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Synthesis of Primaquine Glyco-conjugates as potential tissue-schizontocidal antimalarial agents

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ABSTRACT

Primaquine (**PQ**) is the only drug used to prevent relapse of malaria due to *P. vivax* and *P. ovale*, by eradicating the dormant liver form of the parasite (hypnozoites). The side effects associated with **PQ** limits is uses in treatment of malaria. To overcome the premature oxidative deamination and to increase the life span of drug in the biological system the novel glyco-conjugates of **PQ** were synthesized by coupling of primaquine with hexoses in phosphate buffer. The saccharide part of the hybrid molecules thought to be direct the drug to the liver, where hypnozoites resides. All the synthesized compounds were fully characterized

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and evaluated for their radical curative activities. The three compounds viz glucoside (**15a**), galactoside (**15b**) and mannoside (**15c**) with high activity were tested for their activity in rhesus monkeys where the most active compound **15b** showed two-fold activity (100% radical curative activity at 1.92 mmol/kg) than the standard drug **PQ** diphosphate (3.861 mmol/kg). It is proposed that results from these studies may be advantageous to develop a new potent tissue schizontocide antimalarial compound.

KEYWORDS

Primaquine diphosphate, Phosphate buffer, Tissue-schizontocidal activity, Radical curative activity, 8-amino quinolones.

Malaria in human is caused by five plasmodium species, *viz. P. malariae, P. ovale, P. vivax, P. falciparum* and occasionally *P. knowlesi*. It is the most prevalent among the top 10 diseases in the world.[1] It occurs in tropical and subtropical countries lying between latitude 30°S-40°N.[2] Malaria affecting nearly 200 million persons across the globe and led to 429 thousand deaths in 2015.[3] Most deaths in 2015 are estimated to have occurred in the WHO African Region (92%), and most deaths were in children under 5 years of age which is equivalent to 70% of the total deaths.[3] The global burden of malaria is estimated to have declined by 60% worldwide between 200-2015 and 26 % between 2010 and 2015, nevertheless this situation would be better if there is no presence of anti-malarial drug resistance.[3] In addition to the chloroquine resistance, resistance to the combination of sulfadoxine-pyrimethamine which was already present in South America and in South-East Asia is now incipient in East Africa.[4a] Recently, concern has been raised towards the resistance to artemisinins, which could endanger the artemisinin combination therapies (ACTs).[4b] Intensive research has been done for the discovery of malaria vaccine; and no effective vaccine is introduced in the clinical practice. Till date, GSK RTS,S/AS01 (trade

name Mosquirix) is the only recombinant protein-based malaria vaccine approved for use by European regulators in July 2015, with the limitation of its use in the region specific children.[5]

The most currently used antimalarials are potent blood schizontocidals i.e., they act rapidly against the parasite forms that invade erythrocytes and cause the well-described malaria symptoms. Among the plethora of antimalarial drugs, 8-aminoquinolines are the most potent class of anti-malarial drugs with radical curative activity, having properties to eliminate the hypnozoites from the liver.[6] Considering the historical perspective of aminoquinolines, 8-aminoquinolines are the successor of quinine (**1**) and methylene blue (**2**) (Figure **1**).[7] Among the important 8-aminoquinoline derivatives Primaquine (PQ) is the most effective being the only drug drug against *P. vivax* hypnozoites.[8] PQ acts specifically on the pre-erythrocytic schizonts which are concentrated predominantly in the liver and causes relapse after multiplication.

If PQ is not administered to patient with proven *P.vivax* and *P.ovale* infection, there is a very high likelihood of relapses within weeks or months (sometimes years).[6b] PQ is also effective against mild to moderate Pneumocystis pneumonia (PCP) and is co-administered with clindamycin to AIDS patients.[10] It is the toxicity of the PQ which limits its clinical utility in therapeutic applications. The most serious side effect of the PQ is the haemolysis particularly in individuals with heredity deficiency of G6PD (glucose-6-phophate dehydrogenase).[11] The other significant side effect associated with PQ and 8-aminoquinolines derivatives are methemoglobinemia, leucopoenia, abdominal cramps, and epigastric distress.[12] In addition of its toxicity PQ is readily absorbed metabolized and eliminated, so prolonged doses are required which amplify its undesirable effect thus reduces the interest in the PQ adjunct therapy.[13] So there is a persistent need for safe, long lasting tissue

schizontocide active agent against latent exoerythrocytic forms with prolonged action for the treatment of liver stage of malaria.

To decrease the undesirable side effect of the PQ, modification of primary amine group also play important role because altering this amino group shows tremendous improvement in terms of the bioavailability of PQ. These types of modification at primary amino group involve (i) coupling the N-cycteinyl–PQ to carrier protein, e.g. lactosaminated serum albumin,[14] (ii) N-acylation of PQ with amino acids or oligopeptides,[15] (iii) introduction of an imidazolidin-4-one ring over amine of the PQ,[16] (iv) dimerization of the PQ with N-acylated PQ with amino acids,[17] (v) dihydro-furan-2-one linked PQ.[18] The explanation why the side-chain modified derivatives of PQ have enhanced curative properties is not clear. The most accepted explanation for these modification may be significantly reduce the extent of PQ conversion into carboxyprimaquine which is the important metabolite of the PQ and have been identified in mice, monkeys and humans.[19] PQ is promptly converted into carboxyprimaquine (4) attaining 10 times higher plasma level than those of parent drug, but carboxyprimaquine is significantly less potent than parent drug PQ (Figure **2**). These modifications ongoing as a way to avoid premature PQ inactivation by oxidative deamination to carboxyprimaquine (4). To overcome the two major challenges first inhibit the premature inactivation and second targeting the drug towards the liver where latent tissues exist and responsible for the relapses, we are keen interested to synthesize glyco-conjugate of PQ (7) and then evaluate its biological activity (Figure 3). In continuation of our efforts to design and synthesize new antimalarial agents we designed these molecules on the basis of drug delivery and prodrug approach with improved life span of the drug in the biological system.[20] The saccharide derivative easy hydrolyzed in the biological system to free primaquine at the slow rate, so lesser doses are required and simultaneously inhibit the premature oxidative deammination. The foremost idea is based on the drug delivery system.

It is well known that receptor on plasma membrane of liver parenchymal cells and liver von Kupffer cells recognize hexose like galactose and mannose respectively. From this it would appear that title compound with hexopyranose part as galactose or mannose may direct the PQ to the liver where the latent tissue forms exist. This may result in substantial decrease in its systemic circulation which may reduce the hematological toxicity and increase in its selectivity. Bhadra *et al* designed a drug delivery system in which they intended to deliver primaquine phosphate directly to liver cells using polypropyleneimine (PPI) dendrimers-coated peripherally with galactose. The galactose coating of PPI dendrimers can therefore make the PPI systems more efficient and appropriate for targeted delivery of PQ phosphate to liver.[21]

Experimental

General chemistry

Reagent grade solvents were used for the extraction and flash chromatography. All the reagents and chemicals were purchased from Sigma Aldrich Chemical Co., Lancaster and were used directly without further purification. The progress of reactions was checked by analytical thin-layer chromatography (TLC, Merck silica gel 60F-254 plates). The plates were visualized first with UV illumination followed by charring with 10% H_2SO_4 in CH_3OH. Flash column chromatography was performed using silica gel (230-400 mesh). The solvent compositions reported for all chromatographic separations are on a volume/volume (v/v) basis. All glassware's were dried in an open flame before use in connection with an inert atmosphere. Solvents were evaporated under reduced pressure. Tetramethylsilane (0.0 ppm) was used as an internal standard in ^1H NMR and $CDCl_3$ (77.0 ppm) was used in ^{13}C NMR. The abbreviations used to indicate the peak multiplicity were; s, singlet; bs, broad singlet; d, doublet; dd, double doublet; t, triplet; q, quartet; m, multiplet; Hz, Hertz. FAB MS was recorded on Jeol (Japan)/SX-102. Infrared spectrum was taken with KBr on Perkin-Elmer

RX-1. Melting points were determined on a Buchi 535 digital melting point apparatus and were uncorrected. Elemental analysis was performed on a Perkin-Elmer 2400 C, H, N analyzer and values were within ±0.4% of the calculated values. In this study all the compounds were synthesised in dark.

Bioevaluation methods

For all the experimental studies, laboratory bred Albino Swiss mice of female sex (weight 25 g) were used. All the animals were housed in animal rooms under standard conditions of temperature 22 $\pm 1^0$C and humidity (50-65%) and maintained on commercially available pellet diet supplemented with soaked grains.

Activity in the rodent model

For the radical curative test, compounds were administered orally in three doses on days -1, 0, +1 and animals were challenged intravenously with 1 X 10^4 *P. yoelii nigeriensis* sporozoites on day 0. The thin blood smear was prepared from each animal on day 4. The degree of infection was microscopically recorded in terms of number of *P. yoelli* infected cells per 100 RBC (i.e. percentage Parasitemia) the mean value determined for group of 5 mice was used to calculate the percentage suppression of parasitemia with respect to the untreated control group. Blood smears from experimental animals were observed until day 28 to record the day of patency. The animals which did not show any parasites on day 4 were subsequently monitored twice weekly till day 28 post-infection. The animals which did not develop any infection till day 28 were recorded as cured.

Drugs

The aqueous suspension of each test agent was freshly prepared on the day of initial use and the required drug concentrations were administered orally, once daily in 0.5 ml volume. Pure samples of PQ, were obtained from Sigma, USA.

Radical curative Activity in Rhesus monkeys

Anopheles stephensi mosquitoes were fed on *Plasmodium cynomolgi bastianelli* infected rhesus monkey during the declining phase of secondary peak parasitaemia when mature gametocytes were in circulation (male to female gametocyte ratio of 1:3 is ideal). The mosquitoes were maintained in the insectary under controlled condition of 27 ± 1^{o}C temperature and 75-80 % relative humidity. The monkeys were left untreated till patency. When parasitaemia ranging between 50-100 parasites per 10,000 erythrocytes was reached, the monkeys were treated with the orally administration of the test compounds along with chloroquine phosphate 5 mg/kg (base) per day for 7 days. Monkeys were followed thereafter up to 90 days for reappearance of blood parasites. The absence of any relapse till 90 days after end of treatment indicates complete cure, and partial activity was indicates by delay in appearance of relapse as compared to the control monkeys which were treated with chloroquine only. Primaquine 1mg/kg for 7 days was used as the standard drug for radical curative activity.

The animal studied and experimental protocol were approved by the institutional ethics and usage committee of CSIR-Central Drug Research Institute (CDRI), Lucknow which is specifically designed to review animal research and strictly follows the guidelines of the Committee for the Purpose of Control and Supervision of Experimental on Animals (CPCSEA).

Chemistry

The desired PQ was prepared by classical Skraup's synthesis starting from 4-methoxy- 2-nitroaniline (**8**) which yielded the 6-methoxy-8-nitroquinoline (**9**) , followed by reduction to offer the desired 8-amino-6-methoxyquinoline (8A6MQ, **10**). The reaction of 8A6MQ with phthalimido-2-bromopentane followed by hydrolysis, gave the primaquine (**3**) (Scheme **1**).[22] The reactivity of the hexoses towards PQ in yielding the PQ-glyco-conjugate may depend on the stereochemistry and reactivity of the hexoses. This may influence the formation of mixture of α/β epimers. Thus optimization of the N-glycosylation reaction in terms of yield, reaction time, and diastereoselectivity was a significant challenge. Several attempts were made for the synthesis of glyco-conjugate e.g. condensing the halide donor of hexose with PQ base or PQ diphosphate in DCM or acetone using variety of bases, but non-separable mixture was obtained.

It is well recognized that the reaction between an amine and reducible sugar such as D-glucose in water, at high temperature and in slightly low pH first proceeds through the unstable corresponding N-glycoside, which undergoes the Amadori rearrangement to produce the Amadori product. When the reaction of PQ-base and D glucose in C_2H_5OH was carried out a new compound containing hexose and PQ was formed, this led to the selection of EtOH as a solvent of choice for the model reaction. The formation of the compound containing both PQ and D-glucose was confirmed by the UV absorption and charring (10% H_2SO_4 in CH_3OH) test for the hexose counterpart. This compound also showed the required (ES-MS (M+H) 422.2 m/z.), however its ^{1}H NMR analysis revealed that product was not the N-glycoside, because there was no signal of anomeric proton in the expected region (range 4.5-6.2 ppm) and instead there was two double multiplets at 4.04-4.26 ppm and at 2.66-3.02 ppm of CH_2 which is the indication of formation of Amadori product **13a-c** (Figure **4**).[23] This was further confirmed by the reported spectra corresponding Amadori products and ^{13}C NMR.

The reaction was performed with different hexoses using variety of Lewis/Brϕnsted acid catalysts to achieve the desirable N-glycoside (Table **1**) but in each cases we ended up with the Amadori rearranged products **13a-c** (chromatographed using neutral alumina) (Figure **4**). Afterward, in the pursuit of good and efficient method for the synthesis of N-glycoside a contemporary green methodology reported by Maugard *et al* was used for the synthesis of N-glycosides in phosphate buffer. This novel synthetic modification afforded a new route for the synthesis of N-glycosides of PQ.[24]

Therefore, in the first experiment the condensation between the PQ (**3**, 1.0 mmol) and a D-glucose (**14**, 1.1 mmol) was carried out in aqueous phosphate buffer pH 6.5 and at 50 °C. It resulted in the formation of product which on tlc analysis, showed a bright yellow spot (in iodine) having Rf 0.4 in 30% CH_3OH:$CHCl_3$ suggesting the formation of a product different from the Amadori products **13b**, thus indicating to be possibly the required N-glucoside. This product formed was purified by column chromatography using neutral alumina and the analysis of this product by state of the art techniques like ^{1}H NMR, ^{13}C NMR and mass spectra confirmed it to be the desired N-glycosides. In ^{1}H NMR the anomeric potons appeared as a doublet having coupling constant 7-9 Hz suggesting axial-axial configuration of H1 and H2 and therefore in sugar **15a-b**, and **15e-h**, the glycosidic linkage is β one. In compounds **15c** and **15d** the anomeric potons appeared as a doublet having coupling constant 3.2, 3.6 Hz repectively suggested axial-equatorial configuration of H1 and H2 and therefore glycosidic linkage is α one. The preference for formation of the β isomer in **15-b**, and **15e-h**, can be rationalized in terms of the competition between substitution at the sterically least hindered position (β) against the substitution at a position which sustains stabilization from the anomeric effect (α). The substitution is driven predominantly by the steric factor thus the reaction is find to be stereoselective for the β configuration.

Although the reaction conditions gave the preferred products (**15a-h**, Figure **5**), but in order to optimize them the effect of pH and temperature on the N-glycosylation reaction yield were investigated. When the reaction was carried out at 40^{o}C for 5 h with the same concentrations of D-glucose (**14**) and PQ (**3**) base at different pH, the best yield (60%) of the product was obtained at pH 6.5. The effect of the temperature was also studied with the same concentrations of the reactants at pH 6.5, where the reaction was favored at below 50 ^{o}C. So several N-PQ-glycosides, using D-glucose (**15a**), D-galactose (**15b**), D-mannose (**15c**), D-arabinose, (**15d**), L-rhamnose (**15e**), D-ribose (**15f**), D-xylose (**15g**) and N-acetyl-D-glucosamine (**15h**) were prepared at 40^{o}C and a pH of 6.5. In all reactions hexose was taken in 1.2mol % with respect to PQ-base, acceptable yields from 50-60% were obtained.

Due to the complex requirements for complete parasite development of *Plasmodium vivax*, no *in vitro* model exists for routine screening of causal prophylactic and radical curative compounds against human relapsing malaria, hence the target compounds were evaluated for their tissue schizontocidal (radical curative) activity in Swiss mice and the promising compounds were than tested on rhesus monkeys. The rhesus monkey relapsing malarial model (*P. cynomolgi*) has been used more than four decades and is the most trustworthy model for the screening of antimalarial compounds for both prevention (causal prophylaxis) and treatment of relapse (radical cure).

Radical Curative (RC) Activity in the rodent model

Promising activity was exhibited by synthesized molecules in tissue schizontocidal test in swiss mice.[25] Where glycosyl (**15a, Table 2, entry 1**), galactosyl (**15b, Table 2, entry 2**) and mannosyl (**15c, Table 2, entry 3**) derivatives were 100% curative even at a dose of 30 mg/kg, while the rhamnoside (**15e, Table 2, entry 5**) was only 60% curative indicating that

the synthesized compound **15a-c** are as almost as active as PQ. The corresponding Amadori products (**13a-c**) were inactive (Table **2**).

Radical Curative Activity in rhesus monkeyes.

The promising activity of the compounds **15a-c** fortified for their evaluation in rhesus monkey, since the PQ is effective in Primate Model at 1mg/kg dose which is equivalent to 3.8 mmoles/kg of PQ, and hence all the compounds have been tested in equimolar doses i.e. 3.8, 1.9 and 0.9 mmoles, where the compounds **15a-c** exhibited excellent radical curative activity in rhesus monkey. The galactosyl derivative (**15b, Table 3, entry 2**) was 100% curative even at a dose of 0.81 mg/kg (1.9 mmmol/kg), while at this dose the glucosyl derivative (**15a, Table 3, entry 1**) was approximately 90 % curative. The mannoside **15c (Table 3, entry 3)** was 83% curative at 0.8 mg/kg (1.9 mmol/kg) while at lower dose 0.4mg/kg (0.9 mmol/kg), it was inactive. The comparison of the radical curative activity of these compounds with PQ indicates that the compound **15b** (1.92 mmol/kg) is two times more effective than PQ (3.861 mmol/kg). The higher activity of **15b** may be attributed to the targeted delivery of the drug at the liver where the tissue forms exist and to its action as prodrug because of its easy conversion to PQ in biological System.

The highest activity of galactoside **15b** may be attributed to the higher concentration of galactoside in hepatocytes due to the high binding affinity of galactose with asialoglycoprotein receptors localized in parenchymal liver cells.[26] The lesser activity of mannoside **15c** as compared to **15a** and **15b** may be due to non-recognition of mannose by the asialoglycoprotein receptors on parenchymal liver cells but its moderate activity due to interaction with the receptor generally present on nonparenchymal liver cells and by Kupffer cells. The higher activity of glucoside **15a** than **15c** and lesser than **15b** may be because of the recognition of glucose sugar by the glucose transporter 2 (GLT 2) protein which is found

in cellular membrane of liver which facilitates the bidirectional glucose transport across the hepatocyte plasma membrane.[27] It is interesting to note that these three sugars present in **15a-c** have primary alcoholic group (-CH_2OH) while all other hexoses **15d**, **15f**, and **15g** have except **15h** neither primary alcoholic group nor the extra carbon at this position. Thus indicating the importance of primary alcoholic group which may be phosphorylated in the biological system for further biological transformation.[28] Some activity of rhamnoside **15e** devoid of primary alcoholic group may be due to the presence of $–CH_3$ group at C6 position, which may be necessary for favorable interaction with receptors nevertheless other hexoses **15d**, **15f**, and **15g** missed this type of substitution at C6 position. The inactivity of **15h** with primary alcoholic group at C6 position and acyl-amino at C2 position can be attributed to the importance of free hydroxyl group at C2 position.

Conclusion

To conclude, glyco-conjugates of PQ possessing high tissue-schizontocidal antimalarial activity have been synthesized by the coupling between the primary amino groups of PQ with hexoses in phosphate buffer. Among the three PQ conjugated hexoses **15a-c**, the galactoside conjugate **15b** is twofold active than the PQ. This prominent *in vivo* efficacy of **15b** against *P. cyanomolgi* infected rhesus monkey is indicative of the potential utility of this compound in the therapy of *P. vivax* malaria infection. This enhanced activity can be justified by its targeted delivery to the liver and its action as prodrug. It is intended that results from these studies may be useful to develop a new potent tissue schizontocide antimalarial.

SUPPORTING INFORMATION

Synthesis of compounds and analytical data (^1H and ^{13}C) are given in the Appendix S1.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Figure legends

Figure 1. Precursors of 8-aminoquinoline.

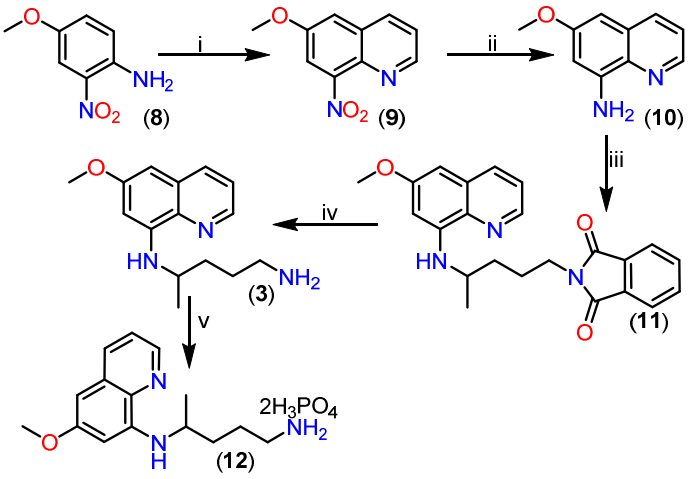
Figure 2. Proposed pathways for side-chain metabolism of Primaquine.

Figure 3. Prototype of the designed Glyco-conjugate of Primaquine.

Figure 4. Amadori products synthesized from the PQ and corresponding hexose.

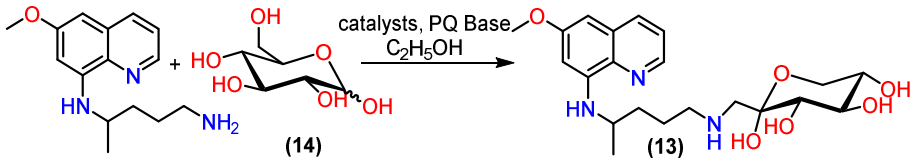
Figure 5. N-Glycosides of PQ synthesized from the direct coupling of PQ with hexose.

Scheme 1.



Scheme 1. Synthesis route for the synthesis of PQ phosphate; Reagent and condition: (i) As_2O_5/H_3PO_4, Glycerine, 100^oC,1 h (ii) Raney-Ni,50 psi,4-5h (iii) phthalimido-2-bromopentane, TEA, 150 ^oC, 6h (iv) NH_2NH_2, EtOH, reflux 72h.(v) ethereal phosphoric acid.

Table 1. Effect of catalyst on synthesis of N-glycosyl PQ. [a]



Entry	Catalyst	Time (h.)	Yield (%)
1	-	10	~
2	Cuprous Bromide	12	25
2	Iron(III) chloride	12	30
3	Copper(II) sulphate	12	25
4	Nickel(IV) chloride	12	20
5	Zirconium(IV) chloride	12	20
6	Zinc(II) triflates	10	25
7	Copper(II) triflates	10	28
8	Zinc acetate	16	25
9	Chloro acetic acid	10	26
10	TFA	10	25
11	Acetic acid	12	28

a. The reaction was conducted with PQ-Base (1 mmol), D-glucose (1.1 mmol) and 15 mol% of catalyst in EtOH at room temperature

Table 2. Tissue schizontocidal activity table of Synthesised compound.*

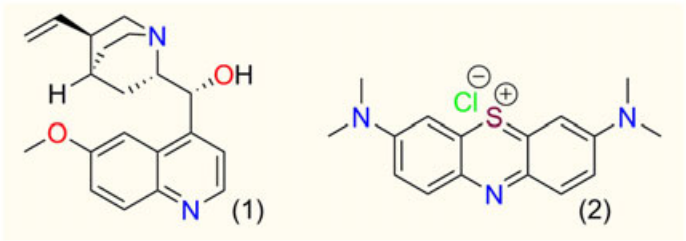
S. no.	Test sample	Dose mg/kg	Percent protection
1	15a	30	100
		22.5	0
		15.0	0
2	15b	30	100
		22.5	0
		15.0	0
3	15c	30	100
		22.5	0
		15.0	0
4	15d	30	0
5	15e	30	60
6	15f	30	0
7	15g	30	0
8	15h	30	0
13	13a	30	0
14	13b	30	0
15	13c	30	0
17	PQ	30	100
		15.0	0
18	Control	--	0
			0
			0

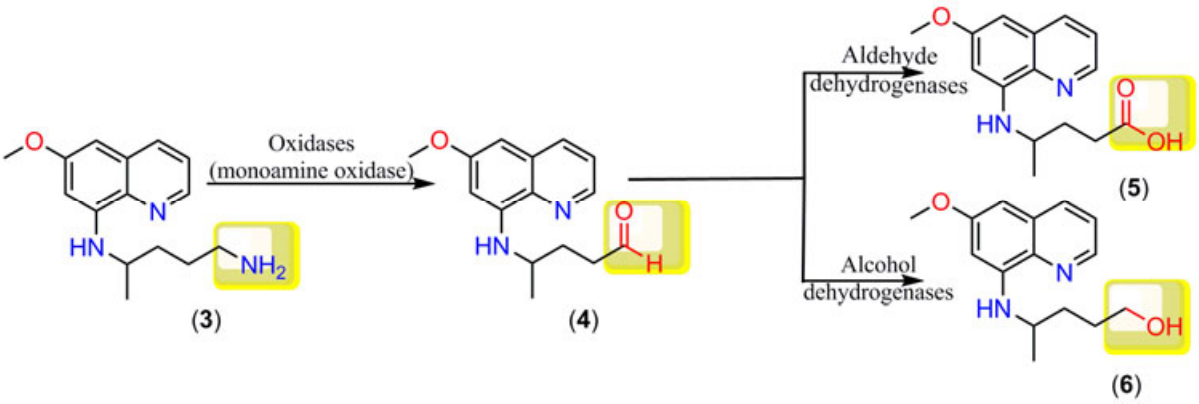
* Swiss mice model, in a group of 4-5 mice.

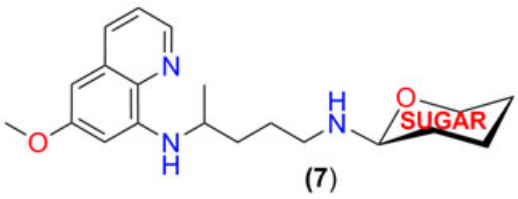
Table 3. Radical Curative activity in rhesus monkey.*

Entry	Test sample	Dose mg/kg (mmol/kg)	Percent protection
1	**15a**	0.82 (1.9)	~90
		0.42 (1)	~72
2	**15b**	3.25 (7.7)	100
		1.62 (3.8)	100
		0.81 (1.9)	**100**
		0.51 (1.2)	50
		0.4 (0.9)	Nil
3	**15c**	1.62 (3.8)	100
		0.8 (1.9)	~83
		0.4 (0.9)	Nil
4	**PQ**	1.0 (3.8)	100
		0.6 (2.3)	40

* Rhesus monkey model, in a group of 3-11 monkies for each dose.







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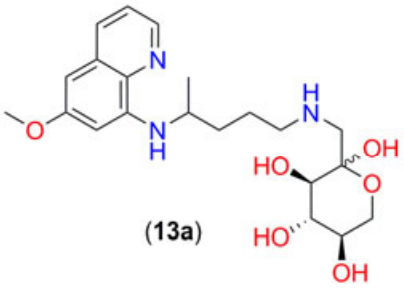

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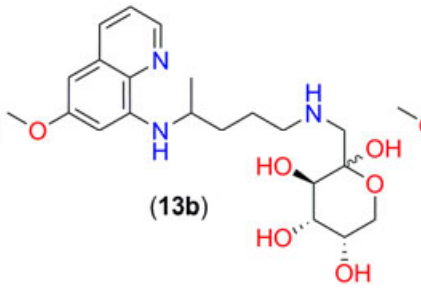

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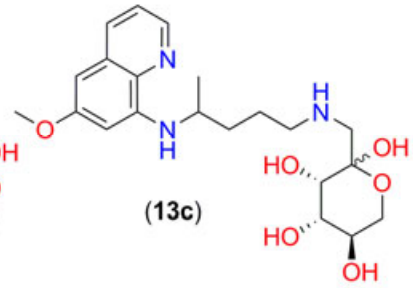

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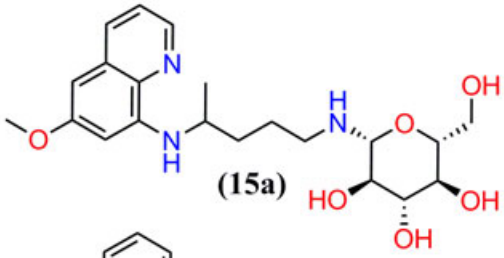

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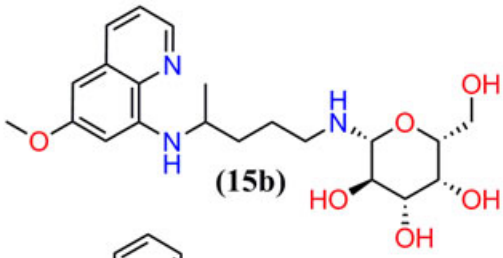

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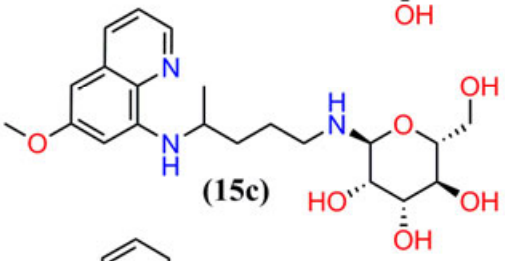

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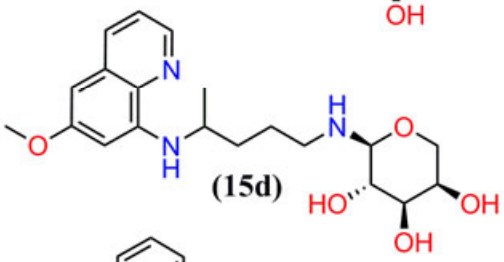

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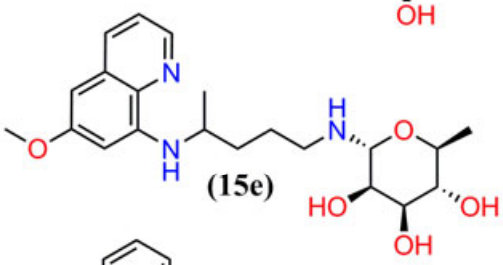

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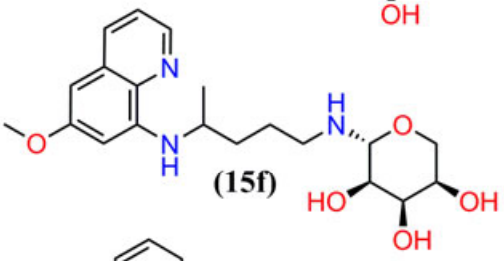

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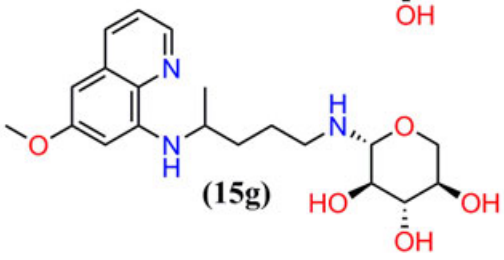

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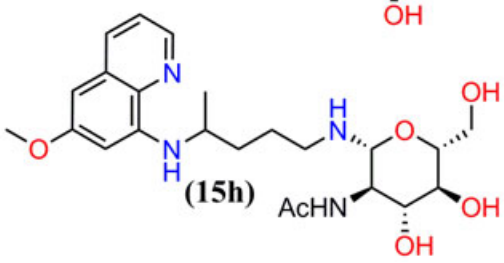

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