120
19.7	9.81	345	145
IsbNH	VC		
0	0	206	0
2.00	20.0	240	34
4.00	30.0	276	70
6.00	40.0	333	127
8.00	50.0	394	188
10.0	60.0	428	222
PtzNH	VC		
0	0	229	0
8.94	30.0	241	12
11.9	40.0	282	53
14.9	50.0	318	89
17.9	70.0	360	131
20.9	80.0	403	174
IsbNH	VC-12		
0	0	193	0
5.18	4.00	238	45
7.78	5.00	266	73
10.4	6.00	305	112
13.0	7.00	330	137
15.6	8.00	353	160
PtzNH	VC-12		
0	0	185	0
7.68	4.00	194	9
11.5	5.00	221	36
15.4	6.00	245	60
19.2	7.00	262	77
23.0	8.00	276	91

to the t_{inh}, whereas this negative effectiveness is rectified by the other antioxidants since n_{other} increases significantly in this case. It can be understood from Scheme 1 that TocH traps $LOO^{\bullet}$ directly to form $Toc^{\bullet}$, then $Toc^{\bullet}$ is repaired by PozNH. So, PozNH prolongs the life span of TocH as that $Toc^{\bullet}$ can be recycled by intracellular VC.[19]

Contrarily, n_{Ar2NH} of CazNH, IsbNH, DpaNH, and PtzNH increase, while n_{other} of TroH decreases in the case of their mixed usage, implying that the mechanism is opposite to that in Scheme 1 completely. So, Scheme 2 illustrates that Ar_2NHs play a major role in trapping

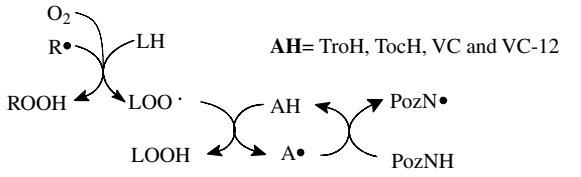
Table 4. The influence of the concentrations of Ar_2NHs and other antioxidants on t_{inh}

Antioxidant	$t_{inh} = A[Ar_2NH] + B[\text{other antioxidant}] + C$[a]	n_{Ar2NH}[b]	n_{other}[b]
CazNH + TroH	$t_{inh} = 28.6[CazNH] - 0.18[TroH] + 3.07$	18.3 (6.56)	−0.12 (0.30)
IsbNH+TroH	$t_{inh} = 45.4[IsbNH] - 2.62[TroH] - 6.05$	29.1 (4.58)	−1.68 (0.30)
DpaNH + TroH	$t_{inh} = 106.6[DpaNH] - 5.58[TroH]$	68.3 (11.9)	−3.58 (0.30)
PtzNH + TroH	$t_{inh} = 17.6[PtzNH] - 1.83[TroH]$	11.3 (2.58)	−1.17 (0.30)
PozNH + TroH	$t_{inh} = -92.5[PozNH] + 15.2[TroH] - 0.04$	−59.3 (13.0)	9.74 (0.30)
CazNH+TocH	$t_{inh} = 22.4[CazNH] + 2.25[TocH] + 0.03$	14.4 (6.56)	1.44 (2.00)
IsbNH + TocH	$t_{inh} = 26.2[IsbNH] - 10.1[TocH] + 0.21$	16.8 (4.58)	−6.50 (2.00)
DpaNH + TocH	$t_{inh} = -16.6[DpaNH] + 45.3[TocH]$	−10.7 (11.9)	29.0 (2.00)
PtzNH + TocH	$t_{inh} = -5.07[PtzNH] + 25.3[TocH]$	−3.25 (2.58)	16.2 (2.00)
PozNH + TocH	$t_{inh} = -2.76[PozNH] + 37.6[TocH]$	−1.77 (13.0)	24.1 (2.00)
CazNH + VC	$t_{inh} = 24.9[CazNH] - 0.44[VC] + 4.77$	16.0 (6.56)	−0.28 (0.25)
IsbNH + VC	$t_{inh} = 34.7[IsbNH] - 2.01[VC]$	22.3 (4.58)	−1.33 (0.25)
DpaNH + VC	$t_{inh} = -17.9[DpaNH] + 5.32[VC]$	−11.5 (11.9)	3.41 (0.25)
PtzNH + VC	$t_{inh} = -12.2[PtzNH] + 5.73[VC] - 13.7$	−7.80 (2.58)	3.67 (0.25)
PozNH + VC	$t_{inh} = -36.8[PozNH] + 6.72[VC] - 0.01$	−23.6 (13.0)	4.31 (0.25)
CazNH + VC-12	$t_{inh} = 3.21[CazNH] + 12.3[VC\text{-}12]$	2.06 (6.56)	7.90 (1.11)
IsbNH + VC-12	$t_{inh} = 0.76[IsbNH] + 18.0[VC\text{-}12] - 7.44$	0.19 (4.58)	11.6 (1.11)
DpaNH + VC-12	$t_{inh} = 69.2[DpaNH] - 41.9[VC\text{-}12]$	44.3 (11.9)	−26.9 (1.11)
PtzNH + VC-12	$t_{inh} = 9.02[PtzNH] - 14.0[VC\text{-}12]$	5.78 (2.58)	−8.94 (1.11)
PozNH + VC-12	$t_{inh} = -69.6[PozNH] + 121.4[VC\text{-}12]$	−44.6 (13.0)	77.8 (1.11)

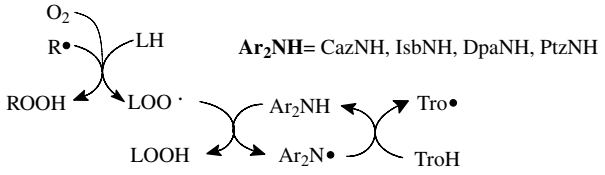
[a] $R_i = 0.641$ μM/min.[17]
[b] Data in parentheses are the n when the antioxidant is used individually, see Table 2.

Table 5. The increase (↑) and decrease (↓) of stoichiometric factors of Ar_2NHs in the case of mixed usage with other antioxidants

	CazNH	IsbNH	DpaNH	PtzNH	PozNH
TroH	↑	↑	↑	↑	↓
TocH	↑	↑	↓	↓	↓
VC	↑	↑	↓	↓	↓
VC-12	↓	↓	↑	↑	↓



Scheme 1. The interaction between PozNH and other antioxidants.



Scheme 2. The interaction between TroH and Ar_2NH.

$LOO^{\cdot}$, then $Ar_2N^{\cdot}$ is recycled by TroH, and the life span of Ar_2NH is prolonged by other antioxidants.

3. Conclusion

In conclusion, the present findings reveal that Ar_2NHs function as efficient antioxidants in protecting human erythrocytes against AAPH-induced hemolysis, in which the activity sequence is PozNH > DpaNH > CazNH > IsbNH > PtzNH. Moreover, they exhibit higher activities when it was used together with TocH, TroH, VC, and VC-12 in this experimental system. But the values of stoichiometric factors of either Ar_2NHs or other antioxidants vary remarkably compared with those of individual usage, demonstrating that a strong interaction between Ar_2NHs and other antioxidants exists in their combinative usage. These findings may be useful for the development of agents for various ROS-mediated diseases in vivo.

4. Materials and methods

4.1. Materials

Human erythrocytes collected from healthy volunteer donors were provided by Red Cross Center for Blood, Changchun, China. After washing three times with phosphate-buffered saline (PBS: 150 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, and 50 μM EDTA, pH 7.4) to remove the residual plasma, the erythrocytes were centrifuged at 1700g for exactly 10 min to obtain compact erythrocytes for experimental use.[20] 2,2′-Azobis(2-amidinopropane dihydrochloride) (AAPH), Trolox, and α-tocopherol were purchased from Aldrich, and carbazole, iminostilbene, diphenylamine, phenothiazine, and phenoxazine were from ACROS. VC was from Shenyang Chemical Ltd Co. China, and VC-12 was synthesized following the literature.[21]

Water-soluble compounds, that is, AAPH, VC, and Trolox, were dissolved in PBS directly. Liposoluble compounds, that is, α-tocopherol, VC-12, and Ar_2NHs, were dissolved in dimethylsulfoxide (DMSO) as the stock solution. It was worthy to point out that the same

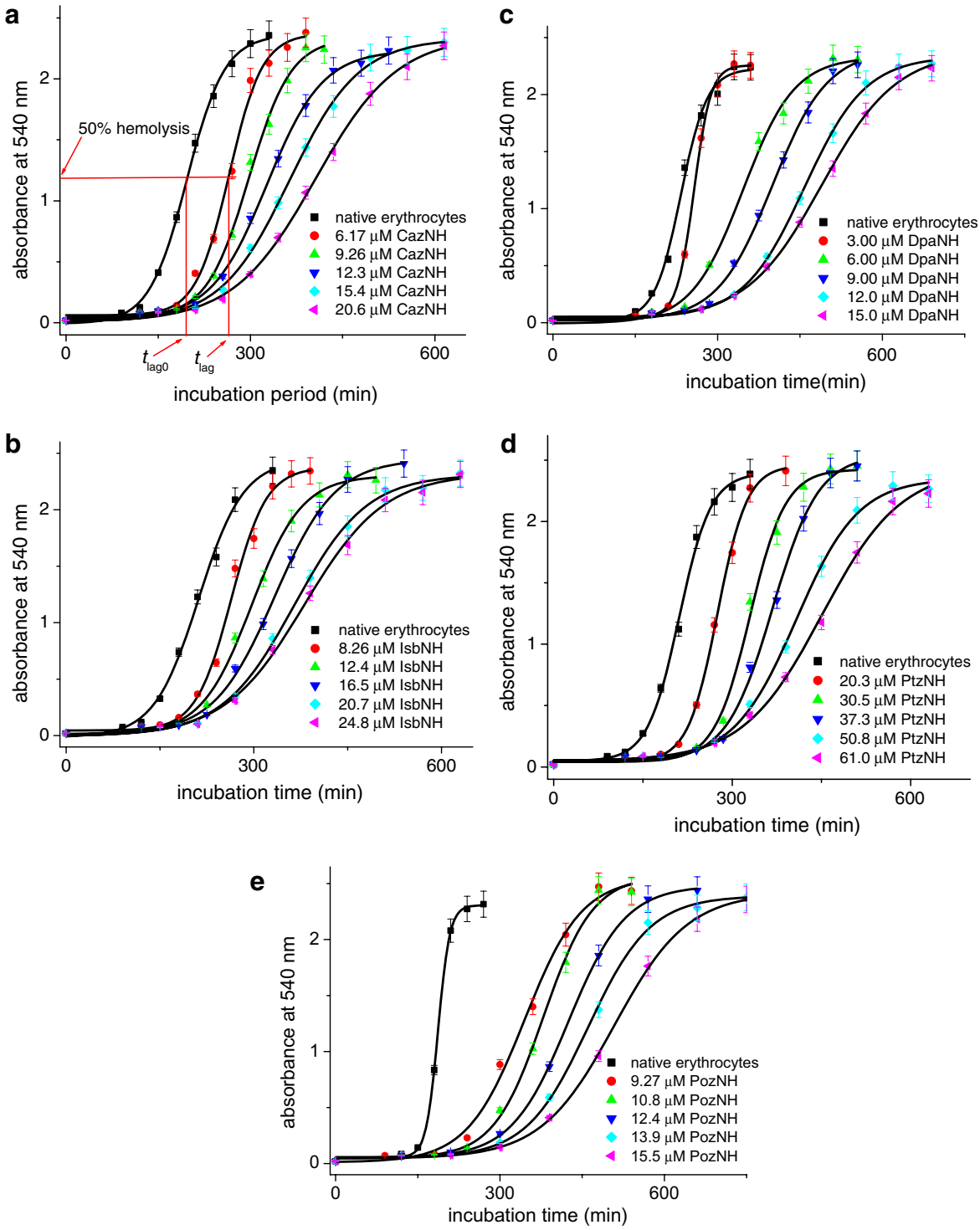


Figure 1. Hemolysis curves of human erythrocytes (3.0% suspension in PBS, pH 7.4) initiated by AAPH (30 mM) at 37 °C in the presence of carbazole (a), iminostilbene (b), diphenylamine (c), phenothiazine (d), and phenoxazine (e) with various concentrations as the inset shows.

amount of DMSO (less than 1.0% to the total volume of hemolysis mixture) was contained in all the experiments in order to avoid its influence on the hemolysis.[22]

4.2. Expression of hemolysis process by Boltzmann equation

The hemolysis experiment followed the description given in the literatures.[7–9,22,23] In brief, antioxidant stock solution and AAPH solution (30 mM as the final concentration) were added successively to the 3.0% erythrocyte suspensions in PBS (v/v). Then, the above mixture was put into a 37 °C thermostatic bath to initiate the hemolysis. Aliquots were taken out from the above mixture at appropriate intervals and centrifuged at 1700*g* for 5 min to obtain the supernatant. Then the absorbance of the supernatant was determined at 540 nm.[19] Therefore, the hemolysis process can be

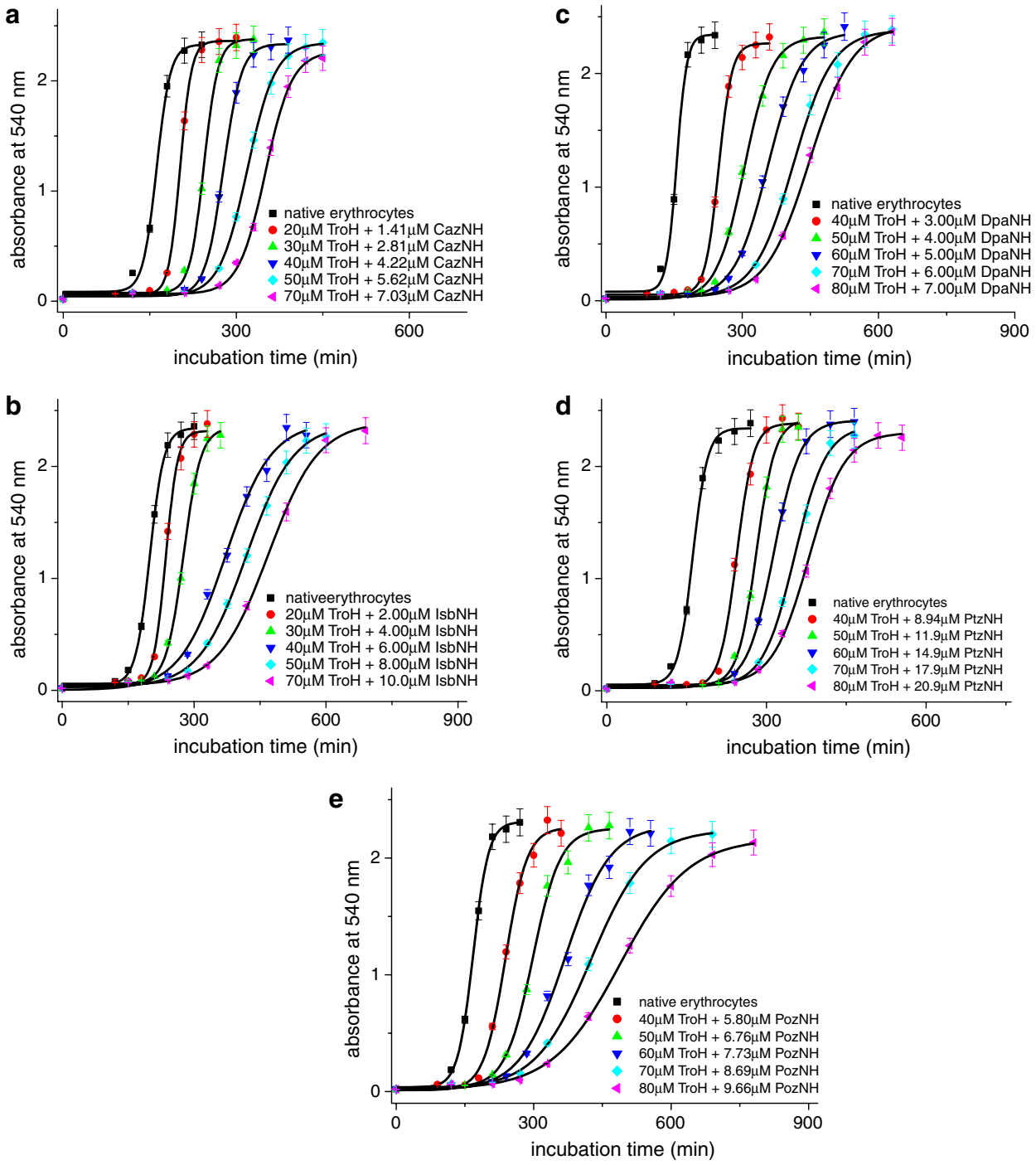


Figure 2. Hemolysis curves of human erythrocytes (3.0% suspension in PBS, pH 7.4) initiated by AAPH (30 mM) at 37 °C in the presence of TroH + CazNH (a), TroH + IsbNH (b), TroH + DpaNH (c), TroH + PtzNH (d), and TroH + PozNH (e) with various concentrations as the inset shows.

illustrated by the relationship between the absorbance at 540 nm (A) and the incubation time (t) as shown in Figures 1–5 (vide post), in which all the A values were the average ones from three independent measurements within 10% experimental error.

The hemolysis curves in Figures 1–5 can be expressed by Boltzmann equation,[22]

$$A = (A_{\text{initial}} - A_{\text{final}})/(1 + e^{(t-t_{\text{lag}})/dt}) + A_{\text{final}} \quad (5)$$

where A_{initial} and A_{final} refer to the absorbance at the beginning and end of the hemolysis, t_{lag} stands for the time when erythrocytes reach the 50% hemolysis. So the t_{lag} involves the period when hemolysis does not take place, and the influence of the antioxidant on the hemolysis rate.

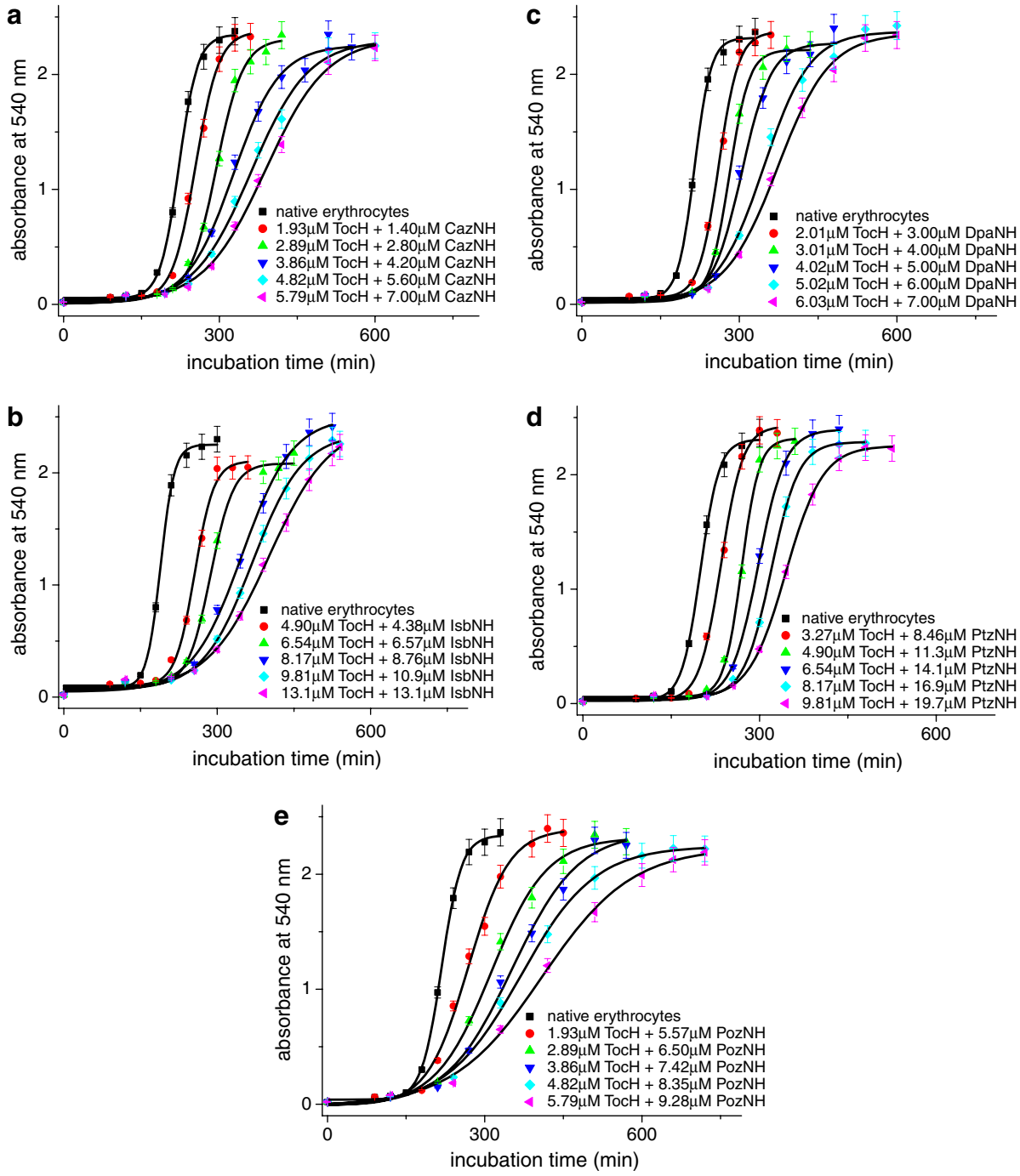


Figure 3. Hemolysis curves of human erythrocytes (3.0% suspension in PBS, pH 7.4) initiated by AAPH (30 mM) at 37 °C in the presence of TocH + CazNH (a), TocH + IsbNH (b), TocH + DpaNH (c), TocH + PtzNH (d), and TocH + PozNH (e) with various concentrations as the inset shows.

4.3. The calculation of the stoichiometric factor (*n*) based on chemical kinetic deduction

The kinetic process of free-radical-induced peroxidation of linoleic acid (LH) in mimic biomembrane, as the following scheme shows,[24,25] has been applied for AAPH-induced peroxidation of human low-density lipoprotein (LDL)[26–28] and erythrocytes successfully.[29,17,18]

$$\mathrm{R{-}N{=}N{-}R} \xrightarrow{R_g} 2\varepsilon \mathrm{R}^{\cdot} + \mathrm{N_2} + (1-\varepsilon)\mathrm{R-R} \qquad (6)$$

$$\mathrm{R}^{\cdot} + \mathrm{O_2} \rightarrow \mathrm{ROO}^{\cdot} \qquad (7)$$

$$\mathrm{ROO}^{\cdot} + \mathrm{LH} \xrightarrow{R_i} \mathrm{ROOH} + \mathrm{L}^{\cdot} \qquad (8)$$

where ε is designated to phase-transfer efficiency to reveal the efficiency of ROO$^{\cdot}$ to transfer into the membrane.[10] Then ROO$^{\cdot}$ attacks LH in membrane (Eq. 8) by a real initiating rate, R_i. Thus, $\varepsilon = R_i/R_g$ implies that not all the ROO$^{\cdot}$ derived from the decomposi-

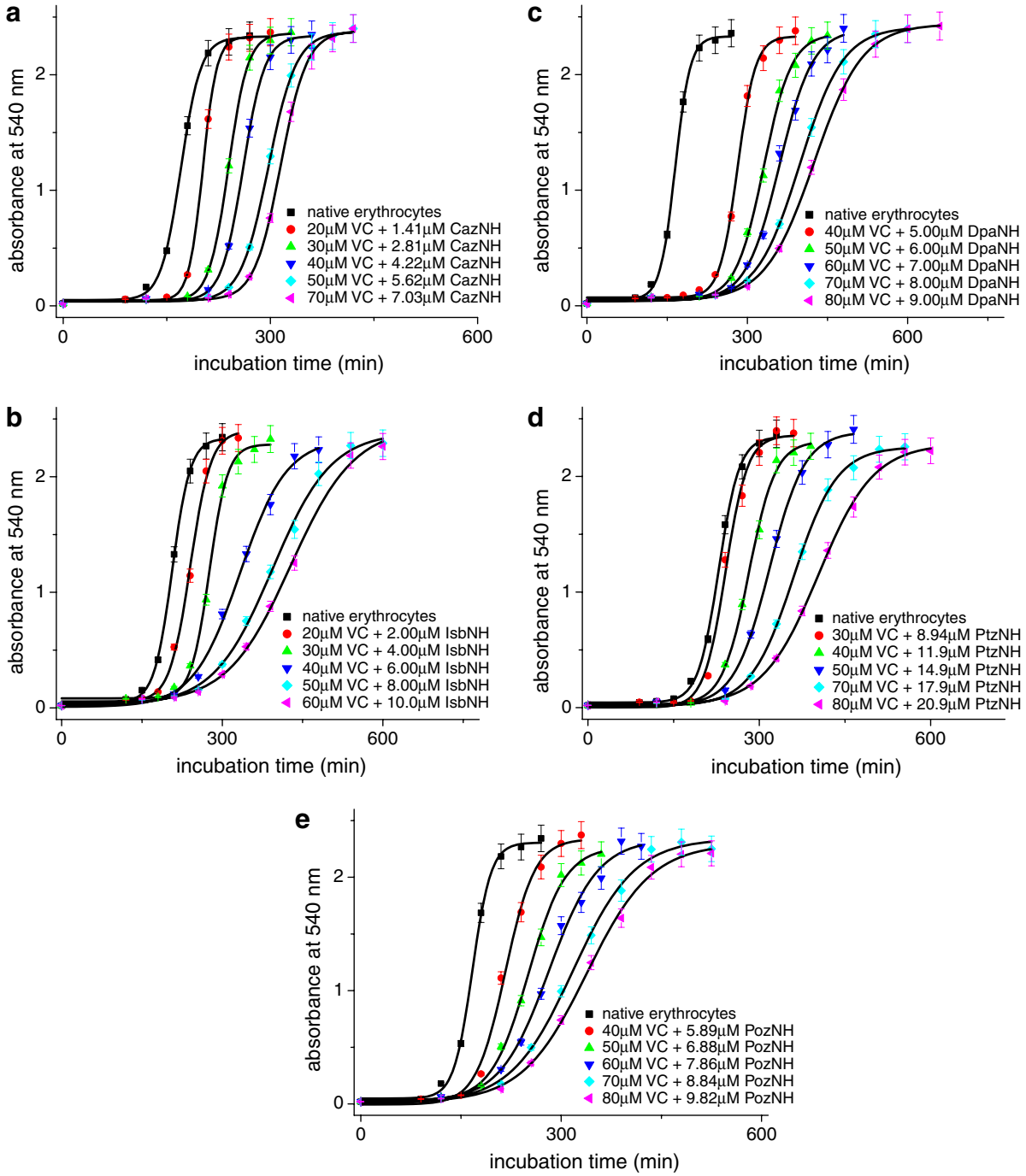


Figure 4. Hemolysis curves of human erythrocytes (3.0% suspension in PBS, pH 7.4) initiated by AAPH (30 mM) at 37 °C in the presence of VC + CazNH (a), VC + IsbNH (b), VC + DpaNH (c), VC + PtzNH (d), and VC + PozNH (e) with various concentrations as the inset shows.

tion of AAPH can initiate the radical-propagation, as Eqs. 9 and 10 show.

$$L^{\cdot} + O_2 \xrightarrow{\text{fast}} LOO^{\cdot} \quad (9)$$

$$LOO^{\cdot} + LH \xrightarrow{k_p} LOOH + L^{\cdot} \quad (10)$$

With an antioxidant (AH) added in the above reaction system, it interacts with $LOO^{\cdot}$ to form an antioxidant radical ($A^{\cdot}$) (Eq. 11), and $A^{\cdot}$ couples with $LOO^{\cdot}$ so rapidly to form a nonradical product (LOOA) (Eq. 12), thus the peroxidation of LH can be inhibited efficiently.

$$LOO^{\cdot} + AH \xrightarrow{k_{inh}} LOOH + A^{\cdot} \quad (11)$$

$$LOO^{\cdot} + A \xrightarrow{\text{fast}} LOOA \quad (12)$$

The treatment of Eqs. 6–12 by the steady-state kinetic deduction yields the correlation of R_i with the concentration of AH and the inhibition period, t_{inh},[11]

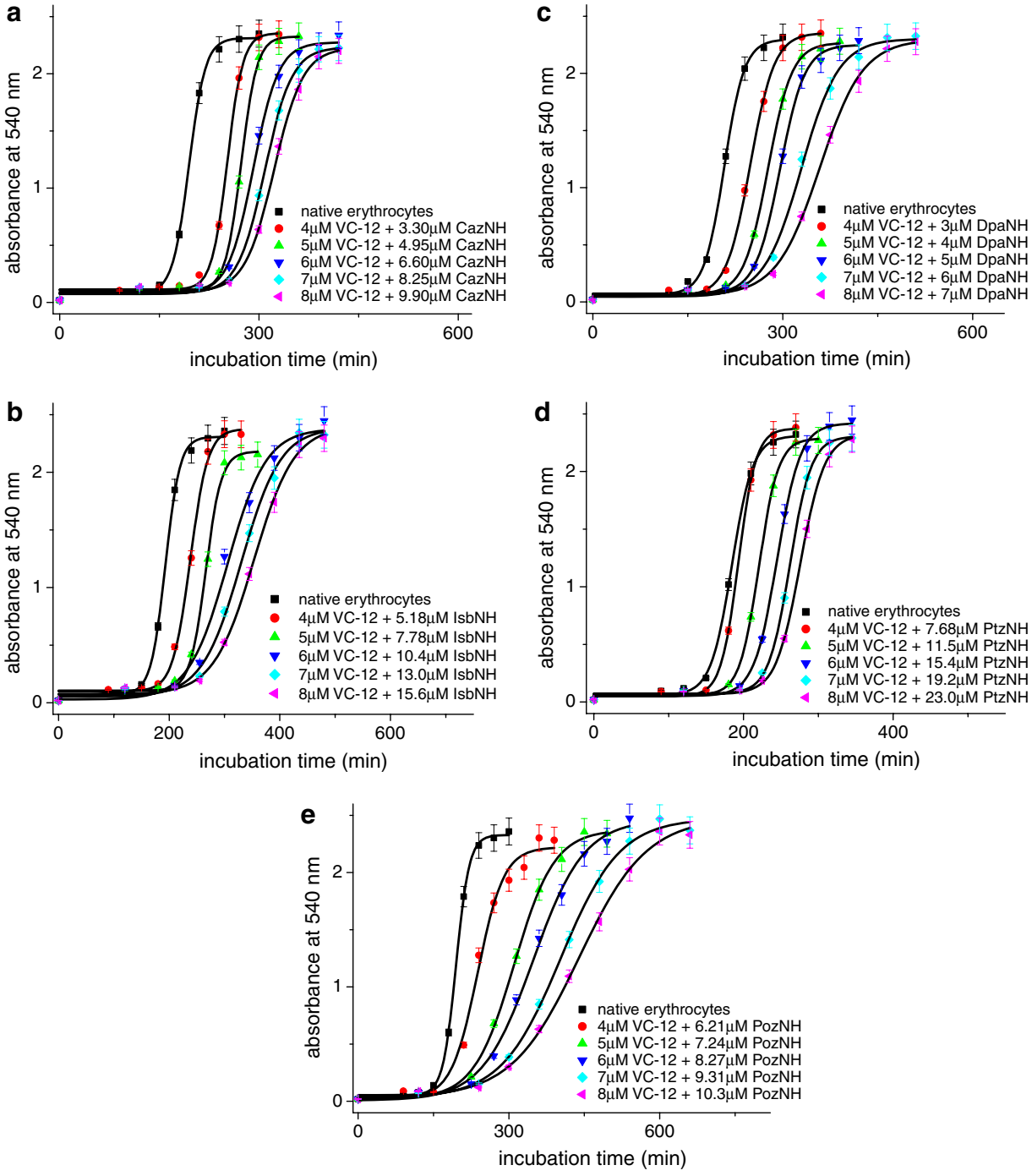


Figure 5. Hemolysis curves of human erythrocytes (3.0% suspension in PBS, pH 7.4) initiated by AAPH (30 mM) at 37 °C in the presence of VC-12 + CazNH (a), VC-12 + IsbNH (b), VC-12 + DpaNH (c), VC-12 + PtzNH (d), and VC-12 + PozNH (e) with various concentrations as the inset shows.

$$R_i = (n[AH])/t_{inh} \quad (13)$$

which can be expressed as the $t_{inh} \sim [AH]$ equation equivalently.

$$t_{inh} = (n/R_i)[AH] \quad (14)$$

The n stands for the stoichiometric factor, meaning the number of $LOO^{\cdot}$ trapped by each AH molecule in a chemical reaction system. Given the R_i is known, the n value of an antioxidant can be obtained by the corresponding relationship of $t_{inh} \sim [AH]$. Nevertheless, it is difficult to determine R_i directly. TocH is generally selected to be the reference antioxidant whose n value is always taken as 2.[10,16] Consequently, R_i can be calculated via the equation of $t_{inh} \sim [TocH]$. Then, n of other antioxidants can be obtained via the corresponding $t_{inh} \sim [AH]$. We have applied Eq. 14 for AAPH-induced hemolysis of human erythrocytes to investigate the

antioxidant activity of hydroxyl Schiff bases.[29,17,18] The n obtained from the complicated erythrocytes system cannot be related to the number of peroxyl radical trapped by an antioxidant as in simple chemical experimental system, hence, the n is a relative value compared with 2 of TocH to exhibit the antioxidant capacity.

4.4. Statistical analysis

All the quantitative relationships involving Boltzmann equations and $t_{inh} \sim [AH]$ equations were performed statistically by one-way ANOVA using Origin 6.0 professional Software, and $P < 0.001$ indicated a significant difference.

Acknowledgment

The authors thank the National Natural Science Foundation, China, for the financial support (20572033).

References and notes

1. Denisov, E.; Khudyakov, I. V. *Chem. Rev.* **1987**, *87*, 1313–1357.
2. GoodmanGilman, A.; Rall, T. W.; Nies, A. S.; Taylor, P. *The Pharmacological Basis of Therapeutics*, 8th ed.; Pergamon press: New York, 1990.
3. Kumar, R.; Ramachandran, U.; Srinivasan, K.; Ramarao, P.; Raichur, S. *Bioorg. Med. Chem.* **2005**, *13*, 4279–4290.
4. Almeida, D. R. P.; Gasparro, D. M.; Pisterzi, L. F.; Torday, L. L.; Varro, A.; Papp, J. G.; Penke, B.; Csizmadia, I. G. *J. Phys. Chem. A* **2003**, *107*, 5594–5610.
5. Almeida, D. R. P.; Gasparro, D. M.; Fulop, F.; Csizmadia, I. G. *J. Phys. Chem. A* **2004**, *108*, 6239–6247.
6. Lucarini, M.; Pedrielli, P.; Pedulli, G. F.; Valgimigli, L.; Gigmes, D.; Tordo, P. *J. Am. Chem. Soc.* **1999**, *121*, 11546–11553.
7. Niki, E.; Komuro, E.; Takahashi, M.; Urano, S.; Ito, E.; Terao, K. *J. Biol. Chem.* **1988**, *263*, 19809–19814.
8. Sato, Y.; Kamo, S.; Takahashi, T.; Suzuki, Y. *Biochemistry* **1995**, *34*, 8940–8949.
9. Zou, C. G.; Agar, N. S.; Jones, G. L. *Life Sci.* **2001**, *69*, 75–86.
10. Bowry, V. W.; Stocker, R. *J. Am. Chem. Soc.* **1993**, *115*, 6029–6044.
11. Fang, J. G.; Lu, M.; Chen, Z. H.; Li, Y.; Zhu, H. H.; Yang, L.; Wu, L. M.; Liu, Z. L. *Chem. Eur. J.* **2002**, *8*, 4191–4198.
12. Tachibana, Y.; Kikuzaki, H.; Lajis, N. H.; Nakatani, N. *J. Agric. Food Chem.* **2003**, *51*, 6461–6467.
13. Rudell, D. R.; Mattheis, J. P.; Fellman, J. K. *J. Agric. Food Chem.* **2005**, *53*, 8382–8389.
14. Eghbal, M. A.; Tafazoli, S.; Pennefather, P.; O'Brien, P. J. *Chem.-Biol. Interact.* **2004**, *151*, 43–51.
15. Moosmann, B.; Skutella, T.; Beyer, K.; Behl, C. *Biol. Chem.* **2001**, *382*, 1601–1612.
16. Antolovich, M.; Prenzler, P. D.; Patsalides, E.; McDonald, S.; Robards, K. *Analyst* **2002**, *127*, 183–198.
17. Tang, Y. Z.; Liu, Z. Q. *J. Pharm. Pharmacol.* **2006**, *58*, 625–631.
18. Tang, Y.Z., Liu, Z.Q. *Cell Biochem. Funct.* **2007** (in press, doi:10.1027/cbf.1287).
19. May, J. M.; Qu, Z. C.; Mendiratta, S. *Arch. Biochem. Biophys.* **1998**, *349*, 281–289.
20. Zavodnik, I. B.; Lapshina, E. A.; Rekawiecka, K.; Zavodnik, I. B.; Bartosz, G.; Bryszewska, M. *Biochim. Biophys. Acta* **1999**, *1421*, 306–316.
21. Cousins, R. C.; Seib, P. A.; Hoseney, R. C.; Deyoe, C. W.; Liang, Y. T.; Lillard, D. W., Jr. *J. Am. Oil Chem. Soc.* **1977**, *54*, 308–312.
22. Liu, Z. Q.; Luo, X. Y.; Liu, G. Z.; Liu, Q. W. *QSAR Comb. Sci.* **2003**, *22*, 859–864.
23. Liu, Z. Q.; Luo, X. Y.; Sun, Y. X.; Wu, W.; Liu, C. M.; Liu, Z. Q.; Liu, S. Y. *J. Pharm. Pharmacol.* **2004**, *56*, 1557–1562.
24. Barclay, L. R. C.; Ingold, K. U. *J. Am. Chem. Soc.* **1980**, *102*, 7792–7794.
25. Barclay, L. R. C. *Can. J. Chem.* **1993**, *71*, 1–16.
26. Liu, Z. Q.; Ma, L. P.; Liu, Z. L. *Chem. Phys. Lipids* **1998**, *95*, 49–57.
27. Liu, Z. Q.; Yu, W.; Liu, Z. L. *Chem. Phys. Lipids* **1999**, *103*, 125–135.
28. Liu, Z. Q.; Ma, L. P.; Zhou, B.; Yang, L.; Liu, Z. L. *Chem. Phys. Lipids* **2000**, *106*, 53–63.
29. Tang, Y.Z., Liu, Z.Q. *Cell Biochem. Funct.* **2007** (in press, doi:10.1027/cbf.1378).