WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 9, 361-390. Research Article ISSN 2277– 7105

DESIGN, SYNTHESIS AND IN-VITRO KINETIC STUDY OF ATOVAQUONE PRODRUG FOR THE TREATMENT OF MALARIA

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Article Received on
03 July 2015,
Revised on 27 July 2015,
Accepted on 22 Aug 2015

DOI:10.20959/wjpr20159-4479

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ABSTRACT

Using DFT molecular orbital at B3LYP 6-31G (d,p) and B3LYP/311+G (d,p) levels and molecular mechanics (MM2) calculations of the hydrolysis of Bruice's di-carboxylic semi-esters **1**–**5** several atovaquone prodrugs were designed**.** It was found that the interconversion rate of the designed atovaquone prodrugs is largely determined on the strain energies of the reaction's tetrahedral intermediates and reactants. Further, no correlation was found between the active parent drug's release and the distance between the nucleophile and the electrophile in the dicarboxylic semi-ester (atovaquone prodrug). Using the half time needed for the interconversion of 50% of di-carboxylic semi-ester **1** and the calculated log k_{rel} values for the designed atovaquone prodrugs the $t_{1/2}$ values for interconversion of those prodrugs to their active parent drug were calculated. The calculated $t_{½}$ value for atovaquone ProD 1 was about 26.4 hours. Utilizing the information gained from the prodrugs design, atovaquone ProD 1 was synthesized and fully characterized. In vitro kinetic study on the interconversion of atovaquone ProD 1 to atovaquone was studied in four different aqueous media mimicking the stomach, intestine and blood circulation. The kinetic results revealed that atovaquone ProD 1underwent hydrolysis in all studied media however with different interconversion rates. The interconversion $t_{1/2}$ values were: in 1N HCl (11.4 hours), pH 2.2 (10.9 days), pH 5.5 (24 hours) and pH 7.4 (28.8 hours).

KEYWORDS: Malaria, prodrugs, atovaquone, dicarboxylic semi-esters, intramolecularity, atovaquone prodrugs.

1-INTRODUCTION

Malaria is a global public health problem, affecting about 40% of the population and causes about 2 million deaths per year.[1]

Most of disease cases are found in tropical Africa, Latin America, Southern Asia and Oceania.[2] World Health Organization (WHO) assesses that 81% of cases and 91% of deaths are found in African regions. Children under 5years old and pregnant women are the most severely affected. This protozoan disease is caused by 5 parasites species of the genus *Plasmodium* that affect humans: *P. falciparum, P. vivax, P. ovale*, *P. malariae* and *P. knowlesi*.[3] Most of death cases are caused by the most severe form, the *P. falciparum*, which dominates in Africa and to which most drug-resistant cases are attributed. Malaria is transmitted to humans via the bite of infected female mosquito of anopheles species.[3] Malaria can exist, in a mild form that most commonly associated with flu-like symptoms; fever, vomiting, and general malaise. While in the sever form caused by *P. falciparum*, a nervous, respiratory and renal complications frequently coexist due to serious organ failure. Despite of being serious infectious disease, malaria is a treatable and preventable illness and a number of treatments are already available.[3]

1.1. Malaria Treatment Medications

1.1.1 Chloroquine

Chloroquine, a 4-aminoquinoline, acts by accumulating inside the digestive vacuole of the infected red blood cell, where it makes complexes with toxic heme moieties and disturbs the detoxification mechanisms that involve heme sequestration into an inert pigment called hemozoin. It is an inexpensive drug used to prevent and treat malaria for decades.[4] However, the emergence of chloroquine resistance in the vast majority of malaria-endemic countries, and the association of tinnitus and central nervous system toxicity with chloroquine treatment limit its use.[5, 6]

1.1.2. Antifolates

Currently used antifolate combinations of sulfadoxine- pyrimethamine and sulfalene-pyrimethamine have long elimination half-lives, 81 hours for sulfadoxine, 62 hours for sulfalene and 116 hours for pyrimethamine.[7, 8] This has both advantages and disadvantages.

On the one hand, it allows single-dose therapy and persistence of the drugs at effective blood levels might protect the patients from reinfections after cure of the initial disease. On the other hand, the latter would be only useful in high transmission areas and the slow elimination favors the selection of resistant parasites.[9] There is also concern with adverse reactions to long-acting sulfonamides,[10] especially in subjects concomitantly infected with human immunodeficiency virus (HIV) infections.[11]

As indicated from the name 'antifolates', they antagonize the action of folic acid by inhibiting dihydrofolate reductase (DHFR) enzyme, hence inhibiting cell division.

1.1.3. Artemisinin

Replacing unsuccessful medications (chloroquine), with well tolerated artesunate monotherapy and artemisinin combinations resulted in decrease in malaria mortality and morbidity.[12] Artemisinin is commonly used in Southeast Asia.[13]

The mechanism of action of these compounds appears to involve the heme-mediated decomposition of the endoperoxide bridge to produce carbon-centered free radicals. [14] In spite of their effectiveness, artemisinin resistance appears in several areas mainly in Pailin and western Cambodia. Moreover, it is associated with reduced cure rates.[15]

It is worth noting that antimalarial drug resistance escalates to the major therapeutic groups used in malaria treatment, which constitutes a major threat to the global malaria control. [16] This can be attributed to the fact that malaria control has significantly dependent on a limited number of chemically related drugs, such as the quinolone or the antifolate groups, which are overused in poor countries due to their low price.[13]

Practice has shown that resistance ultimately shortens the life span of antimalarial drugs. Accordingly, this emphasizes the urgent need to develop alternative medications with a novel chemical structure and mechanisms of action to treat and prevent malaria in one hand, and on the other hand to develop strategies to avoid resistance when new drugs are introduced. [13]
In view of that, efforts were directed toward developing, novel compounds with novel mechanisms of action to maintain an effective malaria control**.**

1.1.4. Atovaquone

Atovaquone (ATQ) is a hydroxynaphthoquinone (Figure 1). Naphthoquinones are known to have antimalarial, anticoccidial and antitheilerial activity.[17] ATQ is relatively a new

treatment option, that has a broad antiprotozoal activity including *Plasmodium spp*,[18 ,19] it has a novel mechanism of action, acts by inhibition of the electron transport system at the level of cytochrome bc1 complex.[20] (Figure 2). In malaria parasites, the mitochondria acts as a sink for the electrons generated from dihydrorotate dehydrogenase; an essential enzyme for pyrimidine biosynthesis; an inhibition of electron transport by ATQ leads to dihydrorotate dehydrogenase inactivation which results in reduced pyrimidine biosynthesis and concomitantly to shutdown in parasite replication.[21] This is because parasites depend on de novo production of pyrimidines and have no salvage pathway; in contrast to humans, thus the final outcome is the prevention of parasite replication.[22] Reports indicate that protozoan electron transport inhibition is about 1000-fold more sensitive than that of mammalian and avian mitochondria.[23]

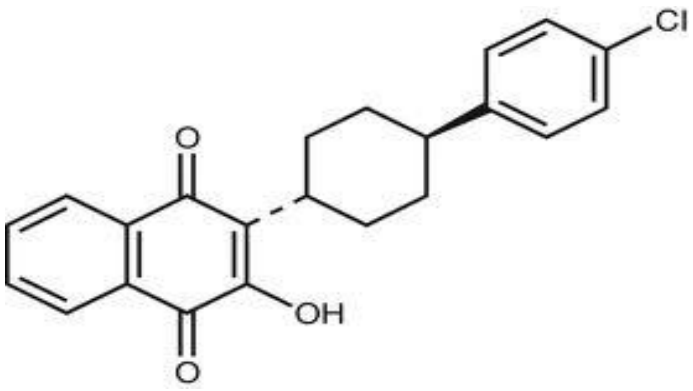


Figure 1. Atovaquone chemical structure.

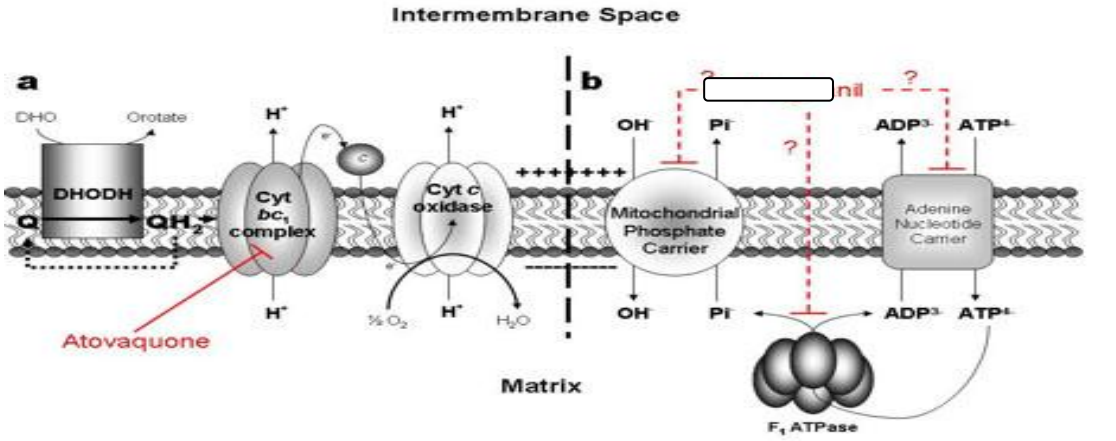


Figure 2. ATQ mechanism of action.

It is well established that ATQ has a long half life (70 to 84 hours), exerts its effects on the parasite within minutes after drug treatment,[24] can be administered via the oral rout and has an excellent safety profile and tolerability. The most common registered side effects are rash,

fever, vomiting, diarrhea, abdominal pain and headache,[25] and there is no registration of any side effects that obligate withdrawal of therapy.[18] The absence of severe side effects of this drug can be attributed to its selectivity.

Despite these advantages, ATQ is associated with some limitations that affect its effectiveness.[26] Atovaquone is a highly lipophilic compound, has low water solubility and low absorption, hence low bioavailability. Thus, increasing atovaquone aqueous solubility will improve its pharmacokinetic profile in particular bioavailability, thus improving its effectiveness and the ability to administer the drug through different routs of administration.
There are a variety of approaches which can be employed to prolong the pharmacological activity, increase oral bioavailability and decrease inter-individual variability of ATQ. In general, the prodrug approach is one of the strategies that can be used to enhance the pharmacokinetic behavior of drugs such as ATQ. It includes the conjugation of the active parent drug to a linker to produce a system that is able to release the parent drug once it reaches the blood circulation or other targeted sites.

1.1.2 Prodrugs

In the past few decades the pharmaceutical industries have been subjected to considerable alterations,[27] in terms of improving drug drawbacks related to pharmacokinetic (absorption, distribution, excretion, and metabolism), pharmaceutical and biological performance of existing drugs which may hinder drug development course.[28]

Overcoming the undesirable physicochemical, biological and organoleptic properties of some existing drugs.[27] can be achieved through the development of new chemical entities with desirable efficacy and safety. However, this is an expensive and time consuming process that needs a screening of thousands of molecules for biological activity.[29] in addition to the rigorous rules and criteria that are applied today for developing new drugs.[13] Therefore, it becomes much more feasible to modify and improve the properties of already existing drugs through exploring the prodrug approach,[29] in order to eliminate their undesirable properties and to increase their commercial life cycle and patentability.[28]

Prodrugs are inactive forms of active drugs that are designed to exhibit pharmacological activity after an enzymatic or chemical reaction when they have been administered into the body.[30] Prodrug approach is a promising and well established strategy for the development

of new entities that possess superior efficacy, selectivity and reduced toxicity over their parent compounds. Hence an optimized therapeutic outcome can be accomplished.[29]

Approximately, 10% of all worldwide marketed medicines can be categorized as prodrugs, and in 2008 alone, 33% of all approved drugs having small-molecular-weights were prodrugs.[31] These statistical numbers confirm the recent successes of the prodrug approach.[28]

In general, prodrugs are designed to (i) improve aqueous solubility, (ii) enhance permeability through modifying lipophilicity, (iii) achieve site specific delivery and increase gastrointestinal (GI) absorption through targeting specific transporters and enzymes and (iv) improve taste, odor and other pharmaceutical and pharmacokinetic properties.[29]

The classic prodrug approach focuses on changing physicochemical parameters. Recently, modern computational methods are being utilized to design linkers for drugs having low bioavailability or suffer from unpleasant taste, poor absorption and permeation or low aqueous solubility.

1.1.2.1. Design of innovative prodrugs using modern computational methods

Similarly to that exploited for drug development and discovery, modern computational methods based on molecular orbital such as ab initio, DFT and semi-empirical and molecular mechanics methods are being utilized for the design of innovative prodrugs for drugs containing hydroxyl, phenol, or amine groups. For this purpose, mechanisms for several enzyme models that have been utilized to understand enzyme catalysis have been recently researched by Karaman's group.[32-54] and used for the design of some novel prodrug linkers. [55-85] The classic prodrug approach is focused on altering various physiochemical parameters, whereas the modern computational approach, considers a design of linkers to be covalently attached to active drugs and upon exposure to physiologic environment undergo a programmed intraconversion to non-toxic moiety and the active parent drug without the involvement of any metabolic enzyme. In addition, since the linkers used are relatively small molecules, it is expected that the prodrugs themselves might be with considerable biological effects before they intraconvert to their active parent drugs.

Using ab initio, DFT, semi-empirical (AM1 and PM3) and molecular mechanics methods, several enzyme models were researched and explored for determining the factors playing

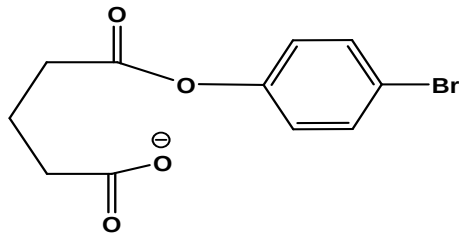
dominant role in governing the reaction rate in such models. Among the enzyme models that have been studied are: (1) proton transfer between two oxygens and proton transfer between nitrogen and oxygen in Kirby's acetals,[86-94] (2) intramolecular acid-catalyzed hydrolysis in N-alkylmaleamic acid derivatives,[86-94] (3) proton transfer between two oxygens in rigid systems as studied by Menger,[95-99] (4) acid-catalyzed lactonization of hydroxyl-acids as investigated by Cohen.[100-101] and Menger, [95-99] and (5) SN2-based cyclization as studied by Brown,[102] Bruice [103-104] and Mandolini.[105]

The interconversion of a prodrug to the active parent drug at the target site is a necessity for the prodrug approach to be successful. [106-107] The major obstacle facing the classical prodrug approach is the difficulty in predicting the bioconversion rates, and thus the pharmacological or toxicological effects of the prodrugs.[108-109]

However, using Karaman's approach which utilizes the above mentioned enzyme models would allow for better design of an efficient chemical device to be used as a prodrug linker that can be attached to a drug moiety which can chemically, and not enzymatically, cleaved to liberate the active drug in a programmable and controlled manner.

Continuing the strategy for exploring enzyme models in the design of novel prodrugs, Bruice's enzyme model (hydrolysis of dicarboxylic semi-esters) was employed in the design of ATQ prodrugs with the potential to be more bioavailable than their active parent drug, ATQ.

Our previous computational study on Bruice's di-carboxylic semi-esters **1**–**5** (Figure 3) revealed that rate of the cyclization of di-carboxylic semi-esters **1**–**5** is solely affected by strain effects and proximity orientation due to the 'reactive rotamer effect was found to be negligible, if any (Figure 4).



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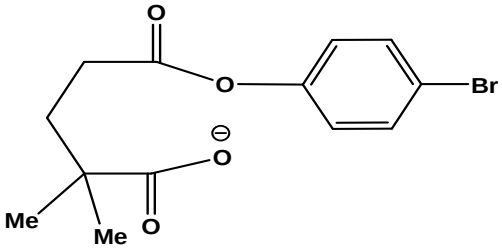

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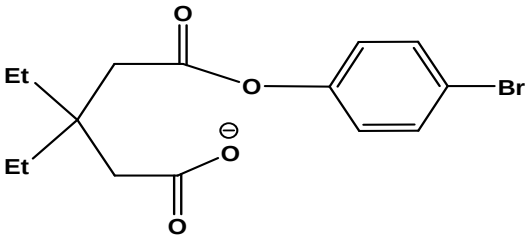

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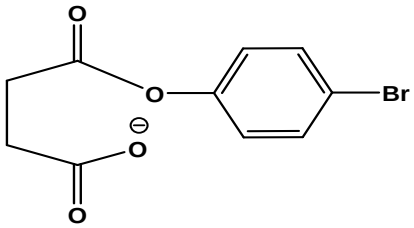

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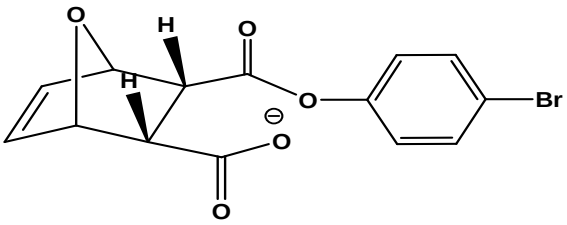

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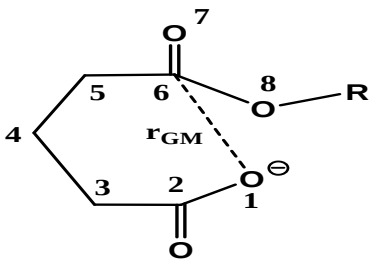
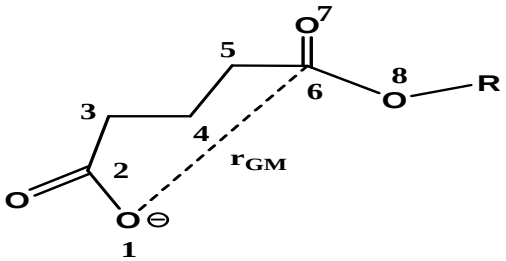
Figure 3. Chemical structures of di-carboxylic semi-esters 1-5.



***Syn* (condensed) conformation**



***Anti* (extended) conformation**

R = *p*-bromophenyl or atovaquone moiety

Figure 4. Schematic representation of the reactants in the cyclization reactions of dicarboxylic semi-esters 1-5. r_{GM} is the distance between the nucleophile (O1) and the electrophile (C6).

Furthermore, it was found that the activation energy in systems **1**-**5** is dependent on the difference in the strain energies of the tetrahedral intermediates and the reactants, and there is no correlation between the cyclization rate and the distance between the nucleophile and the electrophile, r_{GM} (Figure 4). Therefore, the intraconversion rate of atovaquone prodrugs to atovaquone can be programmed according to the nature of the prodrug linker.[79]

Given the severity of malaria, continual development of drug resistance and the undesirable safety profile of some existing medications, efforts were directed toward development of more effective and better tolerated medications with lower propensity to develop resistance, intended for the treatment of this endemic disease.[1, 3-26]

ATQ holds promise in malaria treatment, owing to its unique mechanism of action, effectiveness and safety. However, ATQ has poor oral bioavailability (<10% under fasted condition) and variable oral absorption, and this is due to its poor aqueous solubility (<0.2 μg/ml) that results from its lipophilic structure (log P = 5).[110] Consequently, this results in low and variable plasma and intracellular levels of the drug which is an important determinant of therapeutic outcome.[111] It was demonstrated that low drug plasma concentrations is a powerful means for the promotion of resistant parasites.[112] that leads to an increased morbidity and mortality among children.[113-115] ATQ oral bioavailability can be increased either by fatty food intake.[116-117] or administering larger amount of the drug to recompense for low oral absorption and to reach therapeutic plasma concentrations.[17] This practice is considered to be costly with expensive drugs like ATQ. Altogether, these procedures hinder the use of ATQ in poor developing countries in the time in which ATQ is considered to be the standard antimalarial drug.[25]

Thus, the adoption of strategies to protect ATQ from parasites resistance is an urgent need.[13] In the view of this background and continuing our study on the design and synthesis of ATQ prodrugs,[118] ATQ ProD 1 was synthesized through linking ATQ to a di-carboxylic semi-ester linker, succinic anhydride (Bruice's enzyme model), to produce a system that is more hydrophilic than its parental drug, and is able to release ATQ in a chemically driven controlled manner, once it reaches the blood circulation system.

Consequently, this novel ATQ prodrug has the potential to serve in providing an alternative treatment option to the medical community that may help in addressing the critical need in malaria treatment.

ATQ ProD 1 is expected to fulfill the following requirements: (1) enhanced water solubility, (2) improved oral bioavailability, (3) controlled release rate, (4) predicted plasma levels and (5) improved antiparasitic activity.[79]

Accumulating evidence suggests that ATQ low solubility and hence low oral bioavailability and variable plasma concentration limits ATQ inherent efficacy.[1, 26, 119-120]

In addition, several studies reported that sufficiently high ATQ plasma levels should be achieved to obtain the desired therapeutic response.[26] It was demonstrated in clinical study with a conventional tablet formulation, that the therapeutic response of ATQ against Pneumocystis carinii Pneumonia is reliant on plasma steady-state levels of the drug.[17] Moreover, Chung, Ferreira et al. reported that resistance to ATQ is believed to be a result of low and variable plasma levels that stem from variable oral absorption. This conclusion is supported by the fact that inconsistent drug plasma levels provide the parasite with the opportunity to form resistance against drugs. All of the above mentioned complications result from the lipophilic nature of ATQ.

Despite of this, there is a general agreement that the solution to this problem is feasible.[1, 26, 119-120] Therefore, recently several different techniques were adopted in order to minimize the solubility and bioavailability problems of ATQ.

1.2. Approaches adopted to enhance ATQ aqueous solubility.

Atovaquone was firstly commercialized as tablets (Mepron®), from which complete oral bioavailability can't be achieved. About 21% absolute bioavailability of Mepron® tablets was obtained in HIV seropositive volunteers in the fed state. [121] Therefore, different groups have focused on improving ATQ solubility via several approaches such as improving ATQ formulation. Strategies that were employed focused on an increment of the specific surface area of atovaquone particles and/or its solubility in adequate solvents or micelles to facilitate its dispersion in aqueous media. For example, Cotton developed a micronized ATQ suspension and compared it with ATQ tablets, and he found that the micronized ATQ suspension achieved 2-fold increase in drug bioavailability compared to that with tablet formulation of the same dose. [122] These findings were reported in both the fed and fasted state. [120-123] It was indicated earlier that ATQ absorption can be increased when administered with food [117-120] for both tablet and suspension formulations. An increase of 1.4-fold in ATQ

absorption was obtained in the fed state, compared to that achieved in the fasting state, [117] and this value can be higher depending on the fat content of the meal.[121]

Additional methods to improve atovaquone oral bioavailability have been exploited, including the development of nanosuspensions, [120, 124] self-micro emulsifying drug delivery systems,[125] liposomes.[126] and polymer nano-capsules.[127]

In this context, Dearn and coworkers reported that an administration of ATQ in micro fluidized suspensions of 0.1-2 μm leads to 2.6-fold increase in oral relative bioavailability than when a typical suspensions (of about 3 μm) was used.[124]

In another interesting study, Sek and coworkers examined the influence of a number of surfactants; self-microemulsifying drug delivery systems (SMEDDS), on the oral bioavailability of lipid based formulations of atovaquone.[125] Their study revealed no differences in beagle dogs when comparing two different SMEDDS. In contrast, the relative oral bioavailability in dogs of atovaquone was about 3-fold higher when incorporated in these self-microemulsifying drug delivery systems SMEDDS than when formulated as an aqueous suspension.

Another fascinating option is the association of this drug with bio-adhesive nanoparticles. In this case the strategy combines an increase of the specific surface area of the drug delivery system with the ability of these nanoparticles to develop adhesive interactions within the gut mucosa,[128] which may assist in the formation of a concentration gradient between the dosage form and the gut mucosa, thus enhancing absorption potential.

Despite of being attractive options, the mentioned strategies add other steps to the process, in addition to the increased cost. Another option was to modify the structure of the drug in such a way to enhance poor bioavailability by increasing aqueous solubility of the drug.[17]

Hage et al. have synthesized new atovaquone derivatives, in which ATQ was substituted at the 3-hydroxy group by ester and ether functions. [129] The compounds were assessed in vitro for their activity against the growth of Plasmodium falciparum. It was demonstrated that all the compounds exhibited potent activity, with IC50 values in the range of 1.25-50 nM, comparable to those of atovaquone and much higher than chloroquine or quinine. [17, 129]

On the other hand, Comley and Karaman have shed light on modifying ATQ at the structural level. However, their strategy was to link ATQ to a water soluble moiety to develop prodrugs rather than developing ATQ derivatives to enhance aqueous solubility.

Comley developed the carbamate prodrug of ATQ, 17C91, and compared plasma levels of 17C91 with the micronized ATQ suspension in a severe combined immuno-deficient mouse model of Pneumocystis carinii pneumonia (PCP). Comley found a 3 fold increase in plasma levels of ATQ compared to that with micronized ATQ suspension, which indicates that ATQ prodrugs are superior over both tablets and micronized suspensions.

Comley's prodrug 17C91 system has proved its efficacy and this confirms our expectations for improvement of ATQ physicochemical properties using the linker approach. However, 17C91 releases ATQ very rapidly ($t_{1/2}$ = 3 minutes at pH 7.4),[130] in pH dependent manner without any control on the release rate of the drug.

Another group adopted the development of ATQ prodrugs,[17] in order to improve ATQ aqueous solubility. The new prodrug, 3-(5-methyl-2-oxo-l, 3-dioxol-4-yl) methyloxy-2-trans- [(4-chloro phenyl) cyclohexyl] [l, 4] naphthoquinone, was synthesized by a condensation of atovaquone with 5-methyl-4-chloromethyl dioxalone (III) in a suitable solvent.[17]

The main advantage of Karaman's proposed prodrugs lies in their ability to release ATQ via chemical cleavage in a controlled manner depending on the nature of the linker. This ensures a sufficient ATQ plasma levels that can be maintained for a long time due to controlled ATQ releasing rate, which subsequently increases the probability of ATQ therapeutic success.

It is worth noting that enzymes have a significant role in prodrugs transformation into their active parent forms. Many of the marketed prodrugs undergo hydrolysis to release their active parent drugs, catalyzed by peptidases, phosphatases, carboxylesterases or esterases.[131] However, this pathway is associated with obstacles that hinder its usefulness. For instance, the incomplete absorption obtained with several hydrolytic-enzymes-activated prodrugs of antibiotics, and angiotensin-converting enzyme inhibitors, such as enalaprilate, leads to about 50% bioavailability, because of their premature hydrolysis by esterases during the absorption process. [131] Additional important issue is the bioactivation of the prodrug by cytochrome P450 enzymes. The latter are a class of enzymes that accounts for about 75% of all enzymatic

metabolisms of drugs, including several prodrugs. There is increasing evidence that genetic polymorphisms of prodrug-activating cytochrome P450 enzymes significantly contribute to the variability in prodrug activation and thus to efficacy and safety of drugs utilizing this pathway.[132-135]

All together, the prodrugs chemical approach involving enzyme catalysis is perhaps the most vulnerable and unpredicted approach, because there are many intrinsic and extrinsic factors that can affect the bioconversion mechanisms. For example, the activity of many prodrug activating enzymes may be flocculated due to genetic polymorphisms, age-related physiological changes or drug interactions, leading to adverse pharmacokinetic, pharmacodynamic and clinical effects. In addition, there are wide interspecies variations in both the expression and function of most of the enzyme systems activating prodrugs which could lead to serious challenges in the preclinical optimization phase.[131, 136]

Herein, lies the significance of the of Karaman's prodrugs approach in which the prodrug is intraconverted by chemical means into the active parent drug without the involvement of metabolic enzymes, and so the challenges associated with the enzymatic hydrolysis are avoided. In addition, programming the drug's release rate utilizing Karaman's prodrugs approach allows for sufficient ATQ levels that are maintained long enough in the plasma. Therefore, ATQ frequent administrations can be replaced with once daily dose which can lead to improved patient compliance.

2-EXPERIMENTAL

2.1 General

Inorganic salts were of analytical grade and were used without further purification. Organic buffer components were distilled or recrystallized. Distilled water was redistilled twice before use from all-glass apparatus. Succinic anhydride, anhydrous sodium dihydrogen phosphate, triethylamine, dioxane, acetonitrile (ACN), hexane, ethyl acetate, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), NaOH, methanol (MeOH), magnesium sulfate anhydrous and atovaquone were commercially obtained from sigma Aldrich. HPLC grade solvents of methanol, acetonitrile and water were purchased from Sigma Aldrich. High purity dichloromethane, THF and diethyl ether (> 99%) were purchased from Biolab (Israel). The LC/ESI-MS/MS system used was Agilent 1200 series liquid chromatography coupled with a 6520 accurate mass quadruple-time of flight mass spectrometer (Q-TOF LC/MS). The

analysis was performed in the positive electrospray ionization mode. The capillary voltage was 4.0 kV; the scanned mass range was 200-540 *m/z* (MS).

The high pressure liquid chromatography (HPLC) system consisted of an Alliance 2695 module equipped with 2996 Photodiode array detector from Waters (Germany). Data acquisition and control were carried out using Empower 2 ™ software (Waters, Germany). Analytes were separated on a 4.6 mm x150 mm XBridge® C8 column (5 µm particle size) used in conjunction with a 4.6 x 20 mm, XBridge® C8 guard column. Microfilters of 0.45µm porosity were normally used (Acrodisc® GHP, Waters). pH meter model HM-30G: TOA electronics™ was used in this study to measure the pH value for the buffers. The Sep-Pack C8 6cc (1 g) cartridges were purchased form Waters (Milford, MA, USA). ^{1}H-NMR experiments were performed with a Bruker AvanceII 400 spectrometer equipped with a 5 mm BBO probe. All infrared spectra (FTIR) were obtained from a KBr matrix (4000–400 cm^{-1}) using a PerkinElmer Precisely, Spectrum 100, FT-IR spectrometer.

2.2 Preparation of atovaquone succinic prodrug, ATQ ProD 1 (Figure 5)

In a 250 mL dried round-bottom flask placed on an ice bath, 500 mg of atovaquone (1.363 mmol) was dissolved in THF (50 mL), dry sodium hydride (4.089 mmol) was added to the THF solution and the reaction mixture was stirred at 10 ℃. After 30 minutes, 136 mg (1.363 mmol) of succinic anhydride was added to the reaction mixture, the ice bath was removed and the reaction mixture was stirred at room temperature for 3 days and monitored by TLC using ethyl acetate and hexane (1:5) system as an eluent. Few drops of 1N HCl were slowly added to the reaction mixture to destroy the remaining unreacted sodium hydride and the reaction mixture was evaporated by rotary evaporator to dryness. The product, ATQ ProD 1 was purified using conventional column chromatography. The column was prepared using silica gel and the starting eluent was 10% ethyl acetate and 90% hexane. The polarity of the eluent was gradually increased until a complete recovery of the product. The collected fractions were evaporated by rotary evaporator, then the resulting pale yellow prodrug was dried to yield 69% product which was fully characterized. M.P. 280 $^{\circ}$C; IR (KBr/ν_{max} cm^{-1}), 3379 (-OH), 1750 (C=O), 1647-1659 (C=O), 1596 (aromatic C=C) 727 (arylchloride); 1HNMR (400Hz, DMSO-d_6) δ 7.99(dd, 2H), 7.84(dt, 1H),7.77(dt, 1H), 7.33(s, 4H),3.31(s, 3H),3.07 (t, 1H), 2.56(t, 1H), 2.22(q, 2H),2.12(d, 2H),1.65(d, 2H), 1.56 (q, 2H), 1.49 (s, 1H), *m/z* 467 (M+1).

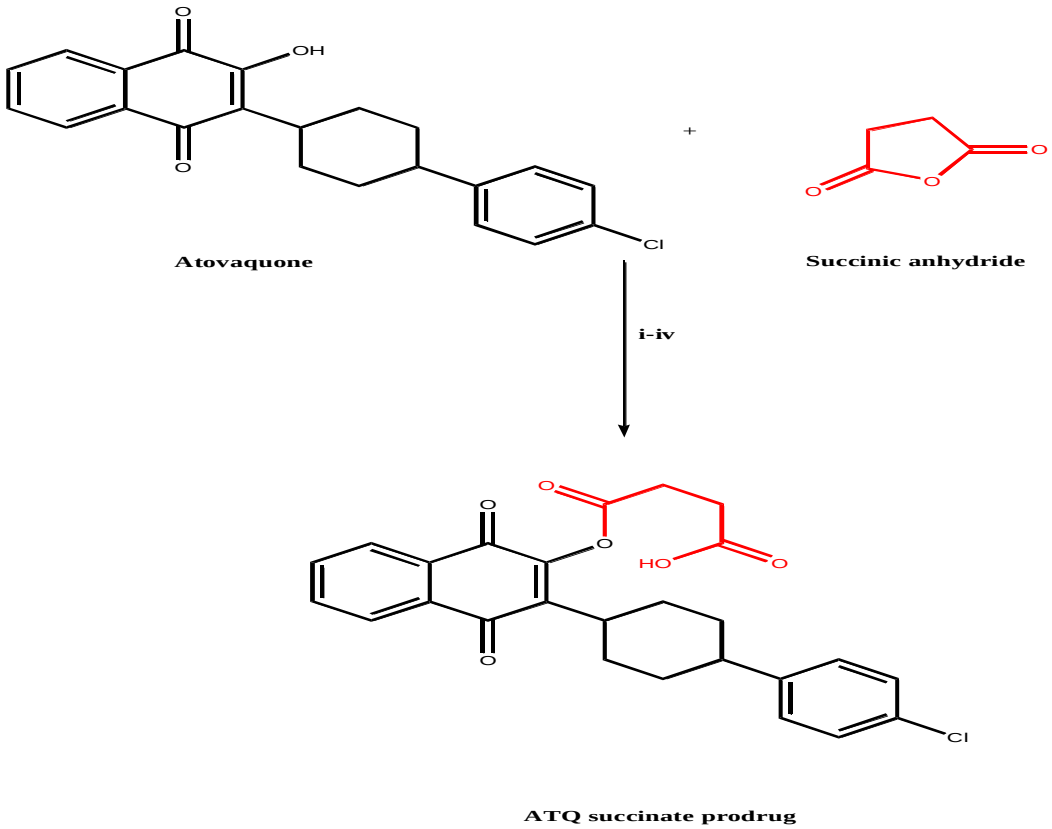


Figure 5. Synthesis of ATQ ProD 1from ATQ and succinic anhydride. Reagents and conditions: (i)-(ii) THF and an excess NaH, (iii) 30 minutes at10 10℃ followed by (iv) room temperature for three days.

2.3 Kinetic study of ATQ succinate prodrug (ATQ ProD 1) at different buffer conditions

2.3.1 Buffer preparation

7 gr. KH_2PO_4 was dissolved in 1L water, this yielded a solution that has a pH of 4.2 then pH was adjusted by either NaOH or 1N HCl to get the desired pH buffer.

2.3.2 Stock solutions

1000 ppm stock solution of each ATQ and ATQ succinate prodrug were prepared by dissolving 25 mg ATQ or ATQ ProD1 in 25 mL DMF placed in a volumetric flask.

2.3.3 Dilution

A 200 ppm of ATQ and ATQ ProD 1 solutions were prepared by transferring 2 mL of 1000 ppm stock solutions of each ATQ and ATQ ProD 1 to a 10 mL volumetric flask and dilution using DMF and the corresponding buffer.

Because both ATQ and ATQ ProD 1have poor solubility in the three buffers, dilution was made by adding 5mL DMF and the volume was completed with each corresponding buffer to achieve clear solutions.

Interconversion of ATQ ProD 1 solution, in 1N HCl, buffer pH 2.2, buffer pH 5.5 or buffer pH 7.4, to its active parent drug, ATQ, was followed by HPLC (C8 column) at a wavelength of 280 nm. The interconversion reactions were run mostly at 37.0 ˚C. Then the disappearance of ATQ ProD 1 with time was followed. Concentration versus time was plotted and the hydrolysis rate at different buffers was calculated.

3- RESULTS AND DISCUSSION

The effective molarity (EM) value is generally used as a measure for intramolecular processes efficiency. It is defined as a ratio of the intramolecular reaction rate and its corresponding intermolecular reaction rate where both reactions are driven by the same mechanism. Values of 10^9-10^{13} M have been measured for the EM in intramolecular processes occurring through nucleophilic addition.[92, 137]

The experimental relative rates for the cyclization reactions of dicarboxylic semi-esters **1**-**5** (Figure 3) were obtained from the division of the intramolecular rate and the corresponding intermolecular reaction. [103-104] For obtaining the relative rates (effective molarity, EM) for ATQ ProD 1 process we assume that its corresponding intermolecular process is similar to that for systems **1**-**5**.

Since an excellent correlation was obtained between the activation free energy values ($\Delta G^{\ddagger}$) for **1**-**5** and the difference in the strain energy values of the reactants and intermediates, $\Delta Es_{(INT-GM)}$ (Eq. 1), the calculated value of $\Delta Es_{(INT-GM)}$ for ATQ ProD 1 was used in Eq. 1 to calculate its corresponding relative rate ($\log k_{rel}$) as shown in equation 1.

$$\log k_{rel} = [(\Delta G^{\ddagger} - 4.6065)/1.1098 - 13.842]/-1.4016 \quad (1)$$

The calculated $\log k_{rel}$ by equation 1 for ATQ ProD 1 was 6.50.

Using the experimental $t_{1/2}$ (the time needed for the conversion of 50% of the reactants to products) value for process **1**, 3.75 hours [103-104] and the calculated $\log k_{rel}$ values for ATQ ProD 1 we have calculated the $t_{1/2}$ value for the degradation of ATQ ProD 1 to its active parent drug, atovaquone, and the value obtained was 26.4 hours.

3.1 Hydrolysis study

The kinetics of the hydrolysis study of atovaquone ProD 1 was carried out in aqueous buffer in the same manner as that done by Bruice and Pandit [103-104] on di-carboxylic semesters **1-5** (Figure 3). This is in order to explore whether the prodrug hydrolyzes in aqueous medium and to what extent or not, suggesting the fate of the prodrug in the system. The hydrolysis kinetics of the synthesized atovaquone ProD 1 was studied in four different aqueous media: 1 N HCl, buffer pH 2.2, buffer pH 5.5 and buffer pH 7.4. Under the experimental conditions the target compounds hydrolyzed to release the parent drug as evident by HPLC analysis. At constant pH and temperature the reaction displayed strict first order kinetics as the k_{obs} was fairly constant and a straight plot was obtained on plotting log concentration of residual prodrug verves time. The rate constant (k_{obs}) and the corresponding half-lives ($t_{1/2}$) for atovaquone ProD 1 in the different media were calculated from the linear regression equation correlating the log concentration of the residual prodrug verses time. The kinetic data are listed in Table 1. The 1N HCl, pH 2.2 and pH 5.5 were selected to examine the interconversion of atovaquone ProD 1 in pH as of stomach, because the mean fasting stomach pH of adult is approximately 1-2 and increases up to 5 following ingestion of food. In addition, buffer pH 5.5 mimics the beginning small intestine pathway. Finally, pH 7.4 was selected to examine the interconversion of the tested prodrug in blood circulation system.

Table 1. $t_{1/2}$ values for atovaquone ProD 1 at different pH values.

Medium	**$t_{½}$ (h)**
1N HCl	11.4 hours
Buffer pH 2.2	10.9 days
Buffer pH 5.5	24 hours
Buffer pH 7.4	28.8 hours

According to Bruice's proposed mechanism of dicarboxylic acid semi ester hydrolysis and based on the results listed in Table 1 it can be concluded that ATQ ProD 1 hydrolysis rate is decreased as the pH of the medium is increased. This is because at basic pH the OH group of the succinic moiety becomes ionized (deprotonated), consequently the nucleophilicity of this group will be enhanced, and this facilitates the nucleophilic attack of the OH group on the electrophilic center (C=O) (Figure 6).

It should be indicated that this explanation can be applied in the cases where the pH of the medium is higher than the pK_a of the carboxylic acid of the succinic moiety, pH 5.5 and pH 7.4. In 1N HCl and pH 2.2 the prodrug transformation into its active parent drug occurs by a

mechanism other than cyclization reaction. It is most likely to proceed by general acid catalyzed hydrolysis. This conclusion is supported by the fact that the hydrolysis rate in 1N HCl is higher than at pH 2.2 due to the former being more acidic (higher H^+ concentration) than the latter.

Based on the results depicted in Table 1, an oral administration of ATQ succinate prodrug is feasible when administered as enteric coated tablets, in order to avoid premature interconversion of the prodrug into its active parent drug in the stomach, thus allowing the prodrug to be released in the small intestine, where the prodrug to drug transformation will take place by chemical means in a controlled manner, without relying upon any involvement of any metabolic enzyme to catalyze the release of the active parent drug.

The combination of releasing ATQ in a controlled manner and the long $t_{1/2}$ of ATQ ProD 1, allows for once daily administration of the drug. This contributes in improving patient compliance and hence the treatment outcomes. Furthermore, a controlled release of the parent drug constitutes a major advantage for highly lipophilic drugs such as ATQ, since a rapid release leads to drug precipitation and poor re-dissolution.[1-6, 136]

Based on the in vitro kinetics of ATQ ProD 1, we can say that the idea of simple administration of effective drugs that treat endemic diseases like malaria becomes feasible and applicable.

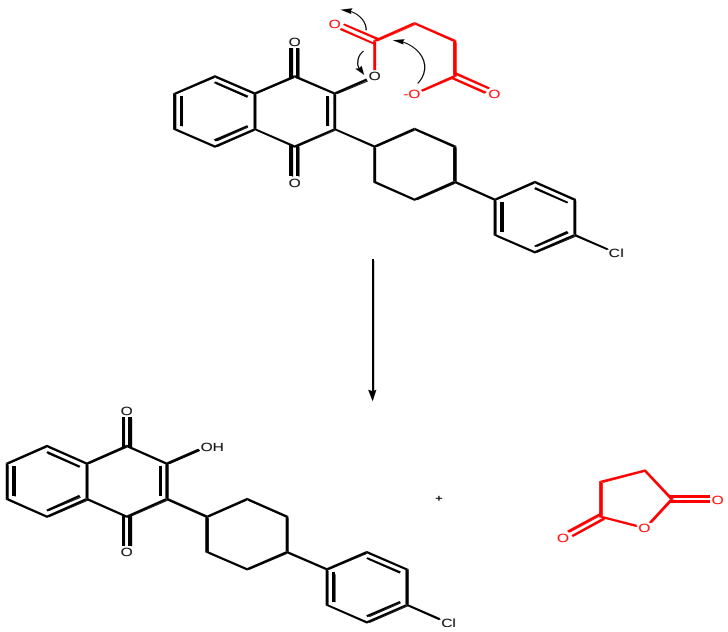


Figure 6. The hydrolysis of ATQ ProD 1 in the body.

It is worth noting that succinic anhydride is particularly considered suitable linkers, because (1) it is produced in the body in cribs' cycle and it is non-toxic in the administered doses of ATQ ProD 1 and (2) it becomes ionized at physiological pH, hence, it contributes to the increased solubility of the prodrug.

4-SUMMARY AND CONCLUSION

Malaria is an endemic global disease and malaria control programs need efficacious drugs that can be used easily by the populations of endemic countries. Several medicines are used to treat malaria; however, the limited efficacy, severe side effects and the appearance of parasitic resistance limit their use. Several lines of evidence indicate that the emergence of drug resistant parasites stimulate research groups to explore different approaches in an attempt to improve the characteristics of existing medications.

Atovaquone, a naphthoquinone and ubiquinone analogue, an effective drug, inhibits the electron transport in the parasite leading to its death resulting in an excellent safety profile. Nevertheless, it has poor bioavailability and it is too expensive.

ATQ formulation improvements have been achieved and showed better bioavailability than conventional tablet dosage form. In addition, new synthesized ATQ prodrugs lead to improved efficacy, pharmacokinetics profile and reduced toxicity. Further, these prodrugs have saved money and time which is an important issue in drug development. For example, ATQ prodrug 17C91 showed a 3-fold increase in bioavailability over the improved ATQ formulation (micronized suspension) and conventional tablets.

Our novel ATQ prodrug, ATQ ProD 1has shown promising results and we hope that such prodrug which possesses superior properties over ATQ and existing ATQ prodrugs, in terms of efficacy, physicochemical properties and drug releasing rate will be effective once tested in vivo.

Based on the planned in vivo testing other ATQ prodrugs might be synthesized for achieving maximum bioavailability and the best clinical profile.

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