Hemolysis induced by artemisinin and its derivatives *in vitro*

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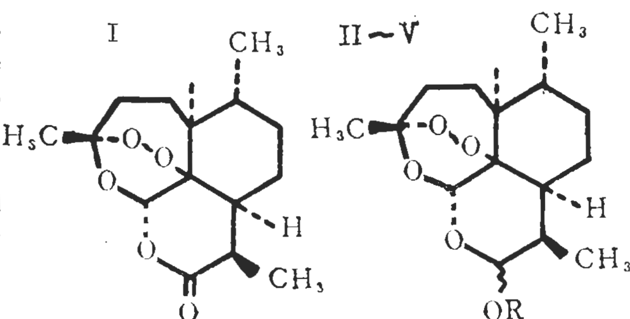
ABSTRACT After exposure of rbc to 1 m*M* artemether, complete lysis of rbc occurred and visible absorption spectrum of the released Hb was unchanged. Similarly, no change was seen of the spectrum of the released Hb from rbc exposed to 0.5 m*M* SM 242. However, slight hemolysis was found in the groups of 1 m*M* QHS, 1 m*M* DHQ, and no significant hemolysis in the group of 1 m*M* CQ. Two shoulder peaks at 502 and 631 nm in the spectrum of the supernatant in 1 m*M* artesunate group replaced the 2 sharp ones at 541 and 573 nm in the spectrum of normal Hb. NaN_3, deferoxamine and superoxide dismutase did not antagonize the hemolytic action by artesunate, artemether or SM 242.

KEY WORDS antimalarials; sesquiterpenes; chloroquine; hemolysis; erythrocytes; hemoglobins; deferoxamine; superoxide dismutase; spectrum analysis; artemisinin

Primaquine causes hemolysis in some patients, particularly in glucose-6-phosphate dehydrogenase deficient ones. The metabolites of primaquine also have a hemolytic action *in vitro*[1]. H_2O_2 was suggested to be a toxic mediator in the toxicity process[2]. Artemisinin-related compounds developed in China are novel antimalarial drugs[3], the oxygen bridge in molecules of the compounds was essential to exert their antimalarial action. Although much work has been done on chemical, preclinical and clinical studies, little data are available on cellular damages. This paper reports the hemolysis and changes of hemoglobin (Hb) by the appropriate concentrations of artemisinin (I), dihydroartemisinin (II), artemether (III), propoxyl dihydroartemisinin (SM 242, IV), and artesunate (V).



		R
I	artemisinin	
II	dihydroartemisinin	-H
III	artemether (methyl dihydroartemisinin)	$-CH_3$
IV	propoxyl dihydro-artemisinin (SM 242)	$-OCO(CH_2)_2CH_3$
V	artesunate (sodium dihydroartemisinin succinate)	$-CO(CH_2)_2CO_2Na$

MATERIALS AND METHODS

Drugs and reagents I–IV were dissolved in *N,N*-dimethyl formamide (DMF, Koch-Light). V, chloroquine (CQ), sodium azide (NaN_3, BDH), deferoxamine mesylate (DFM, Ciba) and superoxide dismutase (SOD, Sigma) were dissolved in isotonic saline. H_2O_2 (BDH) was diluted with isotonic saline.

Red blood cell (rbc) suspension and incubation Human blood (Type A, Rh^+)

Received 1985 Jun 24 Revised 1985 Sep 20

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was centrifuged at $650 \times g$ for 10 min. After removal of the upper plasma and buffy coat, the rbc were washed 3 times with 10 volumes of isotonic saline, then 0.5% and 5% rbc suspensions were made in isotonic saline.

Mixtures consisting of the rbc suspension, drug solution and, if necessary, the additional reagent were incubated at 37℃ under shakes at a frequency of 130/min for 1.5 h. Then the mixtures were spun at $650 \times g$ for 10 min.

Spectrometry Optical spectra of the supernatants in some of the above incubation tubes were recorded with a Perkin-Elmer Ultraviolet-Visible 402 Spectrophotometer. The optical densities (OD) of the samples were read out. The supernatant of the control group was used as a reference of photometry. In the control group drug solution was replaced by equal volume of isotonic saline containing same amount of DMF as in the drug solution.

RESULTS

Two typical extinction maxima at 541 and 573 nm of normal oxyhemoglobin were found by means of visible absorption spectroscopy (Fig 1 A). After exposure of rbc to 1 m*M* III, a complete lysis of rbc (Tab 1) was observed and the optical spectrum of the supernatant was unchanged (Fig 1 B). Similarly, no change was seen of the spectrum of the supernatant from rbc exposed to 0.5 m*M* IV (Fig 1 C) but in a less extent (65%) of hemolysis (Tab 1). However, no significant amounts of Hb leaked out from the rbc exposed to 1 m*M* CQ (Tab 1) and the rbc were not darkened. Addition of the solution of 1 m*M* H_2O_2 to rbc suspension resulted in darkened rbc and release of Hb. Its spectrum (Fig 1 F) was quite different from that of the normal Hb. There was also a difference between the spectrum of the supernatant from the rbc exposed to 1 m*M* V and that

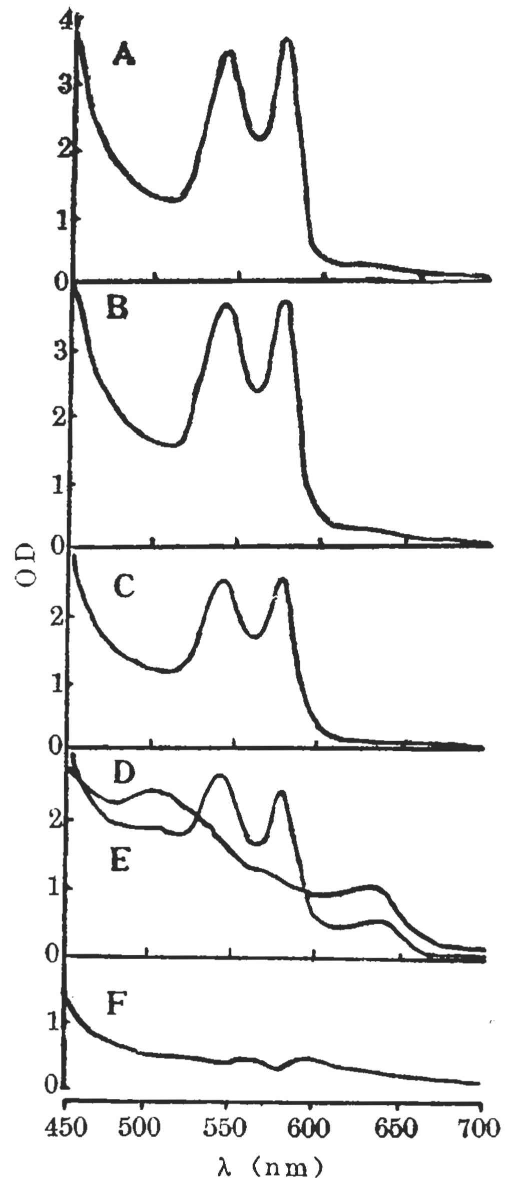

Fig 1. Optical spectra of Hb released by A) distilled water, B) exposure to 1 m*M* III, C) exposure to 0.5 m*M* IV, D & E) exposure to 1 m*M* V, and F) exposure to 1 m*M* H_2O_2. The concentrations of rbc suspension were 0.5% except curve E 5%.

from the normal rbc. Two shoulder peaks at 502 and 631 nm in the former (Fig 1D) replaced the two sharp ones at 541 and 573 nm in the latter (Fig 1 A). Moreover, the spectrum in V group (Fig 1 D) was

Tab 1. Hemolytic action by the derivatives of I. Each tube contained 2.7 ml of 0.5 % rbc suspension, 150 μl isotonic saline and 150 μl drug solution. $\bar{x} \pm SD$, *$p > 0.05$, **$p < 0.05$, ***$p < 0.01$, compared with the group without drug.

Drug	Concn(m*M*)	n	Hemolysis% †
I	1	3	6.6±1.2**
II	1	3	4.9±0.8**
III	1	3	106±11***
IV	0.5	4	65±11***
CQ	1	3	2.4±0.6*

† The hemolysis % was calculated from dividing the OD value measured at 573 nm of the drug group by that of the complete hemolysis group.

Tab 2. Hemolysis by the derivatives of I and their combinations with biological reagent. $\bar{x} \pm SD$, n = 3, *$p > 0.05$

Tube	Drug or reagent	Hemolysis% †	OD(631 nm)
A	III	106±11	
B	NaN_3 + III	93±17*	
C	DFM + III	98±12*	
D	SOD + III	103±8*	
E	IV	65±11	
F	NaN_3 + IV	54±9*	
G	DFM + IV	59±7*	
H	SOD + IV	66±9*	
I	V §		0.23±0.03
J	NaN_3 + V		0.14±0.03*
K	DFM + V		0.24±0.05*
L	SOD + V		0.24±0.03*

Concentrations: III 1 m*M*, IV 0.5 m*M*, V 1 m*M*, NaN_3 1 m*M*, DFM 1 m*M*, SOD 5 μg (15 IU)/ml. Tube A, E and I contained 2.7 ml of 0.5 % rbc suspension, 150 μl isotonic saline and 150 μl drug solution. Tube B–D, F–H and J–L contained 2.7 ml of 0.5 % rbc suspension, 150 μl reagent (NaN_3 for tube B, F and J; DFM for Tube C, G and K; SOD for tube D, H and L) and 150 μl drug solution.

† Same as the note in Tab 1.

§ No significant changes for both pH and osmotic pressure after the addition of V.

also different from that in H_2O_2 group (Fig 1 F). In the V group, a complete hemolysis was also noted.

The results in Tab 2 showed that no apparent effect was seen after addition of the 3 reagents (NaN_3 as an inhibitor of catalase, deferoxamine as a chelator of iron, and SOD as a scavenger of $\cdot O_2^-$) prior to the addition of III-V.

DISCUSSION

In this experiment, 1 m*M* III, 0.5 m*M* IV and 1 m*M* V caused lysis of human rbc and only the Hb exposed to 1 m*M* V changed. The weaker action of both 1 m*M* I and 1 m*M* II to induce lysis of rbc could be due to their weaker intrinsic activity compared to the other 3 derivatives. with different concentrations of rbc, different spectra were depicted, eg, curve 1 D from 0.5% rbc suspension and curve 1 E from 5% rbc suspension. Curve 1 E can be considered as a mixing of spectrum of normal Hb and that of the changed Hb, indicating that only portion of Hb were oxydized.

The cellular damages induced by the above 3 derivatives of I and the changes of Hb induced by V were quite different from those induced by H_2O_2. A prior inhibition of catalase by NaN_3 was necessary for H_2O_2-produced rbc damage, but not the case in III and IV (Tab 2). These indicate the differences in mechanisms of hemolytic actions between them.

The fact that DFM and SOD did not significantly reduce the hemolysis by these derivatives of I suggests that $\cdot O_2^-$[4] did not involved in the hemolysis process.

It is worthy to mention that for the above 3 derivatives of I the hemolytic concentrations were far away from their effective concentrations for killing malaria parasites[5], and for inhibiting nucleic acid[6] and protein[7] synthesis in malaria parasites but may be related to that in the intoxications in animals when treated with high doses of derivatives of artemisinin for

both toxicological tests and experimental therapy for other diseases.

ACKNOWLEDGMENTS This investigation received support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. D. C. W. is supported by the Public Health Laboratory Service.

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中国药理学报　1986年5月; 7 (3) : 269-272

青蒿素衍生物在体外试验中的溶血作用

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提要　人红细胞经与 1 m*M* 蒿甲醚和 0.5 m*M* 丙氧甲酰二氢青蒿素温育后引起不同程度的溶血，所释出的血红蛋白没有改变；经与 1 m*M* 蒿酯钠温育后不但引起溶血，而且血红蛋白也已改变；NaN_3，去铁胺及超氧物歧化酶不能对抗上述三个化合物的溶血作用；经与 1 m*M* 青蒿素和二氢青蒿素温育后有轻度溶血；而与 1 m*M* 氯喹温育后未见溶血。

关键词　抗疟药类；　倍半萜类；　氯喹；　溶血；　红细胞；　血红蛋白类；　去铁胺；　超氧物歧化酶；　光谱分析；　青蒿素

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