Sveučilište u Zagrebu
FARMACEUTSKO-BIOKEMIJSKI FAKULTET

Domagoj Dinter

CITOGENETIČKI UČINCI ATOVAKVONA I PROGVANIL HIDROKLORIDA NA LJUDSKIM LIMFOCITIMA *IN VITRO*

DOKTORSKI RAD

Zagreb, 2020.

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CYTOGENETIC EFFECTS OF ATOVAQUONE AND PROGUANIL HYDROCHLORIDE ON HUMAN LYMPHOCYTES *IN VITRO*

DOCTORAL THESIS

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Mentorice:
prof. dr. sc. Ana-Marija Domijan
prof. dr. sc. Vera Garaj-Vrhovac

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Supervisors:
prof. Ana-Marija Domijan, PhD
prof. Vera Garaj-Vrhovac, PhD

Zagreb, 2020.

Ovaj je doktorski rad izrađen na Sveučilištu u Zagrebu Farmaceutsko-biokemijskom fakultetu te u Jedinici za mutagenezu, Instituta za medicinska istraživanja i medicinu rada pod vodstvom prof. dr. sc. Ana-Marije Domijan, znanstvene savjetnice i prof. dr. sc. Vere Garaj-Vrhovac, znanstvene savjetnice, u sklopu Sveučilišnog poslijediplomskog studija na Sveučilištu u Zagrebu Farmaceutsko-biokemijskom fakultetu.

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SAŽETAK

Malarija je zarazna bolest crvenih krvnih stanica uzrokovana parazitom roda *Plasmodium* koja se na čovjeka prenosi ubodom zaražene ženke komarca vrste *Anopheles*. Gotovo pola svjetske populacije izloženo je riziku od malarije od čega najveći rizik i učestalost zaraze obuhvaća područje sub-saharske Afrike, ali i područja kao što su Jugo-istočna Azija, Južna i Centralna Amerika te Oceanija. Među najugroženijima skupinama su djeca, trudnice, osobe oslabljenog imuniteta te česti putnici koji se zadržavaju ili posjećuju rizična područja bilo zbog posla ili odmora. Najčešće korišteni lijekovi za liječenje i prevenciju malarije su atovakvon i progvanil hidroklorid, no važno je utvrditi rizik od mogućih posljedica produženog i učestalog uzimanja tih lijekova. Samo po sebi se nameće da posljedice uzimanja lijekova ne smiju biti veće od rizika od oboljenja malarijom. Stoga je cilj ovog istraživanja bio procijeniti toksikološku sigurnost te mehanizme djelovanja atovakvona i progvanil hidroklorida, svakoga pojedinačno, ali i u kombinaciji na ne-ciljnim ljudskim stanicama. U tu svrhu, istraživanja su provedena na ljudskim limfocitima periferne krvi kao osjetljivim pokazateljima stupnja izloženosti fizikalnim i kemijskim agensima. U istraživanju su korištene klinički relevantne koncentracije atovakvona i progvanil hidroklorida. To su koncentracije koje su utvrđene u ljudskoj plazmi kada je korištena fiksna doza kombinacije atovakvona i progvanil hidroklorida: 2950, odnosno 130 ng/mL nakon profilaktičkog liječenja te 11800, odnosno 520 ng/mL nakon liječenja malarije. U istraživanju je korišten i izolat jetre štakora (tzv. S9 frakcija) koji sadrži enzime biotransformacije te se kao takav rutinski koristi u citogenetičkim i molekularno-biološkim istraživanjima. Rezultati su pokazali da pojedinačni tretman atovakvonom nije uzrokovao značajni citogenotoksični učinak bez obzira na koncentraciju, vrijeme tretmana ili dodatak S9 frakcije te je zaključeno da atovakvon u ispitivanim klinički relevantnim koncentracijama nije citogenotoksičan te da je siguran sa stanovišta toksikološke sigurnosti. Nasuprot, pojedinačni tretman progvanil hidrokloridom uzrokovao je značajni citogenotoksični učinak u ovisnosti o koncentraciji, vremenu tretmana i dodatku S9 frakcije, ukazujući na potencijalno citogenotoksično djelovanje progvanil hidroklorida i njegovog metabolita ciklogvanila u klinički relevantnim koncentracijama. Kombinacija ovih dvaju lijekova uzrokovala je značajni citogenotoksični učinak u ovisnosti o koncentraciji, vremenu tretmana i dodatku S9 frakcije, ukazujući na potencijalno citogenotoksično djelovanje atovakvona i progvanil hidroklorida, ali i metabolita progvanil hidroklorida, ciklogvanila u klinički relevantnim koncentracijama. Kombinacija ovih dvaju lijekova nije uzrokovala promjene u parametrima oksidacijskog stresa, što pokazuje da

oksidacijski stres nije uključen u njihov mehanizam djelovanja. Iako su klinički relevantne koncentracije atovakvona i progvanil hidroklorida u kombinaciji uzrokovale citogenotoksične učinke na ljudskim limfocitima periferne krvi, dobiveni rezultati pokazuju da je kombinacija ovih dvaju lijekova relativno sigurna sa stanovišta citogenotoksičnosti, naročito ako se ona koristi za profilaksu. Korištene metode pokazale su se učinkovitima u otkrivanju citogenotoksičnog potencijala ove vrste lijekova i mogle bi se koristiti kao alternativa standardnim citogenetičkim testovima u ranom otkrivanju citogenotoksičnosti kandidata za potencijalne lijekove. Unatoč tome, ova studija je potvrdila potrebu za daljnjim citogenetičkim ispitivanjima te redovitim praćenjem pacijenata kako bi se smanjio rizik od neželjenih učinaka, osobito među pojedincima koji često putuju u malarična područja.

Ključne riječi: *atovakvon, progvanil hidroklorid, ciklogvanil, citogenotoksičnost, oksidacijski stres, ljudski limfociti periferne krvi*

SUMMARY

Background: Malaria is a major cause of death in the tropics, and antimalarial drugs have played a key role in controlling its spread through the treatment of patients infected with plasmodial parasites and the control of its transmissibility. On the other hand, antimalarial drugs may exert adverse effects that can sometimes be serious. Therefore, antimalarial agents should fulfil the requirements of efficacy towards the parasite, in addition to being safe for the consumer and not putting them at an additional risk of adverse effects especially when used for prophylaxis. Atovaquone and proguanil hydrochloride is a fixed-dose combination of antimalarial agents used primarily for prophylactic treatment and also for treatment of malaria. This combination interferes with two different pathways. Atovaquone is a selective inhibitor of parasite mitochondrial electron transport, while proguanil hydrochloride primarily exerts its effect by means of the metabolite cycloguanil, a dihydrofolate reductase inhibitor. Inhibition of dihydrofolate reductase in the malaria parasite disrupts deoxythymidylate synthesis.

Aim and methods: The aim of this study was to investigate cyto/genotoxic potential of atovaquone and proguanil hydrochloride, either alone or in combination, towards human peripheral blood lymphocytes *in vitro* and their possible mechanism of toxicity. Since antimalarial drug toxicity is viewed differently depending upon whether the clinical indication is for malaria treatment or prophylaxis, two different concentrations of atovaquone and proguanil hydrochloride were used with and without S9 metabolic activation. The concentrations used were those found in human plasma when a fixed-dose combination of atovaquone and proguanil hydrochloride was used: 2950/130 ng/mL after prophylactic treatment and 11800/520 ng/mL after treatment of malaria, respectively. In this kind of assessment, combinations of different methods may play an important role in the evaluation of cyto/genotoxic damage caused by this type of drugs, and these methods make it possible to evaluate the level of cell and DNA damage even after short-term exposure to potentially cyto/genotoxic agents. Therefore, assessment of cyto/genotoxic potential was performed by means of cell viability (cytotoxicity) assay with acridine orange and ethidium bromide, whereas an alkaline version of the comet assay was applied for the evaluation of the genotoxic potential. Moreover, to explore the possible involvement of oxidative stress in the genotoxicity of this antimalarial drug combination, we used a formamidopyrimidine-DNA glycosylase (Fpg)-modified version of the comet assay that detects oxidative DNA damage, in

addition to assessment of malondialdehyde and glutathione levels as biomarkers of lipid peroxidation and oxidative stress in total.

Results: Atovaquone alone did not have any impact on cell viability and DNA damage based on the cytotoxicity assay as well as comet assay descriptors tested (tail length, tail intensity and tail moment) in clinically relevant concentrations on human peripheral blood lymphocytes *in vitro* regardless of exposure times or addition of S9 metabolic activation. These results indicate that atovaquone and its metabolites have no effect on DNA molecule and are safe from the aspect of cyto/genotoxicity. On the contrary, proguanil hydrochloride alone, in clinically relevant concentrations, had an impact on cytotoxicity and DNA integrity of human peripheral blood lymphocytes *in vitro* especially at higher concentration used for treatment of malaria, longer exposure times and with addition of S9 metabolic activation which shows that proguanil effect on cells and DNA molecule are mainly influenced by its metabolite cycloguanil. When tested in combination, atovaquone and proguanil hydrochloride displayed weak cyto/genotoxicity towards human peripheral blood lymphocytes with no impact on oxidative stress parameters, suggesting that oxidative stress is not implicated in their mechanism of action. Given that the greater part of cyto/genotoxic effect is induced after S9 metabolic activation, it is to presume that principal proguanil hydrochloride metabolite cycloguanil has the major impact on DNA molecule. Moreover, the used methods, especially the comet assay, proved to be useful in detecting cyto/genotoxicity of this type of drugs and could be used as an alternative to standard cytogenetic assays in early cyto/genotoxicity screening of drug candidates.

Conclusions: Overall, the obtained results indicate that the atovaquone and proguanil hydrochloride combination is relatively safe from the aspect of cyto/genotoxicity, especially if used for prophylactic treatment. Nevertheless, the present study has also confirmed the need for further cytogenetic research and regular patient monitoring to minimize the risk of any adverse event especially among frequent travellers to malaria endemic areas. Moreover, the obtained results could also benefit clinicians in patient counselling regarding the selection of most appropriate prophylactic treatment.

Keywords: *atovaquone, proguanil hydrochloride, cycloguanil, cyto/genotoxicity, oxidative stress, human peripheral blood lymphocytes*

SADRŽAJ

1. UVOD

1.1. Malarija

Malarija je teška i po život opasna zarazna bolest crvenih krvnih stanica uzrokovana parazitom roda *Plasmodium.* Naziv je nastao iz lat. *malus aria* (tal. *mal'aria*, „loš zrak"), a označava pretežno tropsku zaraznu bolest koja se na čovjeka prenosi ubodom zaražene ženke komarca vrste *Anopheles* koji je domaćin spolnog ciklusa plazmodija. Osim putem komarca, malarija se može prenijeti i putem transfuzije zaražene krvi ili nečistih igli iako je danas takav način prijenosa bolesti rijedak, a i takav oblik malarije je obično blaži jer se preskače jedan dio razvojnog ciklusa plazmodija. Iznimno rijetko malarija se može prenijeti i sa zaraženog na zdravog pojedinca. Malarija je karakterizirana intermitentnom vrućicom, povećanjem slezene (splenomegalijom), anemijom te rekurirajućim kroničnim tijekom. Ako se pravovremeno ne liječi, bolest može uzrokovati ozbiljne komplikacije pa čak i smrt (1-17).

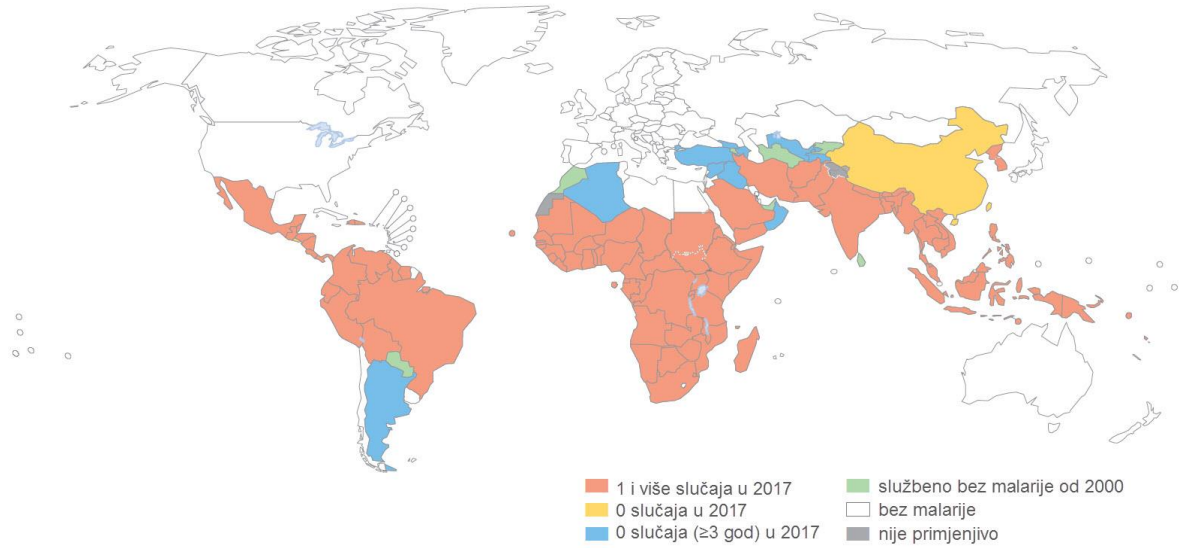
Smatra se da je malarija postojala prije više desetaka tisuća godina, a sam uzročnik *Plasmodium* i prije 30 milijuna godina. Stoga je za pretpostaviti da je uzročnik prvo postojao u praživotinja, a potom u kralježnjacima (18). Malarija je prvi puta opisana još u staro-kineskim medicinskim zapisima Nei Ching 2700 godina pr. n. e. i 1200 godina kasnije u Ebersovom papirusu. U 6. stoljeću pr. n. e. u zapisima Sushruta Samhita, indijskog liječnika Sushruta, pored 1200 različitih bolesti opisana je i malarijska groznica. Kasnije u 5. i 4. stoljeću pr. n. e., Hipokrat je u velikim epidemijama malarije u Grčkoj prepoznao vrućicu kao jedan od osnovnih simptoma ove bolesti, koja se ponavlja u ciklusima i to svakog trećeg ili četvrtog dana. Za pohoda rimske vojske uz močvarna područja Italije malarija se redovito javljala u vojnika kao tzv. rimska groznica za koju se uzrokom smatrao loš zrak (tal. *mal'aria*) te je od tuda bolest i dobila ime. Već u 2 st. pr. n. e. u liječenju malarijske groznice u Kini koristila se biljka slatki pelin (*Artemisia annua*), kineskog naziva Qinghao. U 16. stoljeću španjolski osvajači u Peruu preuzeli su od tamošnjih Indijanaca lijek protiv malarije dobiven iz kore biljke kininovac (*Cinchona succirubra*). Stoga se od 17. stoljeća i u Europi koristi prah kore kininovca kao terapijsko, a potom i kao profilaktičko sredstvo. Iz biljke kininovac su 1820. godine francuski kemičari Pierre Joseph Pelletier i Joseph Bienaimé Caventou izolirali aktivni sastojak kinin (10,15,19-23).

Francuski liječnik Charles Louis Alphonse Laveran 1880. godine je utvrdio da je uzročnik malarije protozoa. 1898. godine engleski liječnik Ronald Ross i talijanski liječnik Giambattista Grassi istodobno su otkrili da je prijenosnik bolesti komarac u kojem se odvija

spolna faza životnog ciklusa plazmodija (sporogonija). Pedeset godina kasnije Shortt, Garnham, Covell i Shute dokazali su da plazmodiji prolaze dio nespolne faze životnog ciklusa (shizogonija) u stanicama jetre, a drugi dio u eritrocitima čovjeka (10,15,24,25). 1970. godine grupa kineskih znanstvenika predvođena Youyou Tu izolirala je iz slatkog pelina aktivnu supstancu artemisinin, antimalarik koji se ubrzo pokazao vrlo učinkovitim u liječenju malarije. Za ovo otkriće, 2015. godine Youyou Tu je dodijeljena Nobelova nagrada za fiziologiju i medicinu. Osim ove, još su četiri Nobelove nagrade dodijeljene za otkrića vezana uz malariju. Ronald Ross 1902. godine dobio je Nobelovu nagradu za otkriće i značenje komarca u biologiji uzročnika malarije, a 1907. godine ona je dodijeljena Charles Louis Alphonse Laveranu za otkriće uzročnika malarije. Julius Wagner-Jauregg 1927. godine dobio je Nobelovu nagradu za indukciju malarije kao piroterapijskog postupka u liječenju paralitičke demencije. 1947. godine Paul Hermann Mülleru dodijeljena je Nobelova nagrada za formulaciju pesticida diklor-difenil-trikloretana, poznatijeg kao DDT (9,23,26-29). DDT se opsežno primjenjivao od 1940-ih do 1960-ih godina te je imao veliku ulogu u iskorjenjivanju malarije u Europi i Sjevernoj Americi. Međutim, otkriveno je da DDT ima vrlo štetne posljedice na okoliš i ljude, pa je već 1972. godine zabranjen u SAD-u, a ubrzo zatim i u većini ostalih zemalja svijeta. Unatoč tome, u određenim iznimnim slučajevima upotreba DDT-a protiv malarije i danas je dopuštena u nekim dijelovima svijeta (30-32). U većini razvijenih zemalja zbog učinkovitosti lijekova i insekticida malarija je danas rijetka bolest, ali je zato česta u tropskim zemljama (1,2,11,13,15,21,33-38).

Prema podacima Svjetske zdravstvene organizacije (WHO, engl. *World Health Organization*) u petogodišnjem razdoblju od 2010. do 2015. godine se stopa novooboljelih od malarije smanjila za 21%, a stopa smrtnosti za 29%. Unatoč tim činjenicama, u svjetskim razmjerima malarija još uvijek predstavlja veliku prijetnju zdravlju ljudi. U 2017. godini u svijetu je zabilježeno 219 milijuna oboljelih od malarije, a od posljedica bolesti umrlo je oko 435 000 osoba, većinom na području sub-saharske Afrike. Za usporedbu, 2016. godine bilo je 451 000 smrtnih slučajeva dok je 2010. godine taj broj iznosio čak 607 000. Iako je u 2017. godini bilo i 20 milijuna manje slučajeva oboljelih od malarije nego u 2010. godini, ipak podaci za razdoblje od 2015. do 2017. godine ističu da nema značajnog napretka u smanjenju slučajeva malarije na globalnoj razini. Prema izvješću WHO-a iz 2018. godine skoro je pola svjetske populacije izloženo riziku od malarije (Slika 1). Procjenjuje se da je riziku od malarije izloženo oko 43% stanovništva u 91 zemlji, a najviše su pogođena najsiromašnija područja tropskih zemalja, posebice u Africi, Aziji, Južnoj i Centralnoj Americi te Oceaniji. Među najugroženijima skupinama su djeca do 5 godina, trudnice, osobe oslabljenog imuniteta

i česti putnici koji se zadržavaju ili posjećuju rizična područja bilo zbog posla ili odmora. Također, procijene utjecaja klimatskih promjena ukazuju na mogućnost povećanja rizika od malarije i širenje na do sada nerizična područja (15,16,39-41).



Slika 1. Karta svijeta koja prikazuje zemlje s više od jednog slučaja malarije godišnje za razdoblje unazad nekoliko godina prema izvješću o malariji Svjetske zdravstvene organizacije iz 2018 (WHO, 2018).

Prema izvješću WHO-a iz 2018. godine, u Europskoj regiji WHO-a je 2015. godine postignut prekid autohtonog prijenosa malarije, pa je 2016. godine Europska regija proglašena slobodnom od malarije. No, zbog učestalih putovanja i velike pokretljivosti ljudi, kao i komaraca u i iz malaričnih područja, u Europi postoji neprekidan unos infekcija (15,16,39-41).

Malarija je u Hrvatskoj iskorijenjena još 1964. godine iako su neki naši krajevi potencijalna malarična područja. Naime, komarci koji prenose malariju nisu kod nas potpuno iskorijenjeni, pa postoji stalna mogućnost ponovnog uspostavljanja autohtonog prijenosa, ali i endemskih žarišta. Takva potencijalno malarična područja nalaze se i u nama susjednim zemljama Makedoniji, Kosovu i Crnoj Gori. Danas se u Hrvatskoj ipak samo bilježe tzv. uneseni slučajevi, nastali u brojnim endemskim područjima malarije u svijetu, u koja stanovnici s područja Hrvatske odlaze zbog poslovnih, turističkih ili raznih drugih razloga. Posljednjih deset godina prijavljuje se prosječno devet oboljelih od malarije godišnje te su ti slučajevi pristigli iz endemskih tropskih i suptropskih krajeva, a takvi bolesnici, sve dok se pravovremeno ne otkriju i izliječe, predstavljaju stalnu opasnost s obzirom na prisutnost

komaraca koji su prenositelji bolesti i sisanjem zaražene krvi osobe koja ima malariju mogu dalje prenositi bolest na druge ljude (10,15,39).

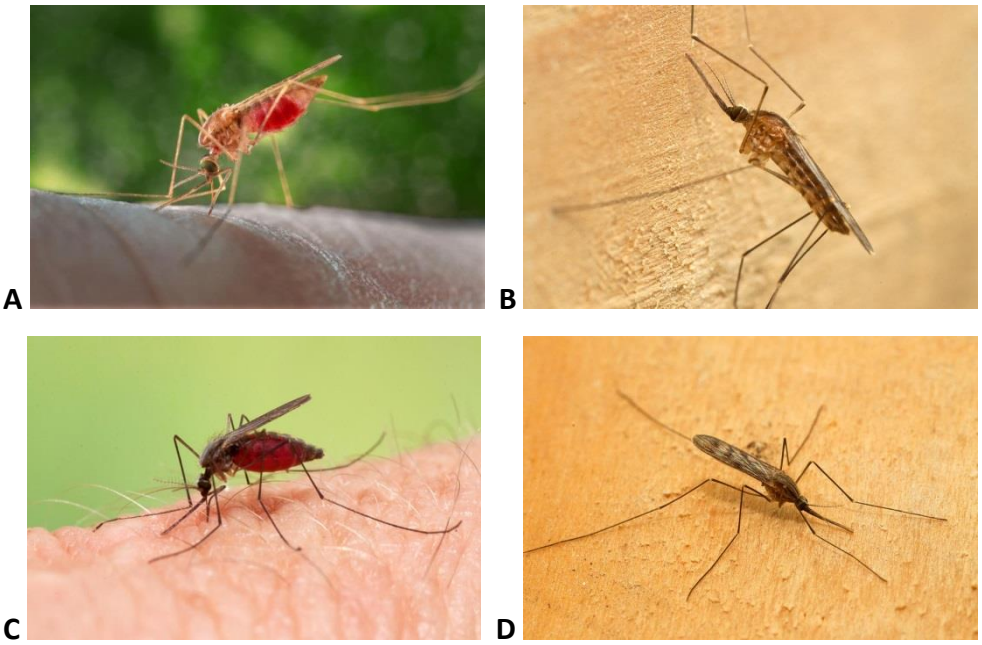
Stoga je vrlo važno djelovati preventivno, ali i pravovremeno otkrivati infekcije, liječiti oboljele, educirati ljude i prevenirati bolest suzbijanjem komaraca kako osobnom zaštitom tako i dezinsekcijom. Putnicima koji zbog posla ili turizma putuju u rizična endemska područja u epidemiološkim ambulantama zavoda za javno zdravstvo savjetuje se kako čuvati zdravlje na putovanju i kako provoditi opće mjere zaštite. Zaštita uključuje nošenje prikladne zaštitne najčešće duge odjeće, primjenu repelenata odnosno sredstava za odbijanje komaraca te korištenje zaštitnih mreža za prozore i vrata i zaštitnih mreža za spavanje koje dodatno mogu biti impregnirane insekticidima. Osim ovakvog vida zaštite propisuje se i kemoprofilaksa, odnosno preventivno uzimanje lijekova protiv malarije prije odlaska, tijekom i nakon povratka s putovanja (15,39,42,43).

1.1.1. Uzročnici malarije

Uzročnici malarije su paraziti iz carstva *Protista*, podcarstvo, *Protozoae*, red *Haemosporidae*, porodica *Plasmodidae*, rod *Plasmodium* koji se na čovjeka prenose ubodom zaražene ženke komarca vrste *Anopheles* (Slika 2) koji je domaćin spolnog ciklusa plazmodija. Danas je oko 130 plazmodija svrstano u životinjske i ljudske patogene. Za razliku od čovjeka, u životinja plazmodiji parazitiraju bez posljedica za domaćina. Kod čovjeka malariju uzrokuje nekoliko vrsta plazmodija, koji su obligatni intracelularni paraziti. To su: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, Plasmodium knowlesi, Plasmodium cynomolgi* i *Plasmodium simium* (neki od njih prikazani su na Slici 3). Posljednja četiri mogu osim čovjeka zaraziti i majmune. Osnovno svojstvo uzročnika malarije je brzo i učinkovito razmnožavanje u ljudskim stanicama i to u stanicama jetre (hepatociti) te crvenim krvnim stanicama (eritrociti). Uzročnici malarije su različito zemljopisno zastupljeni, a također razlikuju se i prema dijelu organizma koji napadaju, vremenu inkubacije, kliničkoj slici, ishodu bolesti te prema osjetljivosti na antimalarike. Morfološke razlike postoje i među razvojnim oblicima plazmodija kada se nalaze u eritrocitima (1,2,4,7,9,11,13,16,17,21,33,36,38,40,44-47).

P. falciparum je uzročnik preko 50% slučajeva malarije na svjetskoj razini. Prevladava u tropskim i suptropskim područjima Afrike i izaziva najteže oblike malarije tzv. malignu malariju, tercijarnu ili tropsku malariju. Inkubacija bolesti traje od 8 do 11 dana. *P. falciparum* brzo se replicira u ciklusima od 36 do 48 sati u zrelim i nezrelim oblicima

eritrocita, ali i unutar tkiva, tako da broj parazita u krvi nije mjera težine bolesti. Pored fatalnog ishoda moguće su i teške kronične posljedice malarije poput mentalne retardacije, hemipareze, kortikalne sljepoće, afazije i cerebralne ataksije (4,7,11,33,38,48).



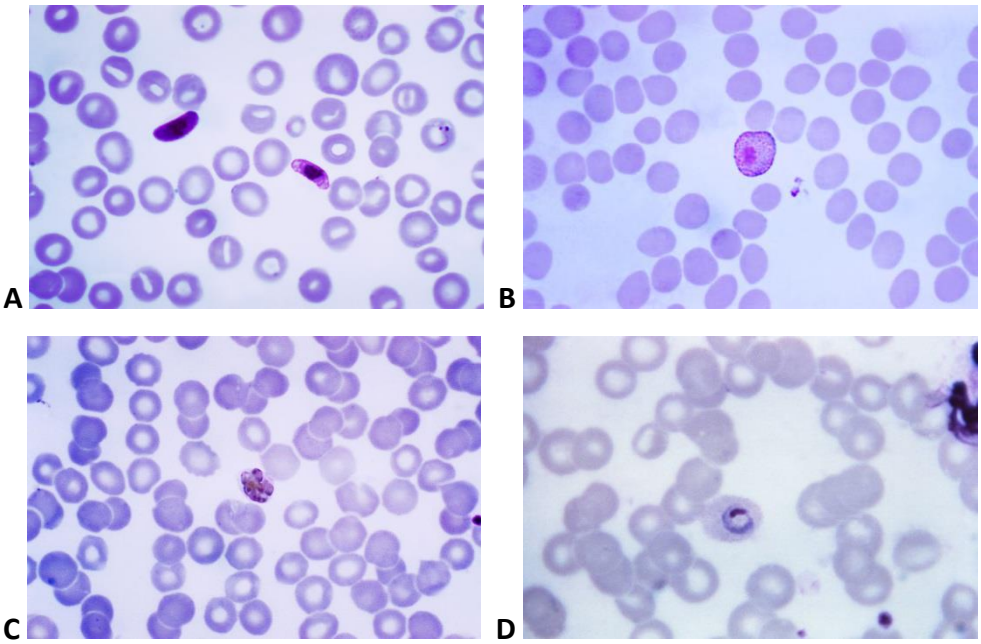
Slika 2. Malarija se na čovjeka prenosi ubodom zaražene ženke komarca vrste *Anopheles* (*A. atroparvus* (A), *A. labranchiae* (B), *A. plumbeus* (C) i *A. sacharovi* (D)) koji je domaćin spolnog ciklusa plazmodija (slike preuzete sa internetskih stranica https://ecdc.europa.eu i https://www.vectorbase.org).

P. vivax rasprostranjen je širom svijeta te izaziva oko 40% svih slučajeva malarije. Prevladava u Aziji i Južnoj Americi i samo nekim dijelovima Afrike. Bolest može u akutnoj fazi biti teška, ali ne kao tropska malarija. *P. vivax* u razvojnom putu u jetri može razviti hipnozoit, formu u kojoj parazit miruje. Inkubacija traje od 10 do 17 dana (mjeseci ili godina). Bolest se nakon 3 do 8 tjedana povlači, ali su recidivi mogući kroz nekoliko mjeseci ili godina u obliku blage ili srednje teške malarije. Od kliničkih simptoma tijekom latentne infekcije prisutna je splenomegalija i leukopenija. Posebnost ovog parazita je veća sklonost prema retikuloctima nego zrelim eritrocitima. Osobe bez Duffy antigena na eritrocitima otporne su na zarazu *P. vivax* (4,7,33,38,48-50).

P. ovale najčešći je uzročnik malarije u tropskim zemljama, zapadnoj Africi, Južnoj Americi i otocima zapadnog Pacifika. Morfološki i biološki najsličniji je *P. vivax*. Može zaraziti Duffy antigen negativne osobe, zbog čega je u Africi učestaliji od *P. vivax*. Izaziva blaže oblike tercijarne malarije. Inkubacijski period traje od 10 do 17 dana, a razvojni ciklus 48 sati (4,33,38,48,51,52).

P. malariae široko je rasprostranjen i dominirao je i u Hrvatskoj. Izaziva oko 7% svih slučajeva malarije koja se zbog dužeg (3 dana) replikacijskog ciklusa naziva kvartarna malarija. Ovaj parazit napada samo zrele eritrocite, a razina razmnožavanja je niska, zbog čega je inkubacija duža i traje od 27 do 40 dana. Iznenadni napadi groznice traju duže nego u infekciji drugim vrstama uzročnika malarije. Ako se ne liječi, parazit se u formi hipnozoita može zadržati u jetri doživotno pri čemu se može s vremenom razviti nefrotski sindrom (4,33,38,48,53).

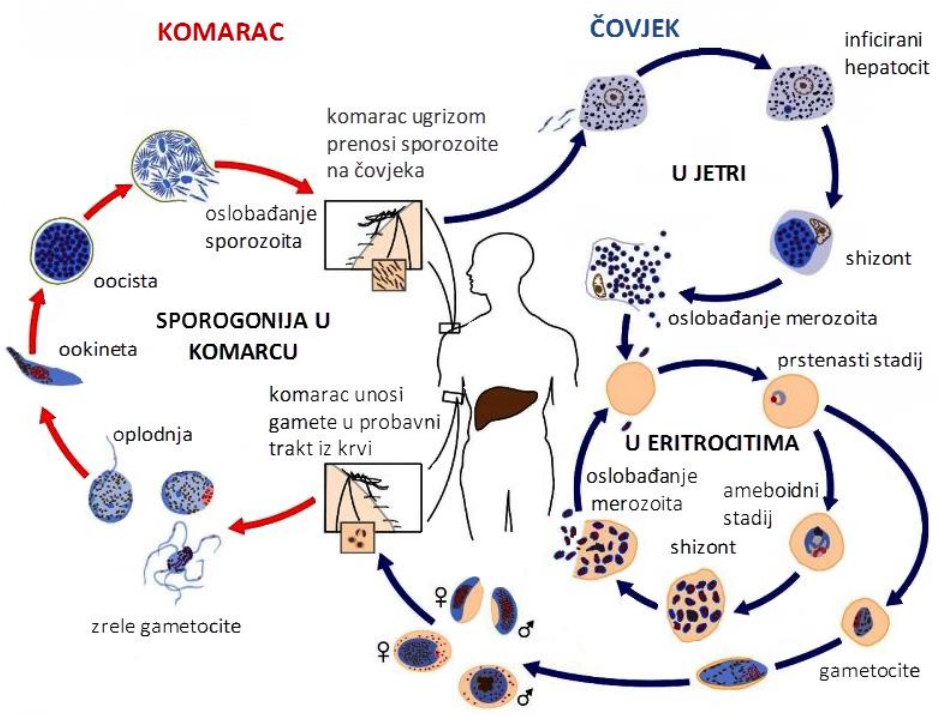
P. knowlesi prevladava u jugoistočnoj Aziji, gdje je odgovoran za 70% slučajeva malarije. Osim u čovjeka parazitira i u kratkorepih i dugorepih makaki (*Macaca*) majmuna. Ima kratak ciklus razmnožavanja od samo 24 sata. Temperatura i groznica praćena hladnim znojenjem je svakodnevna zbog čega može izazvati teže oblike bolesti (4,38,48,54-60).



Slika 3. Nekoliko vrsta parazita roda *Plasmodium* (*P. falciparum* (A), *P. vivax* (B), *P. malariae* (C) i *P. ovale* (D)) koji mogu uzrokovati malariju kod čovjeka. Najčešći i najopasniji od njih je *P. falciparum* (slike preuzete s internetske stranice https://www.cdc.gov).

U životnom ciklusu plazmodija razlikuju se dvije faze, spolna faza (sporogonija) koja se odvija u ženki komarca te nespolna faza (shizogonija) koja se odvija u čovjeku (Slika 4). Spolna faza plazmodija (sporogonija) se odvija izvan čovjeka nakon što ženka komarca usiše krv zaraženog čovjeka i time unese ženske i muške mikrogamete koje se nalaze u kožnim kapilarama. U probavnom sustavu komarca muške mikrogamete i ženske makrogamete stvaraju zigote koje se dalje razvijaju u pokretni oblik ookinta. Ookinete prelaze epitel kao

oocista iz kojih se razmnožavaju i razvijaju sporozoiti u žlijezdama slinovnicama komaraca čime se zatvara ciklus. Nakon toga plazmodij je ponovno spreman za prijelaz na čovjeka ubodom komarca koji prije nego počne sisati krv žrtve ubrizgava slinu koja osim antikoagulansa za sprečavanje zgrušavanja krvi kod zaraženog komarca sadrži i sporozoite i na taj način prenosi bolest malarije na čovjeka. Spolni razvoj plazmodija u komarcu traje otprilike 7 do 10 dana. Nakon što ženka komarca ubodom u krvotok čovjeka/žrtve unese sporozoite oni ubrzo nakon toga dolaze do jetre gdje se počinju dijeliti (izvaneritrocitna faza). Povećanje broja merozoita u stanicama jetre u konačnici dovodi do prsnuća stanica te merozoiti odlaze u krvotok gdje ulaze u eritrocite i prolaze nekoliko faza razvoja unutar eritrocita (prstenasti, ameboidni stadij te stadij shizonta). Također u eritrocitima merozoiti mogu pokrenuti razvojni stadij gameta koji je presudan za ponovno inficiranje komarca i nastavak seksualnog ciklusa plazmodija izvan čovjeka. Razmnožavanje merozoita u eritrocitima traje između 36 do 72 sata (ovisno o vrsti uzročnika) nakon čega dolazi do prsnuća shizonta i otpuštanja merozoita u krvotok koji ponovno ulaze u eritrocite ili u nekim slučajevima mogu ponovno otići u jetru i pokrenuti izvaneritrocitnu fazu. Bolest nastupa kada je ukupan broj parazita u cirkulaciji oko 100 milijuna, a karakteristična povišena temperatura koja se ponavlja svakih 36 do 72 sata je upravo posljedica pucanja eritrocita i oslobađanja velikog broja parazita u krvotok (1,2,4,9,33,38,61,62).



Slika 4. Životni ciklus plazmodija (slika preuzeta i prilagođena sa internetske stranice https://www.cdc.gov).

1.1.2. Prevencija malarije

Nekoliko je načina prevencije malarije. Najznačajnija je kontrola prijenosnika bolesti komarca iz porodice *Anopheles* (kontrola vektora), zatim kemoprofilaksa i potom primjena cjepiva koja su uglavnom tek u predkliničkim i/ili kliničkim ispitivanjima s vrlo neizvjesnom i složenom budućom primjenom (3,4,9,38,63-68).

1.1.2.1. Kontrola vektora

U kontroli komaraca svakako se preporuča isušivanje močvarnih područja. Potom se mogu koristiti razni insekticidi, a koji se mogu primijeniti putem mreža tretiranih insekticidima (ITN engl. *insecticide-treated net*; LLIN, engl. *long lasting insecticidal net*), špricanjem unutrašnjosti prostora (IRS, engl. *indoor residual spraying*), a koji djeluju duže vrijeme i učinkoviti su u sprečavanju širenja zaraze. Tu su i razni repelenti za odbijanje komaraca. Korištenje tretiranih mreža dovelo je do smanjenja pojavnosti bolesti za 50% kao i 55% smanjenje smrtnosti djece do 5 godina u sub-saharskoj Africi (40,66,69,70).

Osim insekticida koji mogu imati nepovoljan učinak na okoliš i uzrokovati razvoj rezistencije, istražuju se i novi načini kontrole. Jedan je genetska modifikacija komaraca kako bi komarac stvarao sterilno potomstvo. Tako bi ugradnja dominantnog gena komarcu, a koji bi uzrokovao sterilnost potomstva, spriječila pojavu malarije (71). Još jedan obećavajući način kontrole vektora je pronalazak simbionta bakterije iz porodice *Wolbachia* spp. koji bi spriječio komarca u prenošenju malarije, a potaknut je sličnim ponašanjem te bakterije na prijenosniku Denge (hemoragijske) groznice (72,73). Također postoje lijekovi kao što je ivermektin koji smanjuje životni vijek komarca nakon što ubode osobu koja ga uzima. To je možda učinkovito za kontrolu komaraca, no nema direktan učinak na čovjeka jer ne liječi bolest pa je upitna opravdanost, a i sigurnosni profil takvih lijekova mora biti izrazito visok kako bi se uopće pojavili na tržištu (9,74).

1.1.2.2. Kemoprofilaksa i SBET (*stand-by emergency treatment*)

Kemoprofilaksa je učinkovita metoda suzbijanja malarije, a posebno se koristi za zaštitu osjetljivih skupina ljudi kao što su trudnice, djeca te putnici u visoko rizična područja. Lijekovi koji se koriste za prevenciju s obzirom da se daju zdravim pojedincima moraju imati povoljan sigurnosni profil te odnos rizika od mogućih štetnih nuspojava lijeka i rizika od

bolesti mora biti negativan tj. njihova primjena mora biti opravdana. Prema učestalosti i trajanju razlikujemo četiri vrste kemoprofilakse: 1) povremeno preventivno liječenje u trudnoći (IPTp, engl. *intermittent preventive treatment in pregnancy*), 2) sezonska kemoprofilaksa (SMC, engl. *seasonal malaria chemoprevention*), 3) kemoprofilaksa fiksnog trajanja te 4) masovna primjena lijekova (MDA, engl. *mass drug administration*) (9,40,75-78).

U posljednjem izvješću, WHO u određenim okolnostima preporuča masovnu primjenu antimalarika kao način kemoprofilakse gdje se svim ljudima koji žive u određenom području (izuzev onih kojima je terapija kontraindicirana) daje puna terapijska doza antimalarika u istom vremenskom razdoblju i često kroz nekoliko intervala (40,70,76-79).

Povremeno preventivno liječenje preporuča se trudnicama, a u tu svrhu se daje pirimetamin–sulfadoksin kod svakog pregleda nakon drugog tromjesečja dok djeca u umjereno rizičnim područjima dobivaju pirimetamin–sulfadoksin zajedno s ostalim rutinskim cjepivima.

Sezonska kemoprofilaksa se provodi među djecom starosti od 3 do 59 mjeseci i to kao kombinacija pirimetamin–sulfadoksina i amodiakina. Za prevenciju malarije kod putnika u malarična područja izbor kemoprofilakse mora uključivati procjenu rizika od malarije s obzirom na područje, vremenski period godine kada se posjećuje kao i poznavanje postojanja rezistencije na određene lijekove u području koje se posjećuje. Kada se potvrdi potreba za uzimanjem kemoprofilakse izbor konačnog lijeka mora uzeti u obzir i rizik od toksičnosti (4,79-83).

Kemoprofilaksa fiksnog trajanja prepisuje se putnicima u rizična područja, a kao najčešći lijekovi iz te skupine se propisuju atovakvon/progvanil hidroklorid, doksiciklin, meflokin ili primakin. Atovakvon/progvanil hidroklorid i doksiciklin se često propisuju putnicima u malarična područja zbog jednostavnog doziranja i uglavnom blagih nuspojava. Meflokin kao profilaksa zbog svog načina primjene koji zahtjeva samo jednu dozu tjedno predstavlja izvrstan izbor, ali zbog neurotoksičnosti i čestih halucinacija nije uvijek prvi izbor za sve putnike u malarična područja. Primakin je iznimno efektivan profilaktički lijek učinkovit i protiv dormantnog oblika plazmodija *P. vivax,* ali nije primjeren za osobe s glukoza-6-fosfat dehidrogenaza (G-6-PD) deficijencijom. Lijekovi iz ove skupine uglavnom nemaju teže nuspojave koje bi zahtijevale prekid terapije, ali malo je podataka o njihovom učinku kada se uzimaju duže vremena (69,80,81,84-89).

SBET (engl. *stand by emergency treatment*), odnosno lijekovi za liječenje malarije, namijenjeni putnicima, koji se uzimaju pri pojavi prvih simptoma malarije, bez liječničke

dijagnoze sve se češće primjenjuju pogotovo za područja gdje je rizik od prijenosa malarije srednji ili nizak odnosno gdje putnici nemaju pristup bolničkom liječenju unutar 24 sata. Lijekovi koji se koriste za SBET su uglavnom isti kao i za kemoprofilaksu s razlikom u režimu doziranja s obzirom da se SBET uzima za liječenje bolesti. Upravo je atovakvon/progvanil hidroklorid jedna o najčešćih kombinacija koja se koristi za SBET s time da je bitno napomenuti da ukoliko je osoba uzimala kemoprofilaksu isti lijekovi se ne bi smjeli koristi i za SBET (75,80,90-92).

1.1.2.3. Cjepiva

Do danas nema cjepiva protiv malarije u širokoj primjeni, ali postoji niz kandidata u različitim fazama istraživanja, razvoja i evaluaciji na ljudima (4,67,93). Trenutno je jedino registrirano cjepivo protiv malarije RTS,S/AS01 koje sadrži rekombinantni protein gena CSP plasmodijuma i gen viralne ovojnice hepatitisa B, trgovačkog imena Mosquirix proizvođača *GlaxoSmithKline & PATH Malaria Vaccine Initiative*. Cjepivo je u fazi ispitivanja u 3 afričke zemlje te se koristi za cijepljenje djece u 4 navrata u dobi od 5 do 17 mjeseci. Ovo cjepivo pokazalo je značajno smanjenje malarije, ali nažalost ne protiv svih uzročnika te je smanjenog djelovanja u kasnijoj dobi djeteta (9,94,95). U Africi je još jedno cjepivo u kliničkim ispitivanjima imena PfSPZ (engl. *P. falciparum sporozite Vaccine*) koje, za razliku od RTS,S/AS01, sadrži oslabljene sporozoite uzročnika malarije *P. falciparum* u njegovom početnom zaraznom stanju te je pokazalo učinkovito djelovanje u odraslih osoba bez težih nuspojava (96). Posljednjih godina fokus je na cjepivima koja izazivaju stvaranje antitijela koja bi pružala zaštitu cijele populacije jer se antitijela mogu prenositi s čovjeka na komarca na isti način kao i parazit malarije. Iz te skupine cjepiva trenutno se najviše istraživanja provodi s konjugiranim cjepivom kojem je ciljno mjesto gametocitni marker Pfs25 ženke komarca (5,9,64,97).

1.1.3. Liječenje malarije

Simptomi nekompliciranе malarije su često nespecifični, a uključuju povišenu temperaturu, drhtavicu, glavobolju, kašalj, proljev, čineći kliničku dijagnozu otežanom. Dijagnoza malarije mora biti pretpostavljena kod svakog febrilnog bolesnika koji je putovao ili boravio u tropskim područjima ili je ondje primio transfuziju krvi. Uzimanje točne epidemiološke anamneze svih pacijenata s vrućicom u ne-endemskim područjima je vrlo bitno

za dijagnosticiranje bolesti. Sigurno se dijagnoza postavlja nalazom parazita malarije u krvnim razmazima bojenim po Giemsa-Romanowskom. Ako se jednom posumnja na malariju, krv treba pregledati odmah i opetovano svakih 12 sati prva tri dana prije nego se bolest isključi. Parazitemija može varirati, a prvih nekoliko dana bolesti broj parazita ne mora dosegnuti dijagnostički nivo. Vrijeme uzimanja krvnog uzorka nije toliko značajno koliko je važno višekratno uzimanje uzoraka. Čak ako su i nalazi negativni, a bolest je vrlo vjerojatna, treba provesti pokušaj liječenja. Kada se jednom postavi dijagnoza malarije, najvažniji daljnji postupak je utvrditi o kojem uzročniku malarije se radi zbog davanja primjerene terapije. Prije same odluke o odgovarajućoj terapiji potrebno je razlučiti infekciju *P. falciparum* od drugih manje virulentnih plazmodija. Zbog izrazite težine bolesti kod falciparum-malarije, mogućeg naglog pogoršanja, većeg mortaliteta kao i moguće otpornosti na lijekove, potrebno je njezino brzo prepoznavanje. Danas ne postoji savršen lijek protiv svih uzročnika malarije odnosno protiv svih oblika koji se javljaju u različitim populacijama stoga je vrlo važan individualan pristup (4,34,38,65,77,98,99).

Klorokin, 4-aminokinolinski derivat, lijek je izbora za eritrocitnu fazu *P. vivax*, *P. ovale* i *P. malariae*, kao i *P. falciparum* osjetljivog na klorokin. Klorokin i drugi shizontocidni lijekovi nedjelotvorni su na izvaneritrocitne hepatalne oblike *P. vivax* i *P. ovale*, pa se kod ovih uzročnika liječenje mora nastaviti 8-aminokinolinskim preparatom primakinom koji djeluje na te oblike parazita. Kod malarija uzrokovanih transfuzijama ne treba dodavati primakin jer tu nema latentne izvaneritrocitne faze. Ako se ne radi o falciparum-malariji, liječenje se može provesti i ambulantno, ali je potrebno promatrati bolesnika u prvih 24 sata zbog moguće zabune u vrsti uzročnika infekcije. Kod vivax-malarije može i unatoč adekvatnom liječenju klorokinom i primakinom doći do recidiva. Tada liječenje treba provesti iznova. Ako je dokazana infekcija s *P. falciparum*, tada se odmah postavlja pitanje je li uzročnik rezistentan na klorokin. Rezistencija na klorokin je široko rasprostranjena u svijetu (Jugoistočna Azija, Južna Amerika, Istočna Afrika te manje Zapadna Afrika). Sumnja na rezistenciju postoji ako bolesnik dolazi iz područja gdje je ona registrirana ili ako je obolio premda je prethodno uzimao klorokin u profilaksi. Kod sumnje na rezistenciju ili ako se radi o teškom obliku bolesti, treba bolesnika obavezno liječiti kininom. Kinin, najstariji antimalarik jedno vrijeme potisnut u pozadinu zbog učinkovitijih derivata, postao je danas lijekom izbora u slučajevima rezistentne tropske malarije (4,65,100-102).

Kombinirana terapija bazirana na artemisininu derivatu iz biljke *Artemisia annua* (ACT, engl. *artemisinin-based combination therapy*) je trenutno najučinkovitija terapija i

često terapija prvog izbora za liječenje malarije (79,103,104). Glavna prednost ACT terapije je što artemisinin vrlo brzo reducira većinu parazita dok drugi lijek iz kombinacije uklanja ostatak. Kinin odnosno njegovi derivati primakin, kinakrin, klorokinin dugo su bili učinkoviti u samostalnoj primjeni, ali se upravo na te derivate kinina među prvima razvila otpornost tako da se sve rjeđe koriste samostalno, no i dalje u kombinaciji s drugim antimalaricima (105). Osim navedenih lijekova i njihovih kombinacija, u terapiji malarije koriste se i neki drugi lijekovi poput amodiakina, halofantrina, dapsona, pamakina, ali i određeni antibiotici poput tetraciklina i doksiciklina. Nadalje, neki su lijekovi još uvijek u fazi kliničkih ispitivanja poput tafenokina.

Iako se većina oblika malarije uspješno liječi postojećim antimalaricima, morbiditet i mortalitet od malarije i dalje je prisutan. To je posljedica sve češćeg razvoja rezistencije parazita na lijekove, ali i porasta rezistencije komaraca na insekticide. Zbog toga su istraživanja na pronalaženju i ispitivanju novih antimalarika, ali i onih koji imaju najmanje nuspojava i dalje vrlo aktualna (3,4,15,34,43,65,77,99,100,106-113,).

1.2. Atovakvon i progvanil hidroklorid

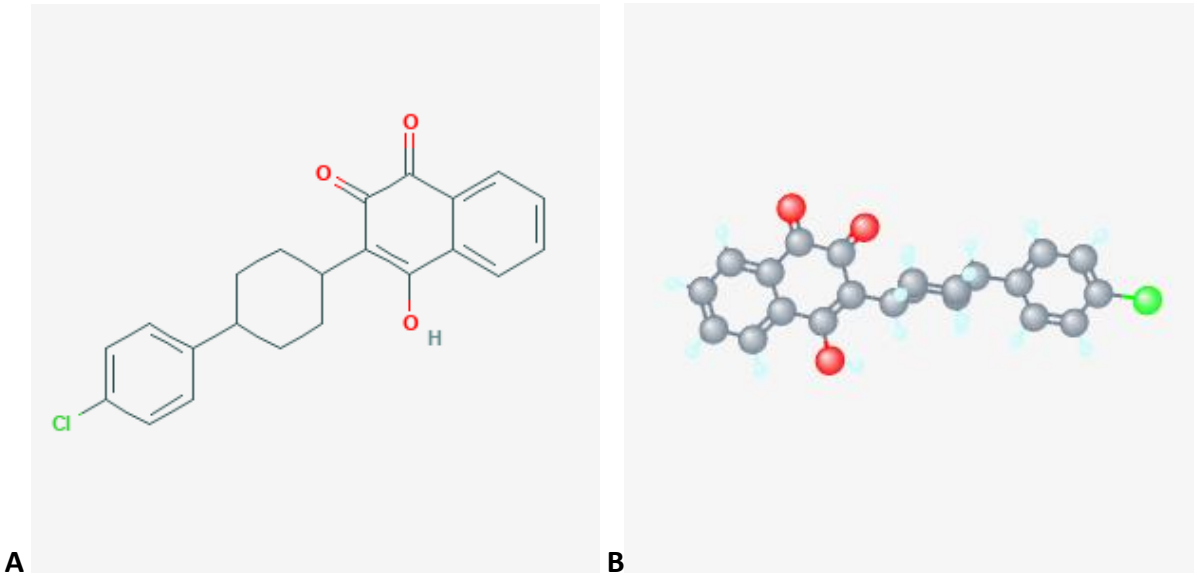
1.2.1. Atovakvon

Kemijski naziv atovakvona je trans-2-[4-(4-klorofenil) cikloheksil]-3-hidroksi-1,4-naftalendion (Slika 5). Atovakvon je žuta kristalna krutina koja je netopljiva u vodi. Molekulska masa atovakvona je 366,84 g/mol, a kemijska formula mu je $C_{22}H_{19}ClO_3$ (114-117). Atovakvon je lipofilni analog ubikvinona, važne komponente mitohondrijskog sustava prijenosa elektrona u stanicama. Antimalarijska aktivnost atovakvona se upravo pripisuje njegovoj interferenciji s mitohondrijskim prijenosom elektrona u samom parazitu. U parazitu atovakvon djeluje na kompleks citokrom c reduktaze što u konačnici rezultira kolapsom potencijala mitohondrijske membrane (118,119).

Atovakvon također inhibira i aktivnost parazitskog enzima dihidroorotat dehidrogenaze, stoga što je dihidroorotat dehidrogenaza ovisna o funkcionalnom mitohondrijskom transportu elektrona. Budući da je dihidroorotat dehidrogenaza ključni enzim u biosintezi pirimidina, njegova inhibicija atovakvonom ometa sintezu i replikaciju plazmodijske molekule DNK (120-123). Osim u terapiji za liječenje malarije uzrokovane *P. falciparum*, atovakvon se koristi i protiv upale pluća uzrokovane *Pneumocystis carinii* te za liječenje toksoplazmoze uzrokovane *Toxoplasma gondii* (124-128). Atovakvon dolazi kao

filmom obložena tableta atovakvon/progvanil hidroklorida koja se sastoji od 250 mg atovakvona u kombinaciji sa 100 mg progvanil hidroklorida (114,115,129-131).

Atovakvon kao pojedinačni lijek uzrokuje tek nekoliko nuspojava koje zahtijevaju prekid terapije s atovakvonom. Najčešće reakcije na atovakvon su osip, vrućica, povraćanje, proljev, bolovi u trbuhu i glavobolja (Tablica 1). Kod bolesnika koji primaju terapiju atovakvonom samo ponekad se javljaju poremećaji u razini serumskih transaminaza i amilaza. Do sada je zabilježeno svega nekoliko akutnih štetnih učinaka, ali za ozbiljniju kliničku sliku potrebne su dodatne procjene, osobito da bi se otkrili mogući rjeđi, neobični ili dugoročni neželjeni toksični učinci atovakvona. Zbog toga atovakvon ne bi trebala uzimati dojenčad, trudnice, dojilje niti bolesnici s teškim oštećenjem bubrega (102,117,132-134).

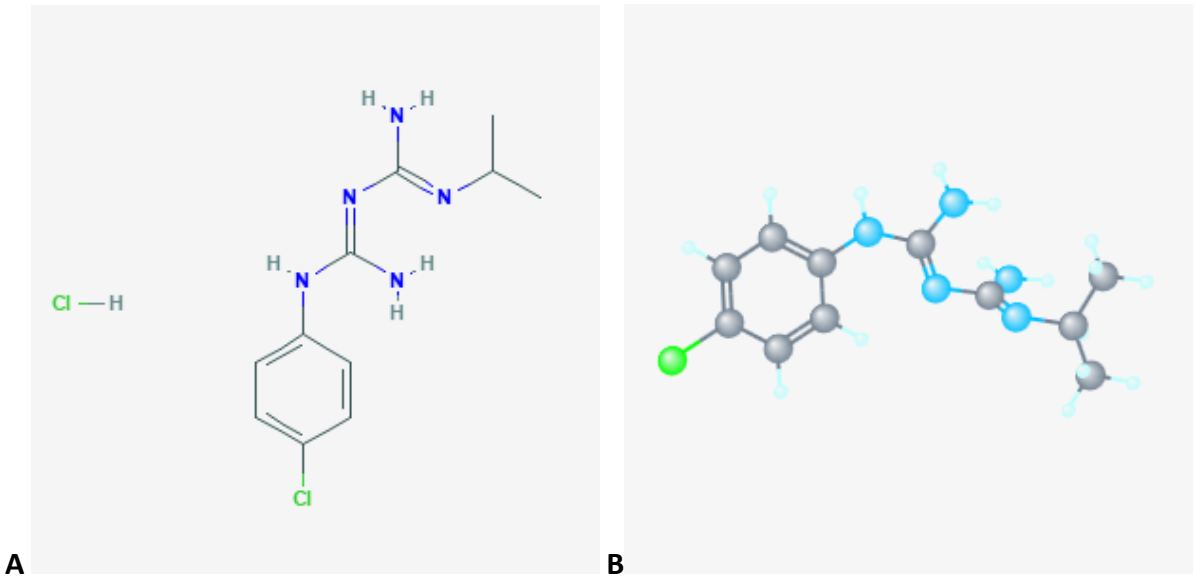


Slika 5. Dvodimenzionalna (A) i trodimenzionalna (B) struktura atovakvona (slike preuzete sa internetske stranice https://pubchem.ncbi.nlm.nih.gov).

1.2.2. Progvanil hidroklorid

Kemijski naziv progvanil hidroklorida je 1-(4-klorofenil)-5-izopropil-bigvanid hidroklorid (Slika 6). Progvanil hidroklorid bijeli je kristalinični prah koji je topljiv u vodi. Molekulska masa progvanil hidroklorida je 290,22 g/mol, a kemijska formula mu je $C_{11}H_{16}ClN_5 \times HCl$ (114,115,135).

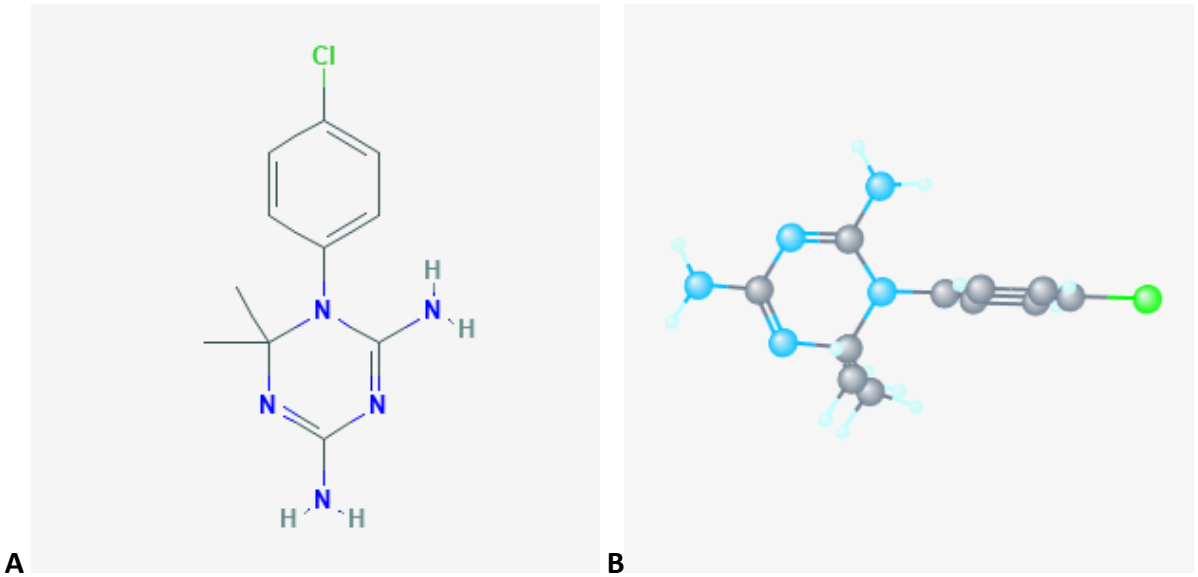
Aktivna forma progvanila je ciklogvanil (Slika 7) koji nastaje metaboliziranjem u jetri putem citokrom P450 izoenzima CYP2C19 i CYP3A4 (136-138). Molekulska masa ciklogvanila je 251,718 g/mol, a kemijska formula mu je $C_{11}H_{14}ClN_5$. Ciklogvanil je taj koji ima antimalarijski učinak tako što djeluje na dihidrofolat reduktazu plazmodija *P. falciparum*. Ciklogvanil se spaja s timidil sintazom u homodimerični bifunkcionalni protein koji katalizira NADPH-ovisnu redukciju dihidrofolata u tetrahidrofolat te pritom osigurava ugljik za metiltransfer reakciju neophodnu za preživljavanje parazita (139,140). Progvanil hidroklorid se koristi za liječenje nekomplicirane malarije uzrokovane *P. falciparum* te za profilaksu blage malarije otporne na klorokin. Progvanil hidroklorid dolazi kao filmom obložena tableta atovakvon/progvanil hidroklorid koja se sastoji od 100 mg progvanil hidroklorida u kombinaciji sa 250 mg atovakvona (114,129-131).



Slika 6. Dvodimenzionalna (A) i trodimenzionalna (B) struktura progvanil hidroklorida (slike preuzete sa internetske stranice https://pubchem.ncbi.nlm.nih.gov).

Najčešće nuspojave prijavljene u bolesnika koji su uzimali progvanil hidroklorid za liječenje malarije su bol u trbuhu, mučnina, povraćanje i glavobolja u odraslih te povraćanje kod male djece (Tablica 1). Kada se koristi u profilaksi, najčešće nuspojave uključuju glavobolju i bol u trbuhu te povraćanje kod male djece. Progvanil hidroklorid dobro se podnosi iako postoji mogućnost nuspojava, poput oralnih afti i čireva, koje su rijetko dovoljno ozbiljne da uzrokuju prekid tretmana ovim lijekom. Iako se progvanil hidroklorid smatra

sigurnim tijekom trudnoće i dojenja dugoročni podaci o sigurnosti u djece su ograničeni. Prilikom dojenja, putem mlijeka se izlučuje nedovoljna količina lijeka da bi mogla zaštiti dojenče pa se takav način prevencije ne preporuča (132,141).



Slika 7. Dvodimenzionalna (A) i trodimenzionalna (B) struktura ciklogvanila (slike preuzete sa internetske stranice https://pubchem.ncbi.nlm.nih.gov).

Atovakvon i progvanil hidroklorid se koriste kao jedna tableta koja sadrži dvije aktivne supstance koja se sastoji od 250 mg atovakvona u kombinaciji sa 100 mg progvanil hidroklorida za odrasle. Kod djece, fiksna doza sadrži 62,5 mg atovakvona u kombinaciji s 25 mg progvanil hidroklorida (114,129-131). Kada se atovakvon i progvanil hidroklorid koriste zajedno u profilaksi ili liječenju ne uzrokuju ozbiljne nuspojave (142,143). Najčešći štetni učinci koji se javljaju pri uzimanju atovakvona i progvanil hidroklorida što za liječenje što za profilaksu su bolovi u trbuhu, glavobolja, mučnina, povraćanje i proljev (Tablica 1). Atovakvon i progvanil hidroklorid se ne smiju koristiti u dojenčadi, trudnica, dojilja ili kod bolesnika s teškim oštećenjem bubrega. Zabilježeno je nekoliko akutnih neželjenih učinaka, ali potrebno je više kliničkih procjena samoga lijeka, posebno za otkrivanje mogućih rijetkih, neuobičajenih ili dugotrajnih nuspojava (102,114,132,144,145). Dugoročni učinci atovakvona i progvanil hidroklorida na rast, pubertet i opći razvoj nisu u potpunosti istraženi (102,115,132,141).

Tablica 1. Nuspojave koje su zabilježene s atovakvonom, progvanil hidrokloridom i/ili kombinacijom atovakvon/progvanil hidroklorida tijekom provedenih kliničkih ispitivanja ili tijekom spontanog izvješćivanja o farmakovigilanciji, navedene prema organskom sustavu i učestalosti. Frekvencije su definirane kao vrlo česte (≥ 1/10), uobičajene (≥ 1/100 i <1/10), rijetke (≥ 1/1000 i <1/100) te kao neodređena frekvencija (učestalost se ne može odrediti iz dostupnih podataka).

Klase organskih sustava	**Frekvencija**			
	Vrlo česte	**Uobičajene**	**Rijetke**	**Nepoznata frekvencija**
	(≥1 / 10)	(≥1 / 100 do <1/10)	(≥1 / 1000 do <1/100)	(ne može se procijeniti iz raspoloživih podataka)
Poremećaji krvi i limfnog sustava		Anemija, Neutropenija		Pancitopenija u bolesnika s teškom bubrežnom insuficijencijom
Poremećaji imunološkog sustava		Alergijske reakcije		Quinckeov edem, anafilaksija, vaskulitis
Poremećaji metabolizma i prehrane		Hiponatrijemija, anoreksija	Povišenje razine amilaze	
Psihijatrijski poremećaji		Neobični snovi	Anksioznost	Kriza panike
		Depresija		Plač
				Halucinacije
				Noćne more
Poremećaji živčanog sustava	Glavobolja	Nesanica		Konvulzije
		Vrtoglavica		
Srčani poremećaji			Palpitacije	Tahikardija
Gastrointestinalni poremećaji	Mučnina		Stomatitis	Žgaravica, dispepsija
	Povraćanje			Afte
	Proljev			
	Abdominalni bolovi			
Jetreni poremećaji		Povišenje jetrenih enzima		Hepatitis
				Kolestaza
Kožni i potkožni poremećaji		Erupcija	Alopecija	Stevens-Johnsonov sindrom
			Urtikarija	Erythema multiforme
				Mjehurići na koži
				Ljuštenje kože
Opći poremećaji organizma		Groznica		
Poremećaji respiratornog sustava		Kašalj		

1.3. Citogenetička i biokemijska istraživanja

1.3.1. Ljudski limfociti periferne krvi

Ljudski limfociti periferne krvi najčešće su korišten stanični model za ispitivanje kemijskih agensa (146). Ljudski limfociti prikladni su testni sustav jer imaju široku primjenu u genetičkoj i okolišnoj toksikologiji te u biomonitoringu populacija u uvjetima *in vitro* i *in vivo* te predstavljaju prikladan i lako dostupan izvor primarnih stanica sa stabilnim genomom. Dobivaju se na relativno neinvazivan način, ne zahtijevaju posebnu pripremu te su se pokazali kao izvrstan model u testovima za procjenu toksičnosti različitih spojeva, a posebno kod komet testa (147,148). Ljudski limfociti periferne krvi metabolički su neaktivne stanice te se u istraživanjima u kojima se oni koriste često upotrebljava izolat jetre štakora (tzv. S9 frakcija) koji sadrži enzime potrebne za biotransformaciju agensa koji se ispituje (149,150).

1.3.2. Citogenetičke i biokemijske metode

U toksikološkim analizama kombinacije različitih metoda mogu odigrati važnu ulogu u procjeni citotoksičnosti i genotoksičnosti uzrokovanoj kombinacijama različitih fizikalnih i kemijskih agensa, ali i lijekova i njihovih kombinacija. Metode koje se koriste u toksikološkim istraživanjima omogućuju procjenu stanične vijabilnosti i razine primarnih oštećenja molekule DNK čak i nakon kratkotrajnog izlaganja potencijalno genotoksičnim spojevima, a njihove modifikacije i komplementarne biokemijske metode omogućavaju uvid u mehanizme kojima spojevi djeluju na brojne ciljne, ali i ne-ciljne stanice.

1.3.2.1. Test stanične vijabilnosti

Potencijalno citotoksično djelovanje fizikalnih i kemijskih agensa procjenjuje se testom stanične vijabilnosti. Jedan od testova u kojem se preživljenje ljudskih limfocita periferne krvi nakon tretmana određenim spojem može pratiti je i test koji koristi istovremeno bojanje fluorescentnim interkalirajućim agensima etidij-bromidom i akridin-oranžom *in situ*. Etidij-bromid polarna je molekula koja ne prolazi kroz membranu živih stanica, pa se na taj način može ugraditi samo u molekulu DNK mrtvih stanica uslijed čega daje crvenu fluorescenciju. S druge strane, akridin-oranž ulazi i u žive i u mrtve stanice te nakon ugradnje

u molekulu DNK daje zelenu fluorescenciju (151,152). Stoga stanice koje daju crvenu fluorescenciju predstavljaju mrtve stanice te se na osnovu toga podatka može utvrditi citotoksičnost spoja.

1.3.2.2. Alkalni i Fpg-modificirani komet test

Potencijalno genotoksično djelovanje fizikalnih i kemijskih agensa može se procijeniti alkalnim komet testom, a kako bi se utvrdio njihov utjecaj na molekulu DNK. Komet test je učinkovita metoda za procjenu stupnja oštećenja i popravka molekule DNK na razini pojedinačnih stanica. U ovoj metodi stanice se uklapaju u tzv. agarozni „sendvič“ gel te se zatim liziraju citoplazma i membranske strukture pri čemu se oslobađa ukupna stanična DNK. Nakon toga, DNK se denaturira u lužnatom ili neutralnom puferu kojim se omogućava detekcija jednolančanih i dvolančanih lomova tijekom elektroforeze. Nakon bojanja fluorescencijskim agensima, pod mikroskopom su vidljivi obrasci putovanja oštećene molekule DNK u gelu koji nalikuju na komete po kojima je sama metoda i dobila ime (147,153-161). Analiza kometa vrši se pod fluorescencijskim mikroskopom, pomoću automatiziranog programa za analizu slike spojenog kamerom za pripadajući mikroskop, gdje se svakom pojedinačnom kometu mjeri različiti set deskriptora kao što su dužina repa, postotak DNK u repu i/ili repni moment (147,160,162-164).

Komet test je relativno brza, jednostavna i osjetljiva metoda za procjenu oštećenja molekule DNK u različitim vrstama stanica uslijed izloženosti brojnim fizikalnim i kemijskim agensima, uključujući lijekove, u uvjetima *in vivo* i *in vitro* (153,155-157,161,165-167). Stoga je *in vitro* komet test i predložen kao alternativa standardnim citogenetičkim testovima u ranom otkrivanju genotoksičnosti kandidata za lijekove (168,169).

Danas su dostupne brojne izvedbe komet testa kao što su komet test u neutralnim ili alkalnim uvjetima za detekciju jednolančanih i dvolančanih lomova molekule DNK, mjesta osjetljivih na lužine te ukriženog povezivanja između molekula DNK-DNK i DNK-proteini, zatim komet-FISH metoda te brojne enzimske modifikacije komet testa koje uključuju enzime kao što su Fpg, Endo III i hOGG1 za detekciju oksidacijskih oštećenja molekule DNK, ali i one za detekciju epigenetskih promjena (166,170-178). Fpg (formamido-pirimidin-DNK-glikozilaza)-modificirana verzija komet testa omogućuje mjerenje stupnja oksidacijskog oštećenja molekule DNK. Fpg enzim prepoznaje oštećenja u molekuli DNK kao što su: 1. forma otvorenog prstena 7-metilgvanin, uključujući i 2,6-diamino-4-hidroksi-5-N-metilformamidopirimidin i 4,6-diamino-5-formamidopirimidin, koji su letalne lezije, 2. 8-

oksogvanin, izrazito mutageno oštećenje i najvažniji biološki supstrat Fpg enzima, 3. 5-hidroksicitozin i 5-hidroksiuracil, 4. aflatoksin-vezani imidazol otvoreni prsten gvanina te 5. imidazolni prsten otvoren na N-2-aminofluorencentnom-C8-gvaninu. Stoga ova modifikacija komet testa daje uvid u potencijalna oksidacijska oštećenja molekule DNK (166,170).

1.3.2.3. Metode za detekciju parametara oksidacijskog stresa

Oksidacijski stres posljedica je neravnoteže nastajanja i uklanjanja reaktivnih kisikovih spojeva (ROS, engl. *reactive oxygen species*) koji djeluju na okolne molekule kao jaki oksidansi. Nastajanje ROS-ova, bilo iz endogenih ili egzogenih izvora, dovodi posljedično do oksidacijskih oštećenja makromolekula u stanici od kojih su najosjetljiviji membranski lipidi, proteini i molekula DNK. Do koje vrste oštećenja će doći ovisi o vrsti ROS-ova, njihovoj reaktivnosti, vremenu trajanja te mjestu njihova nastanka (179-186). ROS-ovi mogu oštetiti DNK na bazama i šećeru što dovodi do degradacije dušičnih baza, lomova i mutacija. Oksidacija baza odvija se najčešće na timinu i gvaninu, dok su adenin i citozin manje osjetljivi na oksidacijski stres. Oksidacijski stres proteina dovodi do modifikacije aminokiselina, fragmentacije peptidnog lanca, povećane osjetljivosti na proteolitičke enzime i formiranje novih reaktivnih grupa. To dovodi do gubitka fluidnosti staničnih membrana, inaktivacije membranskih enzima, ubrzane proteolize, starenja, poremećaja prijenosa signala u stanici, malignih procesa i smrti stanice (182,186-192).

Glutation, kao najzastupljeniji unutarstanični tiol, nalazi se gotovo u svakoj eukariotskoj stanici. U organizmu glutation djeluje kao reducens i antioksidans u različitim biokemijskim reakcijama, služi kao izvor cisteina, štiti stanice od lipidne peroksidacije (LPO, engl. *lipid peroxidation*), vrši detoksikaciju endogenih i egzogenih tvari, sudjeluje u staničnoj proliferaciji i razvoju. Također, neophodan je za sintezu proteina i nukleinskih kiselina, održavanje enzima u njihovoj aktivnoj formi te za održavanje stanične membrane, a njegova se koncentracija smanjuje izlaganjem oksidacijskom stresu (180,182,186,193,194). S druge strane, malondialdehid nastaje kao produkt LPO te kao takav može nastaviti lančanu reakciju oksidacijskog oštećenja. Malondialdehid kao reaktivni elektrofil, stvara kovalentne adukte s proteinima i molekulom DNK te se zbog toga smatra izuzetno mutagenim. No, u usporedbi s ostalim produktima LPO je stabilan te se može koristi kao biomarker u mjerenju razine oksidacijskog stresa u organizmu (182,186,195-198).

2. An alkaline comet assay study on the antimalarial drug atovaquone in human peripheral blood lymphocytes: a study based on clinically relevant concentrations

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An alkaline comet assay study on the antimalarial drug atovaquone in human peripheral blood lymphocytes: a study based on clinically relevant concentrations

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ABSTRACT: Atovaquone, a hydroxynaphthoquinone, is an anti-parasite drug, selectively targeting the mitochondrial respiratory chain of malaria parasite. It is used for both the treatment and prevention of malaria, usually in a fixed combination with proguanil. Although atovaquone has not often been associated with severe adverse reactions in the recommended dosages and has a relatively favorable side effect profile, the present study was undertaken to evaluate its cytogenotoxic potential towards human peripheral blood lymphocytes. Two different concentrations of atovaquone found in plasma when used in fixed-dose combination with proguanile hydrochloride were used with and without S9 metabolic activation: 2950 ng ml^{-1} used for prophylactic treatment and 11 800 ng ml^{-1} used in treatment of malaria. The results showed that lymphocyte viability was not affected after the treatment, suggesting that atovaquone was not cytotoxic in the given concentrations. With the alkaline comet assay we demonstrated that in human peripheral blood lymphocytes no significant changes in comet parameters occurred after the treatment. There were no differences in tested parameters with the addition of S9 metabolic activation, indicating that atovaquone either has no metabolite or it is not toxic in the given concentrations. Since no effects were observed after the treatment, it is to be concluded that atovaquone is safe from the aspect of genototoxicity in the recommended dosages. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: atovaquone; clinically relevant concentrations; human peripheral blood lymphocytes; cytotoxicity; genotoxicity; comet assay

Introduction

Since malaria, which is caused by species of the parasite genus *Plasmodium*, remains a major global health problem, there are various drugs widely used in the prophylaxis and treatment of this disease, one of them being atovaquone, which usually comes in fixed combination with proguanil hydrochloride (Looareesuwan *et al*., 1999; Olliaro, 2001; Patel and Kain, 2005; Pearson, 2001; Wiesner *et al*., 2003). For the prevention of malaria in travelers, antimalarial agents should fulfil the requirements of efficacy towards the parasite, in addition to being safe for the consumer and not putting them at additional risk of adverse effects (Chattopadhyay *et al*., 2007; Dhanawat *et al*., 2009; Nzila and Chilengi, 2010).

The chemical name of atovaquone is *trans*-2-[4-(4-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthalenedione (Fig. 1). Atovaquone is a yellow crystalline solid that is practically insoluble in water. It has a molecular weight of 366.84 and the molecular formula $C_{22}H_{19}ClO_3$ (Looareesuwan *et al*., 1999; Baggish and Hill, 2002; Zhou *et al*., 2009). Atovaquone is used therapeutically to treat *Plasmodium falciparum* malaria, *Pneumocystis carinii* pneumonia and *Toxoplasma gondii* toxoplasmosis (Haile and Flaherty, 1993; Kessl *et al*., 2004; Miller and Trepanier, 2002; Spencer and Goa, 1995; Wiesner *et al*., 2003). Atovaquone is a lipophilic analog of ubiquinone, an important component of the mitochondrial electron transfer system in cells. The antimalarial activity of atovaquone has been attributed to its interference with mitochondrial electron transport in the parasite, specifically at the cytochrome c reductase complex, which results in a collapse of the mitochondrial membrane potential (Fry and Pudney, 1992; Srivastava *et al*., 1999). The activity of the parasitic enzyme dihydroorotate dehydrogenase is also inhibited because of its dependence on a functional mitochondrial electron transport chain. As dihydroorotate dehydrogenase is a key enzyme in pyrimidine biosynthesis, its inhibition by atovaquone disrupts plasmodial DNA synthesis and replication (Korsinczky *et al*., 2000; McFadden *et al*., 2000; Olliaro, 2001; El Hage *et al*., 2009).

Although atovaquone has not been often associated with severe adverse reactions in the recommended dosages, to our knowledge there is no study evaluating its cytogenotoxicity *in vitro*. In this kind of assessment, the combination of different methods may play an important role in the assessment of cytogenotoxic damage caused by this type of drug, and these methods make it possible to evaluate the level of primary DNA damage or the dynamics of its repair even after short-term exposure to genotoxic agents (Abou-Eisha and Afifi, 2004). Considering that, and the lack of data on the cytogenetic status induced

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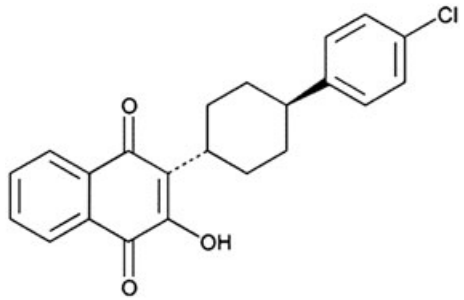

Figure 1. Chemical structure of atovaquone.

by atovaquone, the aim of this investigation was to assess the cytogenotoxic potential of clinically relevant concentrations of atovaquone *in vitro* in human peripheral blood lymphocytes. Although a specific metabolite of atovaquone has not been identified (Mehlotra *et al*., 2009), testing was done with and without metabolic activation. Two different concentrations of atovaquone were used. Plasma concentrations were obtained after administration of a fixed-dose combination with proguanil hydrochloride: 2950 ng ml^{-1}, used for prophylactic treatment, and 11800 ng ml^{-1}, used in the treatment of malaria. Assessment of cytogenotoxic potential was done by means of the cell viability (cytotoxicity) test and an alkaline version of the comet assay.

Materials and Methods

Chemicals

Just before the beginning of the experiment, atovaquone (Glenmark Pharmaceuticals Ltd, Mumbai, India) was dissolved in dimethyl sulfoxide (DMSO; Kemika, Zagreb, Croatia) and added to whole blood samples in the final concentrations of 2950 ng ml^{-1}, and 11800 ng ml^{-1}. RPMI 1640 medium was from Invitrogen (Carlsbad, CA, USA); rat liver S9 mix was from Moltox, Boone, NC, USA; acridine orange was from Heidelberg, Germany; histopaque-1119, ethidium bromide, low melting point (LMP) and normal melting point (NMP) agaroses were from Sigma (St Louis, MO, USA); heparinised vacutainer tubes were from Becton Dickinson (Franklin Lakes, NJ, USA). All other reagents used were laboratory-grade chemicals from Kemika.

Blood Sampling and Treatment

Evaluation of atovaquone was performed on peripheral blood lymphocytes obtained from a young (age 28 years), healthy, nonsmoking, male donor. The donor had not been exposed to ionizing radiation for diagnostic or therapeutic purposes or to known genotoxic chemicals that might have interfered with the results of the testing in the 12 month period prior to the blood sampling. Blood was drawn by antecubital venipuncture into heparinized vacutainers containing lithium heparin as anticoagulant under aseptic conditions. All experiments were conducted on the same blood sample treated with atovaquone in the final concentrations of 2950 and 11800 ng ml^{-1} for 1, 6 and 24 h. The concentrations used were those found in plasma when used in fixed-dose combination with proguanile hydrochloride: 2950 ng ml^{-1} after prophylactic treatment and 11800 ng ml^{-1} after treatment of malaria. The *in vitro* treatment in the present study was performed on nondividing human peripheral blood lymphocytes (G_0). Testing was done with and without S9 metabolic activation (10%, v/v) which is routinely used in cytogenetic assays. Blood samples were incubated *in vitro* at 37 °C in a humified atmosphere with 5.0% CO_2 (Heraeus Heracell 240 incubator, Langenselbold, Germany). After the treatment, all experiments were conducted according to the standard protocols.

Cell Viability (Cytotoxicity) Test

The indices of cell viability and necrosis were established by differential staining of peripheral blood lymphocytes with acridine orange and ethidium bromide using fluorescence microscopy (Duke and Cohen, 1992). Lymphocytes were isolated using a modified Ficoll–Histopaque centrifugation method (Singh, 2000). The slides were prepared using 200 µl of peripheral blood lymphocytes and 2 µl of stain (acridine orange and ethidium bromide). The suspension mixed with dye was covered with a cover slip and analyzed under an epifluorescence microscope AX 70 (Olympus, Tokyo, Japan) using a 60× objective and fluorescence filters of 515–560 nm. A total of 100 cells per repetition were examined. The nuclei of vital cells emitted a green fluorescence and necrotic red fluorescence.

Alkaline Comet Assay Procedure

To detect primary DNA damage, the alkaline comet assay was performed on the whole blood samples according to Singh *et al*. (1988). Fully frosted slides were covered with 1% normal melting point (NMP) agarose. After solidification, the gel was scraped off the slide. The slides were then coated with 0.6% NMP agarose. When this layer had solidified, a second layer, containing the whole blood sample mixed with 0.5% LMP agarose was placed on the slides. After 10 min of solidification on ice, slides were covered with 0.5% LMP agarose. Slides were then immersed for 1 h in ice cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% sodium sarcosinate, pH 10, with 1% Triton X-100 and 10% DMSO) to lyse the cells and allow DNA unfolding. The slides were then placed on a horizontal gel electrophoresis tank, facing the anode. The unit was filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM Na_2 EDTA, pH 13.0) and the slides were placed in this alkaline buffer for 20 min to allow DNA unwinding and expression of alkali-labile sites. Denaturation and electrophoresis were performed at 4 °C under dim light. Electrophoresis was carried out for 20 min at 25 V (300 mA) (20 min at 1 V cm^{-1}). After electrophoresis, the slides were rinsed gently three times with neutralization buffer (0.4 M Tris–HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide and covered with a coverslip. Slides were stored at 4 °C in sealed boxes until analyzed. To prevent additional DNA damage, blood samples handling and all the preparation steps of the slides for the comet assay were conducted under yellow light or in the dark.

Comet Capture and Analysis

One hundred randomly captured comets from each slide were examined at 250× magnification using an epifluorescence microscope (Zeiss, Oberkochen, Germany) connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd, Haverhill, Suffolk, UK). An automated image analysis system was used to acquire images,

compute the integrated intensity profile for each cell, estimate the comet cell components and evaluate the range of derived parameters. Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells and superimposed comets or comets without distinct head ('clouds', 'hedgehogs' or 'ghost cells'). To avoid potential variability, one well-trained scorer performed all scorings of comets. To quantify DNA damage, the following comet parameters were evaluated: tail length, tail intensity (percentage DNA) and tail moment. Tail length (i.e. the length of DNA migration) is related directly to the DNA fragment size and is presented in micrometers. It was calculated from the center of the cell. Tail intensity is defined as the percentage of fluorescence migrated in the comet tail. Tail moment was calculated as (tail length × % DNA in tail)/100. Furthermore, the frequency of apoptotic and necrotic cells was also evaluated on the same slides. The apoptotic and necrotic index was calculated as the percentage of cells with highly spread tail and undefined head of uniform intensity, indicating necrosis, and cells with diffuse fan-like tails and small heads, indicating apoptosis, from a minimum of 100 cells counted per slide (Olive *et al.*, 1993; Henderson *et al.*, 1998; Wada *et al.*, 2003).

Statistics

For the results of the comet assay measured after treatment with atovaquone, statistical evaluation was performed using the STATISTICA 5.0 package (StaSoft, Tulsa, OK, USA). Each sample was characterized for the extent of DNA damage by considering the mean ± SD (standard deviation of the mean), median and range. In order to normalize the distribution and to equalize the variances, a logarithmic transformation of data was applied. Multiple comparisons between groups were done by means of ANOVA on log-transformed data. *Post hoc* analyses of differences were done using the Scheffé test. As for the cell viability, statistical significance was analyzed using the Student's *t*-test. The level of statistical significance was set at $P < 0.05$.

Results

As shown in Figs 2 and 3, viability of peripheral blood lymphocytes, as determined by acridine orange and ethidium bromide staining, using fluorescence microscopy was not significantly affected in both concentrations and in all measured time periods with or without addition of metabolic activator.

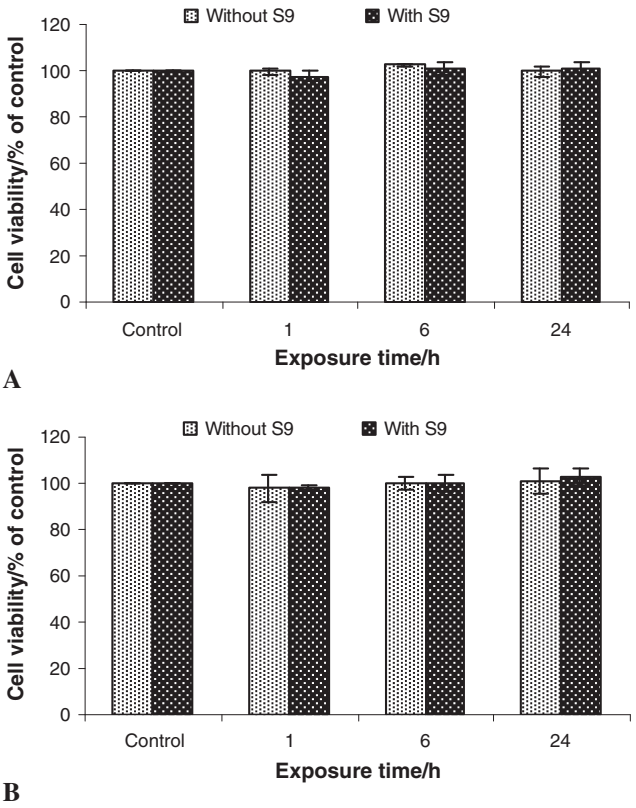

Figure 3. Cell viability after *in vitro* treatment of human peripheral blood lymphocytes with atovaquone in concentrations of 2950 ng ml^{-1} (A) and 11800 ng ml^{-1} (B) with and without S9 metabolic activation, in different time periods. Results are presented as mean values ± SD (standard deviation of the mean).

Basic statistics for the tail length, tail intensity and tail moment parameters of the alkaline comet assay for different concentrations and times of exposure are presented in Table 1. None of the three determined parameters showed significant deviation compared with the corresponding control samples. When comparing mean values between different concentrations and exposure times, there were also no changes. Addition of metabolic activator also did not induce any significant changes in either comet parameter.

The frequency of necrotic and apoptotic cells in peripheral blood lymphocytes was measured on the same slides as comets. As shown in Fig. 4, the necrotic and apoptotic cells were

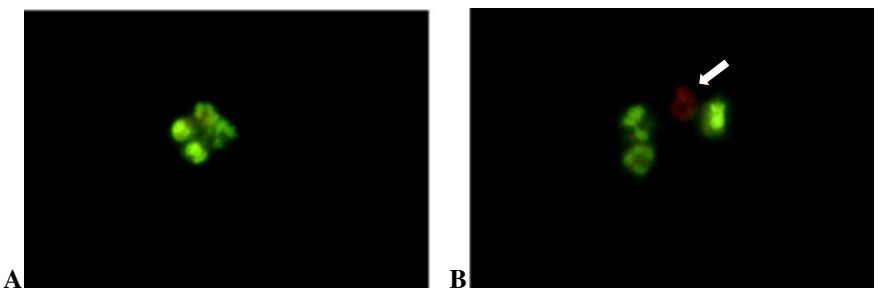

Figure 2. Cell viability microphotographs represent viable lymphocytes from the un-exposed sample (A), and dead (B) cell from sample treated with atovaquone indicated by an arrow. Cells were differentially stained with acridine orange and ethidium bromide. Cells were photographed under the fluorescent microscope, using a 60× objective and fluorescence filters of 515–560 nm.

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Table 1. Results of the alkaline comet assay parameters (tail length, tail intensity and tail moment) in human peripheral blood lymphocytes after treatment with atovaquone in concentrations of 2950 and 11800 ng ml^{-1} with and without S9 metabolic activation in different time periods

Exposure time (h)	Sample concentration (ng ml^{-1})	Metabolic activation	Tail length (μm)				Tail intensity				Tail moment			
			Min	Mean ± SD	Max	Median	Min	Mean ± SD	Max	Median	Min	Mean ± SD	Max	Median
1	Control	−S9	10.90	13.08 ± 1.74	19.23	12.82	0.00	1.03 ± 1.32	8.69	0.55	0.00	0.12 ± 0.16	1.00	0.07
		+S9	10.90	12.97 ± 1.54	17.95	12.82	0.00	1.25 ± 1.62	8.48	0.63	0.00	0.15 ± 0.19	0.98	0.07
	DMSO	−S9	10.90	13.02 ± 1.56	17.95	12.82	0.00	1.56 ± 2.21	9.48	0.68	0.00	0.18 ± 0.26	1.19	0.08
		+S9	10.90	13.13 ± 1.35	17.95	12.82	0.00	1.18 ± 1.60	8.18	0.61	0.00	0.14 ± 0.18	0.92	0.07
	2950	−S9	10.90	12.90 ± 1.41	18.59	12.82	0.00	1.25 ± 1.92	8.41	0.46	0.00	0.15 ± 0.22	1.02	0.06
		+S9	10.90	13.13 ± 1.44	18.59	12.82	0.00	0.96 ± 1.41	8.28	0.38	0.00	0.11 ± 0.17	1.01	0.05
	11800	−S9	10.90	13.01 ± 1.32	16.67	12.82	0.00	1.15 ± 1.64	8.17	0.56	0.00	0.13 ± 0.19	0.97	0.07
		+S9	10.90	13.32 ± 1.64	18.59	12.82	0.00	1.15 ± 1.74	8.15	0.51	0.00	0.14 ± 0.21	0.99	0.06
6	Control	−S9	10.90	13.78 ± 1.60	18.59	13.46	0.00	1.49 ± 1.97	9.96	0.86	0.00	0.18 ± 0.24	1.21	0.11
		+S9	10.90	13.49 ± 1.35	16.67	13.46	0.00	1.35 ± 2.22	9.62	0.46	0.00	0.17 ± 0.26	1.11	0.06
	DMSO	−S9	10.90	13.65 ± 1.60	19.23	13.46	0.00	1.42 ± 1.90	9.51	0.78	0.00	0.17 ± 0.23	1.16	0.09
		+S9	10.90	13.42 ± 1.59	17.95	13.46	0.00	1.70 ± 2.16	9.87	0.70	0.00	0.20 ± 0.26	1.14	0.09
	2950	−S9	10.90	13.81 ± 1.59	18.59	13.46	0.00	1.30 ± 1.90	9.47	0.51	0.00	0.16 ± 0.24	1.27	0.07
		+S9	10.90	13.40 ± 1.49	18.59	12.82	0.00	1.08 ± 1.48	8.21	0.55	0.00	0.13 ± 0.18	1.00	0.07
	11800	−S9	10.90	13.80 ± 1.71	19.23	13.46	0.00	1.10 ± 1.47	9.49	0.53	0.00	0.14 ± 0.18	1.16	0.07
		+S9	10.90	13.84 ± 1.48	19.23	13.46	0.00	1.58 ± 2.35	9.81	0.56	0.00	0.19 ± 0.27	1.14	0.08
24	Control	−S9	10.90	14.18 ± 2.16	19.23	13.46	0.00	1.69 ± 1.98	8.74	0.95	0.00	0.21 ± 0.24	1.12	0.12
		+S9	10.90	13.82 ± 2.45	20.51	12.82	0.00	1.93 ± 1.96	9.66	1.20	0.00	0.23 ± 0.24	1.24	0.15
	DMSO	−S9	10.90	14.01 ± 1.74	18.59	13.46	0.00	1.79 ± 1.94	9.79	1.15	0.00	0.22 ± 0.23	1.07	0.14
		+S9	10.90	14.59 ± 2.85	23.08	13.46	0.00	1.85 ± 2.27	9.86	0.81	0.00	0.24 ± 0.31	1.39	0.10
	2950	−S9	10.90	14.02 ± 2.20	20.51	13.46	0.00	1.92 ± 2.15	9.95	1.16	0.00	0.24 ± 0.27	1.21	0.14
		+S9	10.90	13.76 ± 2.24	22.44	13.46	0.00	1.87 ± 2.00	9.34	1.25	0.00	0.23 ± 0.25	1.26	0.14
	11800	−S9	10.90	13.86 ± 2.26	19.23	13.46	0.00	2.10 ± 2.31	9.96	1.32	0.00	0.24 ± 0.26	1.08	0.15
		+S9	10.90	13.76 ± 2.24	22.44	13.46	0.00	1.87 ± 2.00	9.34	1.25	0.00	0.23 ± 0.25	1.26	0.14

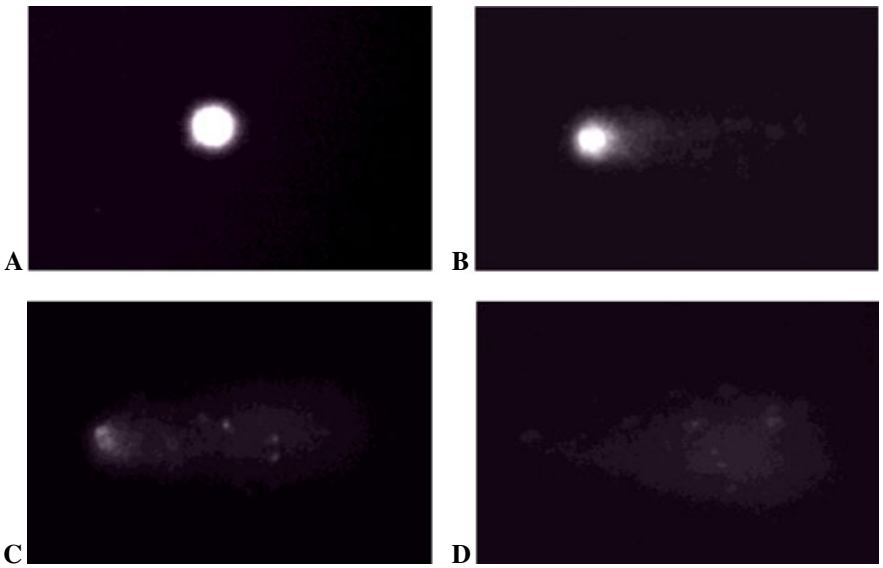

Figure 4. Comet assay microphotographs represent undamaged lymphocyte from the un-exposed sample (A). Image (B) represents damaged lymphocyte that has comet appearance. Necrotic cell (C) with highly spread tail and undefined head of uniform intensity and typical comet undergoing apoptosis (D) that has a small head and diffuse fan-like tail from samples treated with atovaquone. Cells were stained with ethidium bromide. Cells were photographed under the fluorescent microscope using a 60× objective equipped with a 515–560 nm excitation filters and a 590 nm barrier filter.

distinguished on the basis of their appearance. There was no significant enhancement in the percentage of apoptotic or necrotic cell death (Fig. 5) after the treatment with atovaquone with both concentrations, regardless of metabolic activation.

Discussion

Atovaquone represents a class of antimicrobial agents with a broad-spectrum activity against various parasitic infections, including malaria, toxoplasmosis and *Pneumocystis* pneumonia. In malaria parasites, atovaquone inhibits mitochondrial electron transport at the level of the cytochrome bc1 complex and collapses mitochondrial membrane potential. In addition, this drug is unique in being selectively toxic to parasite mitochondria without affecting the host mitochondrial functions (Danis and Bricaire, 2003; McKeage and Scott, 2003; Srivastava *et al.*, 1999). The selective toxicity of atovaquone towards *P. falciparum* is achieved by virtue of the different sensitivities of mammalian and plasmodial electron transport systems to hydroxynaphthoquinones, and also by the fact that *Plasmodium* spp. are

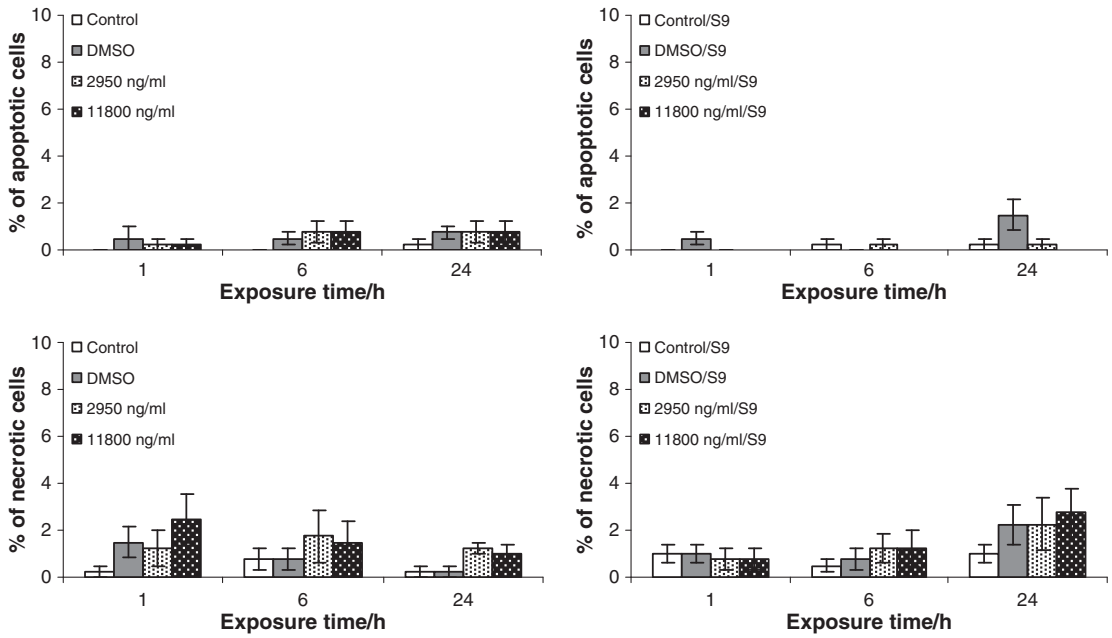

Figure 5. Percentage of apoptotic and necrotic cells evaluated with alkaline comet assay in human peripheral blood lymphocytes after treatment with atovaquone in concentrations of 2950 ng ml^{-1} and 11800 ng ml^{-1} with and without S9 metabolic activation in different time periods. Results are presented as mean values ± SD (standard deviation of the mean).

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dependent on *de novo* pyrimidine biosynthesis while mammalian cells are able to salvage and recycle pyrimidines. The inability of malaria parasites to salvage preformed pyrimidines results in atovaquone blocking nucleic acid synthesis and thus replication (Looareesuwan *et al*., 1999). Additionally, atovaquone has tissue schizonticidal activity, meaning that it kills parasites during the pre-erythrocytic (hepatic) stage of infection; a single 250 mg dose given before challenge is sufficient to prevent infection. This property of killing developing hepatic-stage parasites, known as causal prophylactic, allows discontinuation of atovaquone therapy soon after leaving the area where malaria is endemic (Chulay, 1998). The causal prophylactic activity of the combination agent atovaquone–proguanil has also been demonstrated (Berman *et al*., 2001).

Atovaquone as a single agent has a relatively favorable side effect profile, and causes few side effects that require withdrawal of therapy. The most common reactions are rash, fever, vomiting, diarrhea, abdominal pain and headache. Patients treated with atovaquone only occasionally exhibit abnormalities in serum transaminase and amylase levels. Atovaquone should not be used in infants, pregnant women, women breast-feeding infants or patients with severe renal impairment. Apparently, a few acute adverse effects are caused, but more clinical evaluation of the drug is needed, especially to detect possible rare, unusual or long-term toxicity (Artymowicz and James, 1998; Baggish and Hill, 2002; Kain, 2003; Taylor and White, 2004; AlKadi, 2007).

In the present study, assessment of possible cytotoxic and genotoxic potential of atovaquone was made by determination of lymphocytes viability and by virtue of measuring the DNA migration of treated cells *in vitro* by the use of the alkaline comet assay. These techniques, especially comet assay, are relatively fast, simple and sensitive for the assessment of citotoxicity and genotoxicity in human cells after exposure to different physical and chemical agents including drugs, both *in vivo* and *in vitro* (Collins *et al*., 2008; Dusinska and Collins, 2008; Gajski *et al*., 2008, 2010; Garaj-Vrhovac and Oreščanin, 2009; McArt *et al*., 2009; Piperakis, 2009; Tice *et al*., 2000). Hence, the *in vitro* comet assay is proposed as an alternative to standard cytogenetic assays in early genotoxicity screening of drug candidates (Snyder and Green, 2001; Witte *et al*., 2007).

For atovaquone, the oral median lethal dose (LD_{50}) in rats was greater than 1825 mg kg^{-1}. The intravenous LD50 in rats was 36 mg kg^{-1} and in mice was 26 mg kg^{-1}. Responses included ataxia, decreased activity, prostration and labored breathing. In standard laboratory studies with atovaquone, doses up to 500 mg kg^{-1} per day over 28 days caused no remarkable adverse effects, except for a slight decrease in erythrocyte parameters, in rats. Although atovaquone was not carcinogenic in rats, studies in mice showed treatment-related increases in the incidence of hepatocellular adenoma and hepatocellular carcinoma. Atovaquone was not mutagenic or DNA-damaging when evaluated in the Ames *Salmonella* mutagenicity assay, the L5178Y $TK^{+/-}$ mouse lymphoma cell mutagenesis assay and the cultured human lymphocyte cytogenetic assay. No evidence of genotoxicity was observed in the *in vivo* mouse micronucleus assay (GlaxoSmithKline, 2001, 2009).

Our results demonstrated that atovaquone did not cause any change in the percentage of viable cells, regardless of dose and exposure time, compared with unexposed samples. Samples with or without addition of metabolic activation also did not show any significant difference in percentage of viable cells which was expected since no active metabolite of atovaquone has been identified so far (Mehlotra *et al*., 2009). Results gained by the use of the alkaline comet assay indicate that atovaquone did not cause any DNA damaging effect in peripheral blood lymphocytes after treatment in both concentrations, independent of exposure time or addition of metabolic activation. Thus it is to be presumed that atovaquone in tested concentrations is not cytotoxic or genotoxic.

Conclusion

To our knowledge this study is the first to report a genotoxicity assessment of clinically relevant concentrations of atovaquone on human peripheral blood lymphocytes *in vitro*. Results gained by measuring comet assay parameters in addition to measuring the viability of treated cells are in accordance with available data on toxicity for this drug. Since addition of metabolic activation did not cause any significant changes compared with treatment without metabolic activation, it is to be presumed that atovaquone has no metabolite or it is also not toxic. Results indicate that atovaquone is highly safe for consumption from the aspect of cytogenotoxicity, when used as prophylactic treatment or in the treatment of malaria.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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3. *In vitro* effect of the antimalarial drug proguanil hydrochloride on viability and DNA damage in human peripheral blood lymphocytes

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In vitro effect of the antimalarial drug proguanil hydrochloride on viability and DNA damage in human peripheral blood lymphocytes

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ABSTRACT

This study aimed to evaluate the effect of proguanil, a chemical substance used for treatment and prevention of malaria on viability and DNA integrity in human lymphocytes in vitro. Two different concentrations of proguanil obtained from the plasma concentrations were used: 130 ng/ml used for prophylactic treatment and 520 ng/ml used in treatment of malaria. Testing was done with and without metabolic activation. Viability of lymphocytes decreased in time and dose dependent manner. Comet assay parameters showed similar effects, indicating that some damage to DNA molecule can occur. Frequency of sister chromatid exchanges did not show significant deviation from the control samples. As for the proliferation kinetics no significant changes were noticed. Since majority of DNA damaging effect is induced after metabolic activation it is to be concluded that activity of proguanil is dependent upon the active metabolite cycloguanil and that monitoring should be conducted especially among frequent travellers.

1. Introduction

Malaria, caused mostly by *Plasmodium falciparum*, remains one of the most important infectious diseases in the world. Antimalarial drug toxicity is viewed differently depending upon whether the clinical indication is for malaria treatment or prophylaxis. In the treatment of *P. falciparum* malaria, which has a high mortality if untreated, a greater risk of adverse reactions to antimalarial drugs is inevitable. Nevertheless, drug toxicity must be acceptable to patients and cause less harm than the disease itself (Taylor and White, 2004). Various drugs are widely used in prophylaxis and treatment of malaria, and one of them is proguanil that usually comes in fixed combination with atovaquone (Looareesuwan et al., 1999; Pearson, 2001; Patel and Kain, 2005). In the prevention of malaria in travellers, a careful risk-benefit analysis is required to balance the risk of acquiring potentially serious malaria against the risk of harm from the agent itself (Luzzi and Peto, 1993).

The chemical name of proguanil is 1-(4-chlorophenyl)-5-isopropyl-biguanide hydrochloride. Proguanil is a white crystalline solid that is soluble in water. It has a molecular weight of 290.22 and the molecular formula $C_{11}H_{16}ClN_5 \cdot HCl$ (Looareesuwan et al., 1999; Paci et al., 2002). Proguanil is metabolized in the liver by hepatic cytochrome P450 isoenzymes CYP2C19 and CYP3A4 to the active metabolite cycloguanil, which has been reported to act on *P. falciparum* dihydrofolate reductase (Armstrong and Smith, 1974; Kaneko et al., 1999; Toteja et al., 2001). This enzyme, which in *P. falciparum* is fused with thymidylate synthase as a homodimeric bifunctional protein, catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, thus providing a source of one-carbon donors for methyl transfer reactions and deoxythymidine synthesis required for parasite survival, and thus prevents multiplication of the plasmodium (Foote et al., 1990; Fidock et al., 1998; Sweetman, 2002).

Proguanil is used for both treatment of uncomplicated *P. falciparum* and prophylaxis of mild chloroquine-resistant malaria. The most common adverse effects reported in patients taking proguanil for treatment of malaria are abdominal pain, nausea, vomiting, and headache in adults, and vomiting in children. For prophylaxis of malaria adverse effects include headache and abdominal pain and vomiting in children. It is well tolerated, and although oral aphthous ulcerations are not uncommon, they are rarely severe enough to warrant discontinuing this medication. Proguanil is considered safe during pregnancy and breastfeeding, but insufficient drug is excreted in the milk to protect a breastfed infant (Schlagenhauf, 1999; AlKadi, 2007).

Although proguanil has not been often associated with severe adverse reactions in the recommended dosages there are only few studies regarding the effect of this drug on the genotoxic status of the cell (Bygbjerg, 1985; Bygbjerg and Flachs, 1986). Combining different methods may play an important role in the assessment of

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genotoxic damage caused by this type of drugs and these methods make it possible to evaluate the level of primary DNA damage or the dynamics of its repair even after short-term exposure to genotoxic agents (Abou-Eisha and Afifi, 2004). Considering that, and the lack of data on the cytogenetic status induced by proguanil, the aim of this investigation was to assess the genotoxic potential of proguanil in vitro in human peripheral blood lymphocytes with and without metabolic activation. Two different concentrations of proguanil were used. Concentrations were obtained from the plasma concentrations after administration of fixed-dose combination with atovaquone: 130 ng/ml used for prophylactic treatment and 520 ng/ml used in treatment of malaria. Assessment of genotoxic potential was done by means of the alkaline comet assay (SCGE) and sister chromatid exchange (SCE) analysis. In addition, viability and proliferation kinetics of treated peripheral blood lymphocytes were also measured.

2. Materials and methods

2.1. Blood sampling

Evaluation of proguanil was performed on peripheral blood lymphocytes obtained from a young (age 28 years), healthy, non-smoking, male donor. The donor had not been exposed to ionizing radiation for diagnostic or therapeutic purposes or to known genotoxic chemicals that might have interfered with the results of the testing in the 12-month period prior to the blood sampling. Blood was drawn by antecubital venipuncture into heparinized vacutainers containing lithium heparin as anticoagulant (Becton Dickenson, Franklin Lakes, NJ) under aseptic conditions.

2.2. Agent

Proguanil was administered as proguanil hydrochloride (Divis Laboratories Ltd, Hyderabad, India). Just before the beginning of the experiment, proguanil was dissolved in sterile redistilled water and added to lymphocyte cultures in the final concentrations of 130 ng/ml, and 520 ng/ml. The concentrations used are concentrations found in plasma when used in fixed-dose combination with atovaquone: 130 ng/ml after prophylactic treatment, and 520 ng/ml after treatment of malaria.

2.3. Experimental design

All experiments were conducted on the same blood sample treated with proguanil hydrochloride in the final concentrations of 130 ng/ml, and 520 ng/ml for 1 h, 6 h and 24 h. Testing was done with and without metabolic activation (10% (v/v) using rat liver S9 mix; Moltox, Boone, NC). Blood samples were incubated in vitro at 37 °C in a humified atmosphere with 5.0% CO_2 (Heraeus Heracell 240 incubator, Langenselbold, Germany). After the treatment, all experiments were conducted according to the standard protocols.

2.4. The cell viability test

Cell viability was determined by differential staining with acridine orange and ethidium bromide and by fluorescence microscopy (Duke and Cohen, 1992). Lymphocytes were isolated using a modified Ficoll-Histopaque (Sigma, St Louis, MO) centrifugation method (Singh, 2000). The slides were prepared using 200 μL of human peripheral blood lymphocytes and 2 μL of stain (acridine orange and ethidium bromide, both diluted in phosphate-buffered saline, PBS). A total of 100 cells per repetition were examined with an Olympus AX-70 microscope (Tokyo, Japan), using a 60× objective and fluorescence filters of 515–560 nm. The cells were divided into two categories: live cells with a functional membrane, with uniform green staining of the nucleus and dead cells with uniform orange staining of the nucleus.

2.5. The alkaline comet assay (SCGE)

DNA damage in lymphocytes was analyzed with the alkaline version of the comet assay detecting single-strand breaks, alkali labile and incomplete excision repair sites due to enhanced DNA migration (Singh et al., 1988). Fully frosted slides were covered with 1% normal melting point (NMP) agarose (Sigma). After solidification, the gel was scraped off the slide. The slides were then coated with 0.6% NMP agarose. When this layer had solidified, a second layer, containing the whole blood sample mixed with 0.5% low melting point (LMP) agarose (Sigma) was placed on the slides. After 10 min of solidification on ice, slides were covered with 0.5% LMP agarose. Slides were then immersed for 1 h in ice cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% sodium sarcosinate (Sigma), pH 10) with 1% Triton X-100 (Sigma) and 10% dimethyl sulfoxide (Kemika, Zagreb, Croatia) to lyse the cells and allow DNA unfolding. The slides were then placed on a horizontal gel electrophoresis tank, facing the anode. The unit was filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM Na_2 EDTA, pH 13.0) and the slides were placed in this alkaline buffer for 20 min to allow DNA unwinding and expression of alkali-labile sites. Denaturation and electrophoresis were performed at 4 °C under dim light. Electrophoresis was carried out for 20 min at 25 V (300 mA) (20 min at 1 V/cm). After electrophoresis, the slides were rinsed gently three times with neutralization buffer (0.4 M Tris–HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide and covered with a coverslip. Slides were stored at 4 °C in sealed boxes until analyzed. To prevent additional DNA damage, handling blood samples and all the steps included in the preparation of the slides for the comet assay were conducted under yellow light or in the dark.

2.6. Comet capture and analysis

One hundred randomly captured comets from each slide were examined at 250× magnification using an epifluorescence microscope (Zeiss, Oberkochen, Germany) connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd, Haverhill, Suffolk, UK). An automated image analysis system was used to acquire images, compute the integrated intensity profile for each cell, estimate the comet cell components and evaluate the range of derived parameters. Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells, and superimposed comets or comets without distinct head ("clouds", "hedgehogs", or "ghost cells"). To avoid potential variability, one well-trained scorer performed all scorings of comets. To quantify DNA damage, the following comet parameters were evaluated: tail length, tail intensity (% of DNA), and tail moment. Tail length (i.e. the length of DNA migration) is related directly to the DNA fragment size and is presented in micrometers (μm). It was calculated from the centre of the cell. Tail intensity is defined as the percentage of fluorescence migrated in the comet tail. Tail moment was calculated as (tail length × % of DNA in tail)/100. Moreover, the frequency of apoptotic and necrotic cells was also evaluated on the same slides. The apoptotic and necrotic index was calculated as the percentage of cells with highly spread tail and undefined head of uniform intensity indicating necrosis and cells with diffuse fan-like tails and small heads indicating apoptosis from a minimum of 100 cells counted per slide (Olive et al., 1993; Henderson et al., 1998; Wada et al., 2003).

2.7. Sister chromatid exchange (SCE) analysis

The sister chromatid exchange analysis was performed in agreement with the standard guidelines described by Latt et al. (1981). Blood samples were cultivated in Euroclone medium (Chromosome kit P, Euroclone, Italy). Cultures were set up and incubated at 37 ± 1 °C for 72 h. For SCE demonstration 5-Bromodeoxyuridine (0.06 ml) was added in the beginning of the culture period. To arrest dividing lymphocytes in metaphase, colchicine (0.004%) was added 4 h prior to the harvest. Cultures were centrifuged at 2000 rpm for 4 min, the supernatant was carefully removed, and cells were resuspended in a hypotonic solution (0.075 M KCl) at 37 °C. After centrifugation for 4 min at 2000 rpm, the cells were fixed with a freshly prepared fixative of ice cold methanol/glacial acetic acid (3:1, v/v). Fixation and centrifugation were repeated several times until the supernatant was clear. Cells were pelleted and resuspended in a minimal amount of fresh fixative to obtain a homogenous suspension. The cell suspension was dropped onto microscope slides and left to air-dry. To obtain harlequin chromosomes, slides were stained using a modified fluorescence plus Giemsa method (Perry and Wolff, 1974), technique that is used to distinguish between cells in their first (M_1), second (M_2) and third (M_3) in vitro metaphases based on the relative proportions of light- and dark-stained chromatids. A total of 50 randomly selected second division metaphases were analyzed blindly. Every point of exchange was counted as an SCE. The number of SCE per metaphase and range of SCE were determined.

2.8. Lymphocyte proliferation kinetics (PRI)

The 5-Bromodeoxyuridine (BrdU) differential staining technique was used to assess the effects of proguanil on cell replication. Cells dividing for the first (M_1), second (M_2) or third (M_3) time in culture containing BrdU were determined by the differential staining pattern of sister chromatids. Lymphocyte proliferation kinetics was studied on 50 differentially stained metaphases per each sample. The proliferation rate index (PRI) was calculated according to the formula: $PRI = (M_1 + 2M_2 + 3M_3)$/total number of cells scored (Lamberti et al., 1983).

2.9. Statistical analysis

Each experimental set contained duplicated slides. For the results of the comet assay and sister chromatid exchange analysis measured after treatment with proguanil statistical evaluation was performed using Statistica 5.0 package (StaSoft, Tulsa, OK). Each sample was characterized for the extent of DNA damage by considering the mean ± SD (standard deviation of the mean), median, and range. In order to normalize the distribution and to equalize the variances, a logarithmic transformation of data was applied. Multiple comparisons between groups were done by means of ANOVA on log-transformed data. Post hoc analyses of differences were done by using the Scheffé test. As for the cell viability and proliferation kinetics, statistical significance was analyzed using the Student's *t*-test. The level of statistical significance was set at $P < 0.05$.

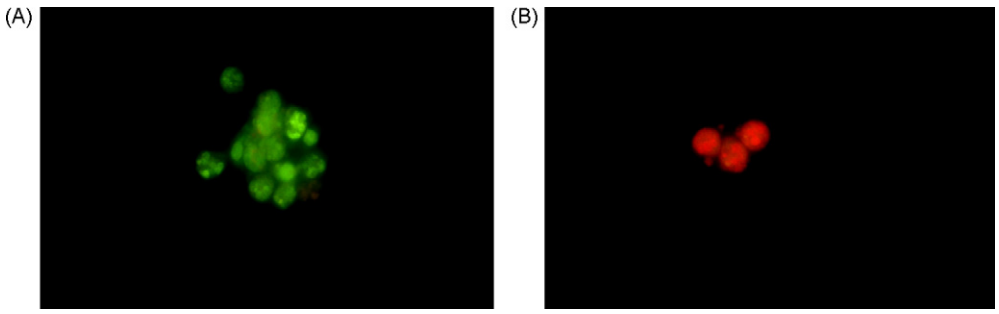

Fig. 1. Cell viability microphotographs represent viable lymphocyte from the un-exposed sample (A), and dead (B) cell from sample treated with proguanil hydrochloride. Cells were differentially stained with acridine orange and ethidium bromide. Cells were photographed under the fluorescent microscope, using a 60× objective and fluorescence filters of 515–560 nm.

3. Results

As shown in Figs. 1 and 2, viability of the cells, as determined by acridine orange and ethidium bromide staining, using fluorescence microscopy decreased in time and dose dependent manner, but statistically significant decrease was noticed only for 130 ng/ml treatments with metabolic activation on 6 h and 24 h whereas 520 ng/ml treatments decreased viability with and without metabolic activation on 6 h and 24 h ($P < 0.05$). These data indicate that proguanil inhibited cell viability, resulting in a 19.15 ± 11.4%, and 39.16 ± 14.5% reduction of viable cells at 130 ng/ml, and 520 ng/ml without metabolic activation along with 23.03 ± 11.5%, and 49.4 ± 6.93% reduction of viable cells at 130 ng/ml, and 520 ng/ml with metabolic activation, respectively.

Basic statistics for the tail length, tail intensity and tail moment parameters of the alkaline comet assay for different concentrations and times of exposure are presented in Tables 1–3. All three determined parameters showed significant deviation from normal distribution in the assays only for higher concentration and exposure time with addition of metabolic activation. When comparing mean values between different concentrations and exposure times, all three parameters increased with concentration and exposure time. This difference was higher when the metabolic activation was administered. The results of Scheffé's post hoc comparison test showed a significant difference between control values and all three exposure times for the 520 ng/ml concentration for the tail length parameter, and for the 6 h and 24 h for the tail moment while difference in tail intensity was only significant for the 24 h treatment. Significant difference was also found between lower concentration and corresponding control, but only for the 24 h treatment for the tail length and tail moment parameters. Significance was higher with addition of metabolic activation.

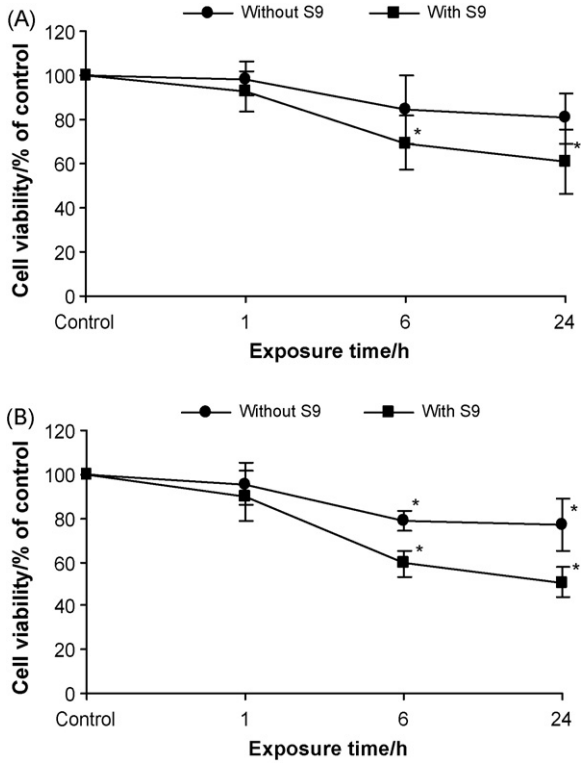

Fig. 2. Cell viability after in vitro treatment of human peripheral blood lymphocytes with proguanil hydrochloride in concentrations of 130 ng/ml (A) and 520 ng/ml (B) with and without S9 metabolic activation in different time periods. Results are presented as mean values ± SD (standard deviation of the mean). *Statistically significant decrease compared to the corresponding control ($P < 0.05$).

The frequency of necrotic and apoptotic cells in human peripheral blood lymphocytes was measured by the use of the alkaline comet assay. As shown in Fig. 3, the necrotic and apoptotic cells were distinguished on the basis of their appearance. Treatment of cells with 130 ng/ml, and 520 ng/ml concentrations of proguanil resulted in slight enhancement of necrotic cell death compared to the corresponding controls whereas there was no enhancement in the percentage of apoptotic cell death (Fig. 4). The increase was dose and time dependent. The highest level of necrosis was observed after 24 h with metabolic activation.

Results of the frequencies of sister chromatid exchanges in human peripheral blood lymphocytes are summarized in Table 4. The mean SCE per cell in all the exposed samples was slightly higher than that in the corresponding controls and was time and dose dependent, but there was no statistical significance among measured values. Results of the proliferation kinetics on peripheral blood lymphocytes, as determined by 5-Bromodeoxyuridine (BrdU) differential staining technique revealed that proguanil did not inhibit lymphocyte proliferation in significant manner without or with metabolic activation.

4. Discussion

This study aimed to evaluate the effect of proguanil, a chemical substance used for treatment and prevention of malaria caused by *P. falciparum*, on DNA integrity in human peripheral blood lymphocytes in vitro. Proguanil is a prodrug of cycloguanil, a dihydrofolate reductase inhibitor, which in malaria parasites primarily blocks the conversion of deoxyuridine monophosphate to deoxythymidine in the thymidylate synthase reaction (Sabchareon et al., 1998; McKeage and Scott, 2003). Proguanil undergoes two main oxidation reactions to an active triazine metabolite cycloguanil, principally via CYP2C19 and partly via CYP3A4 and to a minor extent to an N-dealkylated product, 4-chlorophenylbiguanide via CYP2C19, which has no antimalarial activity (Helsby et al., 1993; Dorne et al., 2003;

Table 1
Results for the tail length parameter of the alkaline comet assay in human peripheral blood lymphocytes after treatment with proguanil hydrochloride in concentrations of 130 ng/ml and 520 ng/ml with and without S9 metabolic activation in different time periods.

Exposure time (h)	Sample concentration (ng/ml)	Metabolic activation	Tail length (μm)			
			Min	Mean ± SD	Max	Median
1	Control	−S9	11.54	14.17 ± 1.86	21.79	14.10
		+S9	10.90	14.14 ± 2.15	21.79	13.46
	130	−S9	10.90	14.19 ± 2.16	21.79	13.46
		+S9	10.90	14.39 ± 2.73	24.36	13.46
	520	−S9	11.54	14.90 ± 2.40	23.72	14.10
		+S9	11.54	15.53 ± 3.09*,#	26.28	14.74
6	Control	−S9	10.90	14.40 ± 1.83	19.87	14.10
		+S9	11.54	14.94 ± 2.13	21.15	14.42
	130	−S9	11.54	14.64 ± 1.84	20.51	14.10
		+S9	11.54	14.70 ± 1.99	21.15	14.10
	520	−S9	11.54	15.13 ± 1.85	20.51	14.74
		+S9	11.54	16.60 ± 3.18*,#	26.92	15.38
24	Control	−S9	11.54	16.42 ± 3.17	26.28	15.38
		+S9	11.54	17.03 ± 3.17	25.64	16.67
	130	−S9	11.54	18.94 ± 5.32*	36.54	17.31
		+S9	12.82	21.00 ± 6.88*	42.95	19.23
	520	−S9	12.18	20.89 ± 7.41*	42.95	17.31
		+S9	12.82	24.31 ± 9.78*,#	46.79	21.47

* Statistically significant increase compared to the corresponding control ($P < 0.05$).
\# Statistically significant increase compared to the treatment without S9 metabolic activation ($P < 0.05$).

Gardiner and Begg, 2006; Kerb et al., 2009; Zhou et al., 2009; Soyinka and Onyeji, 2010).

In this study, evaluation of DNA damage was made by virtue of measuring comet assay parameters and frequency of sister chromatid exchanges, in addition to determination of lymphocyte viability. Because of the differential staining, SCE technique also allows metaphases in first division, second division, and third division to be recognized after culturing (Degrassi et al., 1989). For this reason, the SCE analysis was also employed in estimation of proliferation kinetics after the proguanil treatment. These techniques are relatively fast, simple, and sensitive for the analysis of DNA damage in mammalian cells. Therefore, the in vitro comet assay and SCE analysis are proposed as an alternative to standard cytogenetic assays in early genotoxicity screening of drug candidates (Snyder and Green, 2001; Witte et al., 2007).

Our results demonstrated that proguanil decreased the percentage of viable cells in a dose and time dependent manner, but significant decrease was noticed only for 130 ng/ml treatments with metabolic activation, whereas 520 ng/ml treatments decreased viability with and without metabolic activation, respectively.

The alkaline comet assay is now a well-established genotoxicity test for the estimation of DNA damage at the individual cell level both in vivo and in vitro (Collins et al., 2008; Piperakis, 2009). In human studies, the comet assay has widely been used to quantify DNA damage in isolated lymphocytes after exposure to different physical and chemical agents including drugs (Piperakis, 2009; Tice et al., 2000; Dusinska and Collins, 2008; Gajski et al., 2008). Results gained by using the alkaline comet assay indicate that proguanil causes some DNA damage in human lymphocytes after treatment with higher concentration of proguanil in lower exposure periods whereas, after long exposure even concentrations measured for prophylaxis can induce genetic alterations. In addition, DNA damage was more prominent with addition of metabolic activation.

Another technique used in this study to investigate the impact of proguanil on DNA molecule is sister chromatid exchange analysis,

Table 2
Results for the tail intensity parameter of the alkaline comet assay on human peripheral blood lymphocytes after treatment with proguanil hydrochloride in concentrations of 130 ng/ml and 520 ng/ml with and without S9 metabolic activation in different time periods.

Exposure time (h)	Sample concentration (ng/ml)	Metabolic activation	Tail intensity			
			Min	Mean ± SD	Max	Median
1	Control	−S9	0.00	1.09 ± 1.29	6.67	0.60
		+S9	0.00	1.57 ± 1.94	8.83	0.85
	130	−S9	0.00	1.49 ± 2.15	13.32	0.56
		+S9	0.00	1.55 ± 2.20	9.46	0.53
	520	−S9	0.00	1.60 ± 2.06	11.61	1.00
		+S9	0.00	2.19 ± 2.79	14.00	1.00
6	Control	−S9	0.00	2.35 ± 2.36	9.87	1.55
		+S9	0.00	2.00 ± 2.15	9.78	1.30
	130	−S9	0.00	1.91 ± 2.07	9.33	1.22
		+S9	0.00	1.95 ± 2.47	10.99	1.09
	520	−S9	0.00	1.73 ± 2.02	9.24	0.97
		+S9	0.00	2.84 ± 3.16#	14.68	1.89
24	Control	−S9	0.00	2.63 ± 2.27	9.54	1.93
		+S9	0.00	2.93 ± 2.53	9.53	2.37
	130	−S9	0.00	4.11 ± 4.15	17.58	2.50
		+S9	0.00	4.53 ± 4.58	19.72	3.04
	520	−S9	0.00	4.42 ± 4.16*	20.70	2.91
		+S9	0.00	5.89 ± 5.40*	20.80	3.59

* Statistically significant increase compared to the corresponding control ($P < 0.05$).
\# Statistically significant increase compared to the treatment without S9 metabolic activation ($P < 0.05$).

Table 3
Results for the tail moment parameter of the alkaline comet assay on human peripheral blood lymphocytes after treatment with proguanil hydrochloride in concentrations of 130 ng/ml and 520 ng/ml with and without S9 metabolic activation in different time periods.

Exposure time (h)	Sample concentration (ng/ml)	Metabolic activation	Tail moment			
			Min	Mean ± SD	Max	Median
1	Control	−S9	0.00	0.14 ± 0.17	0.90	0.07
		+S9	0.00	0.20 ± 0.25	1.13	0.11
	130	−S9	0.00	0.19 ± 0.28	1.79	0.07
		+S9	0.00	0.21 ± 0.33	1.58	0.06
	520	−S9	0.00	0.21 ± 0.27	1.56	0.12
		+S9	0.00	0.29 ± 0.38[#]	1.86	0.13
6	Control	−S9	0.00	0.29 ± 0.29	1.21	0.19
		+S9	0.00	0.26 ± 0.27	1.19	0.16
	130	−S9	0.00	0.24 ± 0.25	1.08	0.15
		+S9	0.00	0.25 ± 0.30	1.48	0.14
	520	−S9	0.00	0.23 ± 0.26	1.24	0.13
		+S9	0.00	0.39 ± 0.44[*,#]	2.21	0.24
24	Control	−S9	0.00	0.36 ± 0.32	1.46	0.25
		+S9	0.00	0.41 ± 0.35	1.52	0.32
	130	−S9	0.00	0.62 ± 0.66[*]	2.89	0.35
		+S9	0.00	0.73 ± 0.77[*]	3.24	0.48
	520	−S9	0.00	0.73 ± 0.77[*]	3.98	0.48
		+S9	0.00	1.04 ± 1.01[*,#]	4.03	0.54

[*] Statistically significant increase compared to the corresponding control ($P < 0.05$).
[#] Statistically significant increase compared to the treatment without S9 metabolic activation ($P < 0.05$).

employed as a sensitive biomarker in evaluation of DNA damage since SCE is known to increase as a result of the exposure to various genotoxic agents such as drugs and seem to reflect repair of DNA lesions by homologous recombination (Tucker et al., 1993; Wilson and Thompson, 2007; Garcia-Sagredo, 2008). In this study, frequency of sister chromatid exchanges did not show statistically significant deviation from the control samples even though mean SCE per cell in all the exposed samples was slightly higher than that in corresponding control and was more prominent with addition of metabolic activation.

In the study done by Bygbjerg (1985) regarding effect of both, proguanil and cycloguanil on human lymphocytes in vitro, proguanil had no effect on ^{14}C-thymidine incorporation or on the number of cells. On the contrary, cycloguanil, in concentrations corresponding to the plasma levels found in clinical practice, blocked the endogenous synthesis of thymidine and decreased the number of mitogen- and antigen-stimulated cells. The effect on phytohaemagglutinin-stimulated cells was temporary. Higher concentrations of cycloguanil, corresponding to intralymphocytic levels in clinical practice, permanently suppressed the growth of lymphocytes. Also the effect of cycloguanil could be reversed by low doses of folinic acid and high doses of folic acid suggesting that the mechanism of action of cycloguanil is competitive blocking of dihydrofolate reductase. In addition the shortage of thymidine can lead to uracil misincorporation into DNA, its subsequent excision, and resultant strand breakage (Curtin et al., 1991; Fidock and Wellems,

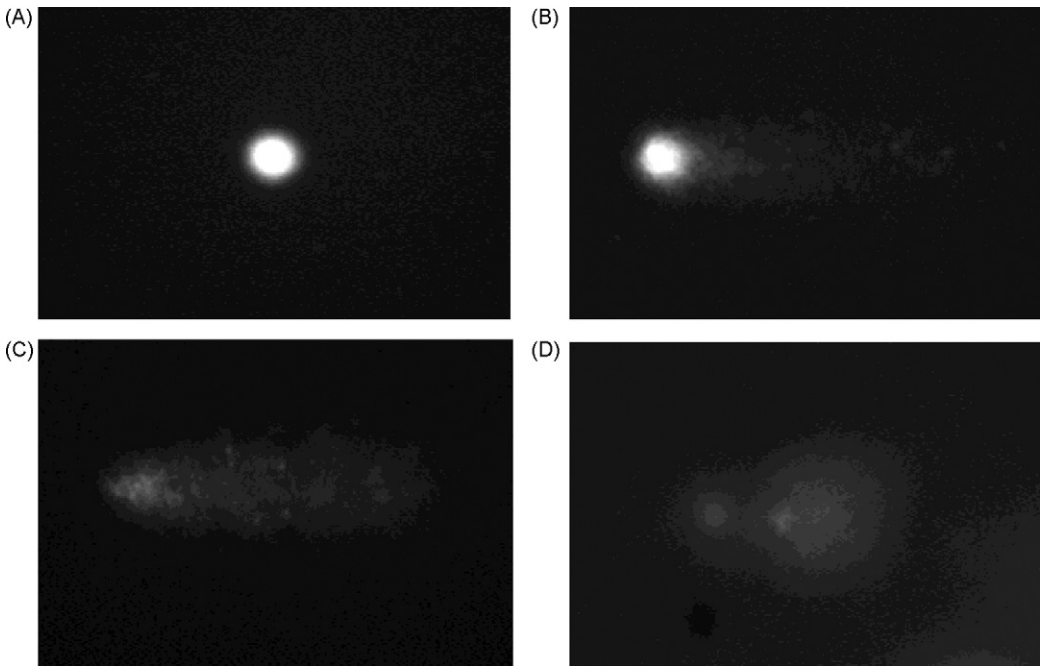

Fig. 3. Comet assay microphotographs represent undamaged lymphocyte from the un-exposed sample (A). Image (B) represents damaged lymphocyte that has comet appearance. Necrotic cell (C) with highly spread tail and undefined head of uniform intensity and typical comet undergoing apoptosis (D) that has a small head and diffuse fan-like tail from samples treated with proguanil hydrochloride. Cells were stained with ethidium bromide. Cells were photographed under the fluorescent microscope using a 60× objective equipped with a 515–560 nm excitation filters and a 590 nm barrier filter.

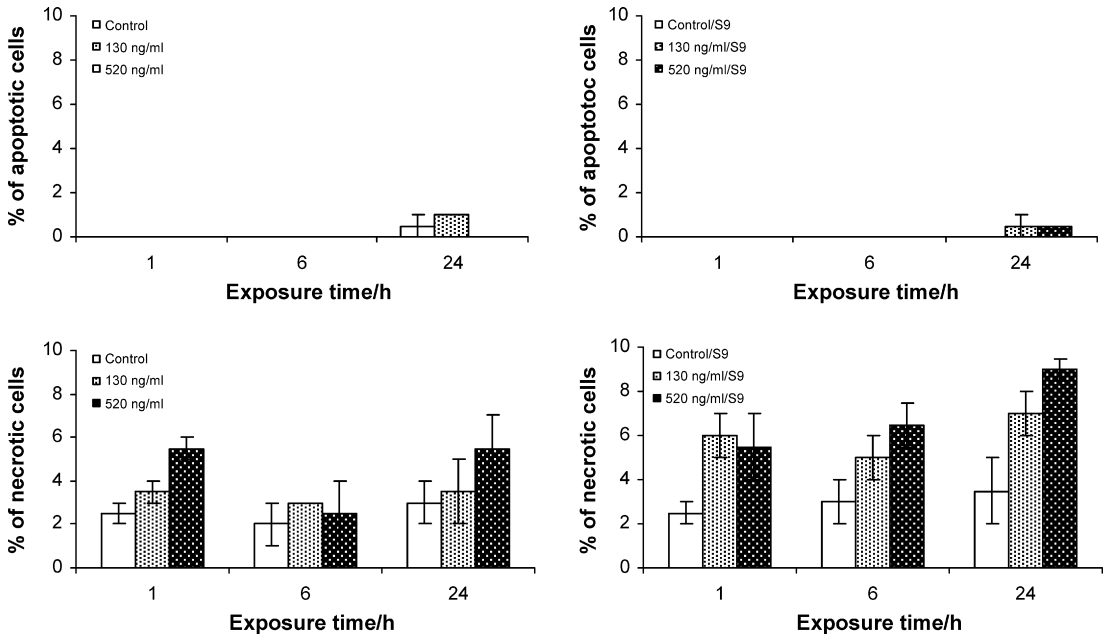

Fig. 4. Percentage of apoptotic and necrotic cells evaluated with alkaline comet assay in human peripheral blood lymphocytes after treatment with proguanil hydrochloride in concentrations of 130 ng/ml and 520 ng/ml with and without S9 metabolic activation in different time periods. Results are presented as mean values ± SD (standard deviation of the mean).

Table 4
Results for the frequency of sister chromatid exchanges in human peripheral blood lymphocytes after treatment with proguanil hydrochloride in concentrations of 130 ng/ml and 520 ng/ml with and without S9 metabolic activation in different time periods.

Exposure time (h)	Sample concentration (ng/ml)	Metabolic activation	SCE frequency			
			Min	Mean ± SD	Max	Median
1	Control	−S9	1	3.28 ± 2.11	9	2.00
		+S9	1	3.72 ± 2.33	10	3.00
	130	−S9	1	4.02 ± 2.19	9	4.00
		+S9	1	4.56 ± 2.40	10	4.50
	520	−S9	1	4.26 ± 1.68	7	4.00
		+S9	2	4.90 ± 1.93	10	5.00
6	Control	−S9	1	3.74 ± 2.31	10	3.00
		+S9	1	3.80 ± 1.86	10	4.00
	130	−S9	0	4.18 ± 2.54	12	4.00
		+S9	0	4.72 ± 2.12	12	4.50
	520	−S9	1	4.72 ± 2.24	10	4.00
		+S9	1	5.18 ± 2.80	13	5.00
24	Control	−S9	0	3.78 ± 2.21	12	3.00
		+S9	1	4.08 ± 2.04	9	4.00
	130	−S9	1	4.48 ± 2.06	10	4.00
		+S9	1	4.88 ± 2.37	10	5.00
	520	−S9	1	4.88 ± 1.85	10	5.00
		+S9	2	5.46 ± 2.18	11	5.00

1997; Fidock et al., 1998). Another study investigated effect of oral proguanil on human lymphocyte proliferation. Prophylactic dosage of proguanil did not alter the number of mononuclear cells in peripheral blood, but the number of neutrophils was slightly reduced (Bygbjerg and Flachs, 1986). In addition, Kharazmi et al. (1983) have shown that a therapeutic level of proguanil in serum can inhibit chemotaxis by neutrophils.

5. Conclusions

Our study is the first to report that proguanil and its metabolites have impact on DNA integrity what was shown by measuring comet assay parameters. Alkaline comet assay has proven useful in detecting genotoxicity of this type of drug. Given that greater part of DNA damaging effect is induced after metabolic activation it is to presume that principal metabolite cycloguanil has the major impact on DNA molecule. Another technique employed in this research showed no significant changes in the frequency of sister chromatid exchanges and there was no impact on the proliferation kinetics of treated lymphocytes. Results indicate that proguanil is relatively safe for consumption from the aspect of genotoxicity, especially if used as prophylactic treatment, since majority of DNA damaging effect is induced after the treatment with concentrations used in treatment of malaria that are four times higher than the ones used for prophylaxis. Present study has also confirmed the need for further cytogenetic research and regular patient monitoring to minimise the risk of adverse events especially among frequent travellers.

Conflict of interest

The authors declare that there are no conflicts of interest.

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4. Cytogenetic and oxidative status of human lymphocytes after exposure to clinically relevant concentrations of antimalarial drugs atovaquone and proguanil hydrochloride *in vitro*

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ORIGINAL ARTICLE

Cytogenetic and oxidative status of human lymphocytes after exposure to clinically relevant concentrations of antimalarial drugs atovaquone and proguanil hydrochloride in vitro

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ABSTRACT

Atovaquone (ATO) and proguanil hydrochloride (PROG) is the fixed combination for the prevention and treatment of *Plasmodium falciparum* malaria. As safe and effective antimalarial drugs are needed in both the treatment and the prophylaxis of malaria, this study was performed to investigate their possible cyto/genotoxic potential towards human lymphocytes and the possible mechanism responsible for it. Two different concentrations of ATO and PROG were used with and without S9 metabolic activation. The concentrations used were those found in human plasma when a fixed-dose combination of ATO and PROG was used: 2950/130 ng/mL after prophylactic treatment and 11 800/520 ng/mL after treatment of malaria, respectively. Possible cellular and DNA-damaging effects were evaluated by cell viability and alkaline comet assays, while oxidative stress potential was evaluated by formamidopyrimidine-DNA glycosylase (Fpg)-modified comet assay, in addition to measuring malondialdehyde and glutathione levels. According to our results, the ATO/PROG combination displayed only weak cyto/genotoxic potential towards human lymphocytes with no impact on oxidative stress parameters, suggesting that oxidative stress is not implicated in their mechanism of action towards human lymphocytes. Given that the key portion of the damaging effects was induced after S9 metabolic activation, it is to presume that the principal metabolite of PROG, cycloguanil, had the greatest impact. The obtained results indicate that the ATO/PROG combination is relatively safe for the consumption from the aspect of cyto/genotoxicity, especially if used for prophylactic treatment. Nevertheless, further cytogenetic research and regular patient monitoring are needed to minimize the risk of adverse events especially among frequent travellers.

INTRODUCTION

Malaria has always been a major cause of death in the tropics, and antimalarial drugs have played a key role in controlling its spread through the treatment of patients infected with plasmodial parasites and the control of its transmissibility. On the other hand, antimalarial drugs may exert adverse effects that can sometimes be serious [1–3]. In that manner, antimalarial agents should fulfil the requirements of efficacy

towards the parasite, in addition to being safe for the consumer and not putting them at an additional risk of adverse effects [4–8].

Atovaquone (ATO) and proguanil hydrochloride (PROG) is a fixed-dose combination of antimalarial agents. This combination interferes with two different pathways. ATO is a selective inhibitor of parasite mitochondrial electron transport, while PROG primarily exerts its effect by means of the metabolite cycloguanil (CYC), a dihydrofolate reductase inhibitor. Inhibition of dihydrofolate reductase in the malaria parasite disrupts deoxythymidylate synthesis [9–20]. Chemical structures of ATO, PROG and its active metabolite CYC are presented in *Figure 1*.

Both ATO and PROG have a favourable side effect profile [21,22], and although they have not been often associated with severe adverse reactions in the recommended dosages, to the best of our knowledge, cyto/genotoxicity studies have not been performed with ATO in combination with PROG in vitro. Previously, we reported that PROG alone in clinically relevant concentrations has an impact on cytotoxicity and DNA integrity of human peripheral blood lymphocytes (HPBLs) in vitro [23]. On the contrary, ATO alone did not have any impact on cell viability and DNA damage in clinically relevant concentrations in HPBLs in vitro [24].

In this kind of assessment, combinations of different methods may play an important role in the evaluation of cyto/genotoxic damage caused by this type of drugs, and these methods make it possible to evaluate the level of primary DNA damage or the dynamics of its repair even after short-term exposure to potentially genotoxic agents [25]. Considering the aforementioned and the lack of data on the cytogenetic status induced by ATO and PROG combination, this study aimed to investigate their possible cyto/genotoxic potential and the potential mechanism responsible for it. This was done by using clinically relevant concentrations of ATO and PROG on HPBLs in vitro, as those cells are sensitive biomarkers of exposure to different physical and/or chemical agents. Testing was performed with and without S9 metabolic activation. Two different concentrations of ATO and PROG were used. The concentrations used were those found in human plasma after administration of a fixed-dose combination of ATO/PROG: 2950/130 ng/mL, when used for prophylactic treatment, and 11 800/520 ng/mL, when used in the treatment of malaria, respectively. Assessment of cyto/genotoxic potential was performed by means of cell viability (cytotoxicity) assay with acridine orange (AO) and ethidium bromide (EtBr), whereas an alkaline version of the comet assay was applied for the evaluation of the genotoxic potential. Moreover, to explore the possible involvement of oxidative stress in the genotoxicity of this antimalarial drug combination, we used a formamidopyrimidine-DNA glycosylase (Fpg)-modified version of the comet assay that detects oxidative DNA damage, in addition to measuring malondialdehyde (MDA) and glutathione (GSH) levels as biomarkers of lipid peroxidation (LPO) and oxidative stress in total.

MATERIALS AND METHODS

Chemicals

Just before the beginning of the experiment, ATO (Glenmark Pharmaceuticals Ltd., Mumbai, India) was dissolved in dimethyl sulfoxide (DMSO; Kemika, Zagreb, Croatia), while PROG (Divis Laboratories Ltd., Hyderabad, India) was dissolved in sterile redistilled water. AO, disodium EDTA, EtBr, histopaque, metaphosphoric acid (MPA), 5,5′-dithiobis-2-nitrobenzoate (DTNB), 1,1,3,3-tetramethoxy propane (TMP), thiobarbituric acid (TBA), low melting point (LMP) and normal melting point agaroses and Triton X-100 were purchased from Sigma (St Louis, MO, USA). Potassium dihydrogen phosphate (KH_2PO_4) was purchased from Merck (Darmstadt, Germany); RPMI 1640 medium was purchased from Invitrogen (Carlsbad, CA, USA);

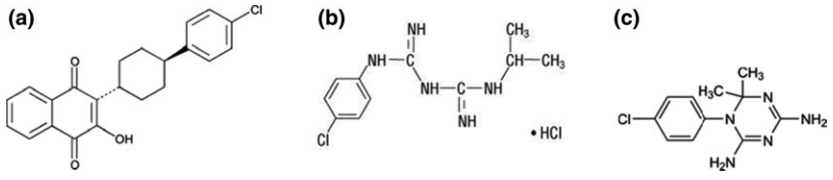

Figure 1 Chemical structures of atovaquone (a), proguanil hydrochloride (b) and the proguanil hydrochloride active metabolite cycloguanil (c).

rat liver S9 mix was purchased from Moltox (Boone, NC, USA), and heparinized vacutainer tubes were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). All other reagents used were laboratory-grade chemicals from Kemika.

Blood sampling and treatment

To overcome possible interindividual variability as a response to the treatment, blood samples were obtained from one young (age 28 years), healthy, non-smoking, male donor. The donor had not been exposed to ionizing radiation for diagnostic or therapeutic purposes or to known genotoxic chemicals that might have interfered with the results of the testing in the 12-month period prior to the blood sampling. Blood was drawn by antecubital venipuncture into heparinized vacutainers containing lithium heparin as an anticoagulant under aseptic conditions. The donor gave informed consent to participate in this study. The study was approved by the institutional ethics committee and observed the ethical principles of the Declaration of Helsinki. All experiments were conducted on the same blood sample treated jointly with ATO in the final concentrations (C_{max}) of 2950 and 11 800 ng/mL (8042 and 32 166 nM) and PROG in the final concentrations (C_{max}) of 130 and 520 ng/mL (512 and 2049 nM) for 1, 6 and 24 h. The concentrations used were those found in human plasma when a fixed-dose combination of ATO and PROG is used: 2950/130 ng/mL after prophylactic treatment and 11 800/520 ng/mL after treatment of malaria [26]. In each experiment, a nontreated solvent control was included. The in vitro treatment in this study was performed on HPBLs. Testing was performed with and without S9 metabolic activation (10%, v/v), which is routinely used in cytogenetic assays. Blood samples were incubated in vitro at 37 °C in a humidified atmosphere with 5% CO_2 (Heraeus Heracell 240 incubator, Langenselbold, Germany). After the treatment, all experiments were conducted according to the standard protocols.

Cell viability (cytotoxicity) test

Cytotoxicity of the tested ATO and PROG combination was established by differential staining of HPBLs with AO and EtBr using fluorescence microscopy [27]. Lymphocytes were isolated using a modified Ficoll–Histopaque centrifugation method. The slides were prepared by adding AO and EtBr (both diluted in phosphate-buffered saline, PBS) to HPBL suspension in the final concentration of 100 μg/mL (1 : 1; v/v). The suspension mixed with dyes was covered with a coverslip and analysed under an epifluorescence microscope (Olympus AX 70, Tokyo, Japan) using a 40× objective and fluorescence filters of 515–560 nm. A total of 100 cells per repetition were examined visually based on their appearance. The nuclei of vital cells emitted a green fluorescence and of dead cells red fluorescence.

The alkaline comet assay

The alkaline comet assay procedure followed the protocol by Singh et al. [28] with minor modifications [29]. Briefly, after the exposure, whole-blood samples were embedded in agarose matrix and lysed (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% DMSO; pH 10) overnight at 4 °C. After lysis, the slides were placed into alkaline solution (300 mM NaOH, 1 mM Na_2EDTA; pH 13) for 20 min at 4 °C to allow DNA unwinding and subsequently electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralized in 0.4 M Tris buffer (pH 7.5) for 5 min three times, stained with EtBr (20 μg/mL) and analysed at 250× magnification using an epifluorescence microscope (Zeiss, Göttingen, Germany) connected through a black-and-white camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd., Haverhill, Suffolk, UK). A total of 100 randomly captured comets were examined automatically from each slide. To quantify DNA damage, the following comet parameters were evaluated: tail length, tail intensity (percentage of DNA in tail) and tail moment. Furthermore, the frequency of apoptotic and necrotic cells was also evaluated visually on the same slides based on their appearance. The apoptotic and necrotic index was calculated as the percentage of cells with highly spread tail and undefined head of uniform intensity, indicating necrosis, and cells with diffuse fan-like tails and small heads, indicating apoptosis, from a minimum of 100 cells counted per slide [30–32].

Fpg-modified comet assay

The analysis of the formation of oxidized purines was performed using a modified formamidopyrimidine-DNA glycosylase (Fpg) FLARE™ (Fragment Length Analysis using Repair Enzymes) Assay Kit (Trevigen Inc., Gaithersburg, MD, USA) with minor modifications [29]. Fpg enzyme catalyses the excision of the following forms of DNA damage: (i) open-ring forms of 7-methylguanine, including 2,6-diamino-4-hydroxy-5-N-methyl-

formamidopyrimidine, and 4,6-diamino-5-formamidopyimidine, a lethal lesion; (ii) 8-oxoguanine, a highly mutagenic lesion and probably the most important biological substrate of Fpg; (iii) 5-hydroxycytosine and 5-hydroxyuracil; (iv) aflatoxin-bound imidazole-ring-opened guanine and (v) imidazole-ring-opened N-2-aminofluorene-C8-guanine. Therefore, by Fpg-modified comet assay, oxidative DNA damage can be detected. For each sample and control, whole-blood samples were mixed with LMP agarose (provided with the FLARE™ assay kit) and placed on the slides. After solidification on ice, the slides were covered with another layer of LMP agarose, then immersed in a pre-chilled lysis solution (provided with the FLARE™ assay kit) and finally lysed overnight at 4 °C. After lysis, the slides were treated with 100 μL of Fpg enzyme freshly diluted in REC dilution buffer (1 : 500) or with 100 μL of REC dilution buffer only (control) and were covered with a cover slip and incubated at 37 °C for 30 min. Subsequently, the slides were immersed in an alkali solution (0.3 M NaOH, 1 mM Na_2EDTA; pH 12.1) for 40 min and electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralized and stained with EtBr (20 μg/mL), and the comets were analysed automatically as described in alkaline comet assay. To quantify DNA damage, tail intensity (percentage of DNA in tail) parameter was evaluated.

Glutathione assay

The concentration of GSH in whole-blood samples was determined using Ellman's method [33]. Blood samples (800 μL) were previously hydrolysed with 3.1 mL of re-H_2O. Hydrolysed samples (3.5 mL) were treated with an equal volume of 1% MPA to precipitate proteins, and samples were subsequently centrifuged for 10 min at 3500 g. To the 500 μL of collected supernatant, 300 μL phosphate buffer (pH 7.4) and 50 μL DTNB were added. Absorbance was measured against the blank on a spectrophotometer (Cecil 9000; Cecil Instruments Ltd., Cambridge, UK) at 412 nm. The unknown concentration of GSH was calculated according to the molar extinction coefficient that was re-evaluated and calculated to be 14.15×10^3/M/cm at 412 nm and 25 °C [34] and expressed as μM.

Lipid peroxidation assay

The level of LPO was measured by determining the MDA concentration. The measurement of MDA level in blood samples was performed according to the method described in Gajski et al. [35]. The treated blood samples (800 μL) were first hydrolysed with 3.1 mL of re-H_2O, and afterwards, hydrolysed samples (3.5 mL) were treated with an equal volume of 1% MPA (to precipitate proteins) and were subsequently centrifuged for 10 min at 3500 ***g***. To 50 μL of supernatant or standard (TMP was employed as MDA standard), 400 μL H_3PO_4 (0.1%, v/v) and 100 μL TBA (0.6%, w/v) were added. Samples were mixed and incubated in thermostatic block for 30 min at 90 °C. To stop the reaction, samples were placed on ice before analysing with high-performance liquid chromatography (HPLC) on an HPLC (Knauer, Berlin, Germany) with fluorescent detector (Hitachi Merck, Darmstadt, Germany). The mobile phase consisted of 50 mM KH_2PO_4 and methanol (60 : 40, v/v, pH 6.8), and flow rate was set at 1 mL/min. Separation was performed on analytical column C-18 reverse-phase (LiChrospher; Merck) with 5-μm particles (125.0 × 4.0 mm), and the fluorescent detector wavelengths were set at λ_{ex} 527 nm and λ_{em} 551 nm. The MDA levels in the tested samples were calculated using the calibration curve ($r^2 = 0.9923$), and the results were expressed as μM.

Statistics

The results are presented as means ± SD (standard deviation of the mean). Statistical analyses were performed with STATISTICA 5.0 package (StaSoft, Tulsa, OK, USA). For the comet assay, multiple comparisons between groups were performed by means of ANOVA on log-transformed data. To normalize distribution and to equalize the variances, a logarithmic transformation of data was applied. Post hoc analyses of differences were performed using the Scheffé test. The difference in the cell viability and oxidative stress markers in control and treated samples was analysed using the Student's *t*-test. The level of statistical significance was set at $P < 0.05$.

RESULTS

The viability (cytotoxicity) of HPBLs exposed to ATO/PROG was determined by AO and EtBr in situ fluorescent assay. Changes were determined visually according to the differential staining of the nucleus. As shown in *Figure 2*, viability of the cells decreased in a time- and dose-dependent manner, but a statistically significant decrease was noticed for 2950/130 ng/mL treatment only for S9 metabolic activation after 6 and 24 h, whereas 11 800/520 ng/mL treatment decreased viability after 6 h with S9 metabolic activa-

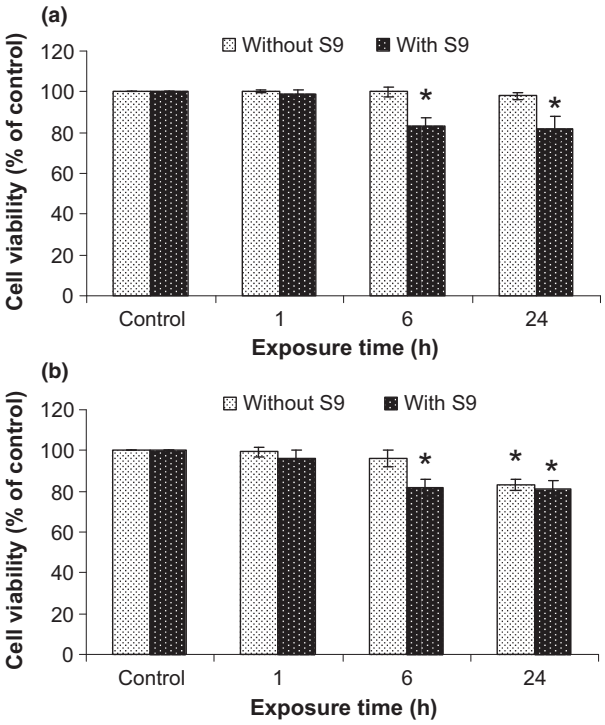

Figure 2 Cell viability after joint in vitro treatment of human peripheral blood lymphocytes with atovaquone and proguanil hydrochloride in concentrations of 2950/130 ng/mL (a) and 11 800/520 ng/mL (b) without and with S9 metabolic activation, in different time periods. Data are presented as mean values ± SD (standard deviation of the mean). *Statistically significant decrease compared to the corresponding control ($P < 0.05$).

tion and 24 h with and without S9 metabolic activation ($P < 0.05$). The IC_{50} (the concentration that reduces the viability of treated cells to 50%) values were greatly above the tested concentrations after all exposure periods. This is considered to be in the acceptable range for conducting further genotoxic analysis [36].

Results for the tail length, tail intensity and tail moment parameters of the alkaline comet assay for different concentrations and exposure periods are presented in *Figure 3*. All three of the determined parameters showed statistically significant increase compared to corresponding controls only for higher concentration after 6 and 24 h of exposure. The effect was more pronounced with the addition of S9 metabolic activation and was time- and dose dependent. The results of Scheffé's post hoc comparison test showed a significant difference between corresponding control values and 24 h of exposure for the 11 800/520 ng/mL concentration for all tested parameters without S9 metabolic activation, while significant difference with S9 metabolic activation was observed after 6 and 24 h of exposure. Significance was higher with the addition of S9 metabolic activation for tail length only.

The frequency of necrotic and apoptotic cells in HPBLs was also measured by the use of the alkaline comet assay. The necrotic and apoptotic cells were distinguished visually on the basis of their appearance. Treatment of cells with both 2950/130 and 11 800/520 ng/mL concentrations of ATO/PROG resulted in slight enhancement of necrotic cell death compared to the corresponding controls, whereas there was no enhancement in the percentage of apoptotic cell death (*Figure 4*).

Induction of oxidative DNA damage was studied with the modified comet assay with the purified DNA-damage-specific enzyme, Fpg, which recognizes and excises oxidized purines. Although we did observe an increase in DNA migration in Fpg-modified comet assay compared to corresponding controls, our results failed to show significant difference between undigested and Fpg-digested samples (*Figure 5*). This shows that the DNA damage induced by ATO/PROG was unlikely to be due to oxidative stress.

To further evaluate whether oxidative stress is involved in ATO/PROG toxicity, GSH and MDA levels were determined in blood samples after the treatment. The levels of both GSH and MDA were not significantly affected by ATO/PROG treatment regardless of concentration, exposure time or addition of S9 metabolic activation (*Figure 6*), suggesting that oxidative stress is not implicated in their mechanism of action against human lymphocytes.

DISCUSSION

Malaria, which affects a large number of international travellers each year, can be prevented through anti-mosquito measures and drug prophylaxis. ATO and PROG is a fixed-dose combination of antimalarial agents used for the prevention and treatment of malaria. However, antimalarial drugs may sometimes cause adverse effects [1–3]. Both ATO and PROG have a favourable side effect profile [21,22]. ATO causes few side effects that required withdrawal of therapy. Patients treated with ATO rarely exhibit abnormalities in serum transaminase and amylase levels. ATO/PROG should not be used in infants, pregnant women, women breastfeeding or patients with severe renal

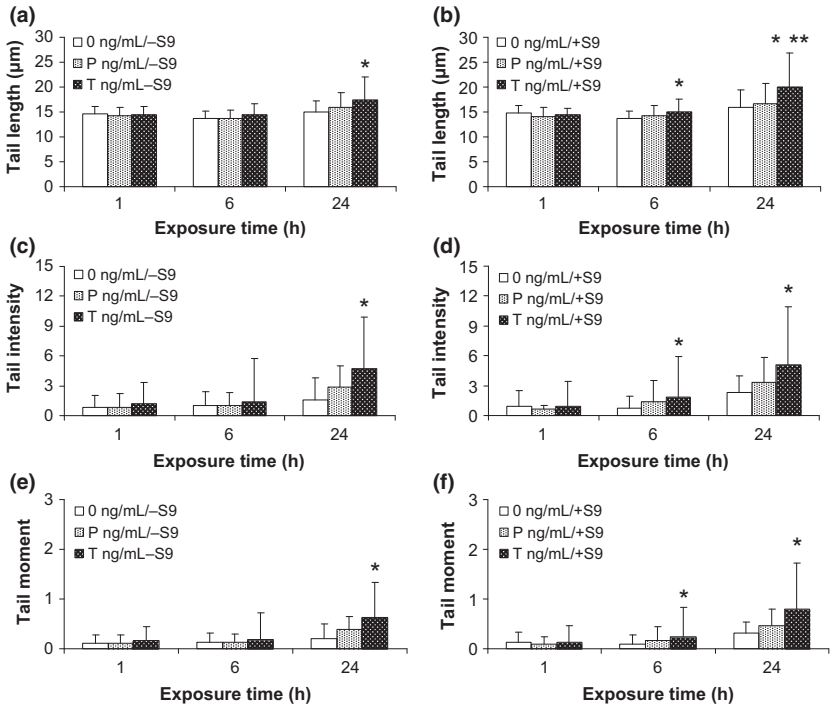

Figure 3 Results of the alkaline comet assay parameters [tail length (a, b), tail intensity (c, d) and tail moment (e, f)] in human peripheral blood lymphocytes after joint treatment with atovaquone and proguanil hydrochloride in concentrations of 2950/130 ng/mL (P – prophylactic treatment) and 11 800/520 ng/mL (T – treatment of malaria) without and with S9 metabolic activation in different time periods. Data are presented as mean values ± SD (standard deviation of the mean). *Statistically significant increase compared to the corresponding control ($P < 0.05$); **statistically significant increase compared to the treatment without S9 metabolic activation ($P < 0.05$).

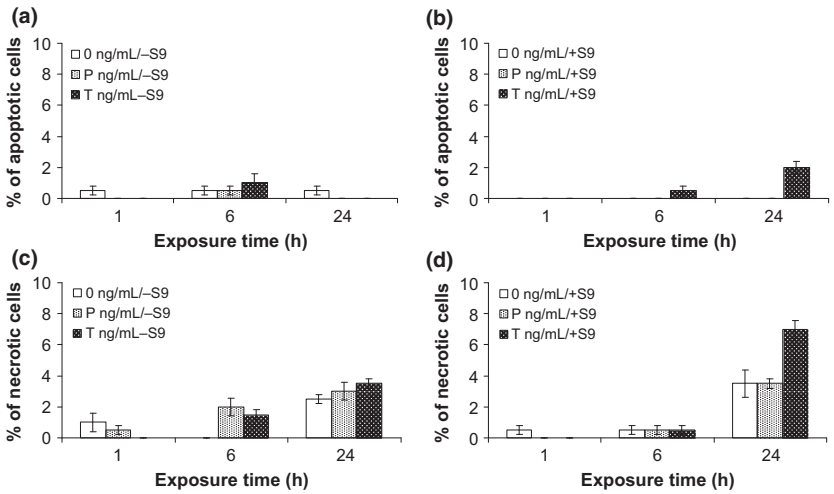

Figure 4 Percentage of apoptotic (a, b) and necrotic cells (c, d) evaluated with alkaline comet assay in human peripheral blood lymphocytes after joint treatment with atovaquone and proguanil hydrochloride in concentrations of 2950/130 ng/mL (P – prophylactic treatment) and 11 800/520 ng/mL (T – treatment of malaria) without and with S9 metabolic activation in different time periods. Data are presented as mean values ± SD (standard deviation of the mean).

impairment. Apparently, a few acute adverse effects have arisen, but more clinical evaluations of the drug are needed, especially to detect possible rare, unusual or long-term toxicity [14,37–39]. The most common adverse effects reported when taking ATO/PROG either for treatment or prophylaxis are abdominal pain, nausea, vomiting and headache. PROG is considered safe during pregnancy and breastfeeding [38–41].

Although ATO and PROG have not been often associated with severe adverse reactions in the recommended dosages, this study aimed to investigate the possible cyto/genotoxic potential of their combination on HPBLs in vitro. This was done by the evaluation of HPBL viability using AO and EtBr in situ fluorescent assay and by virtue of measuring DNA damage using alkaline comet assay. These techniques, especially comet assay, are relatively fast, simple and sensitive tools for the investigation of cytotoxicity and DNA damage in a variety of cell types in response to a range of DNA-damaging agents including drugs, both in vivo

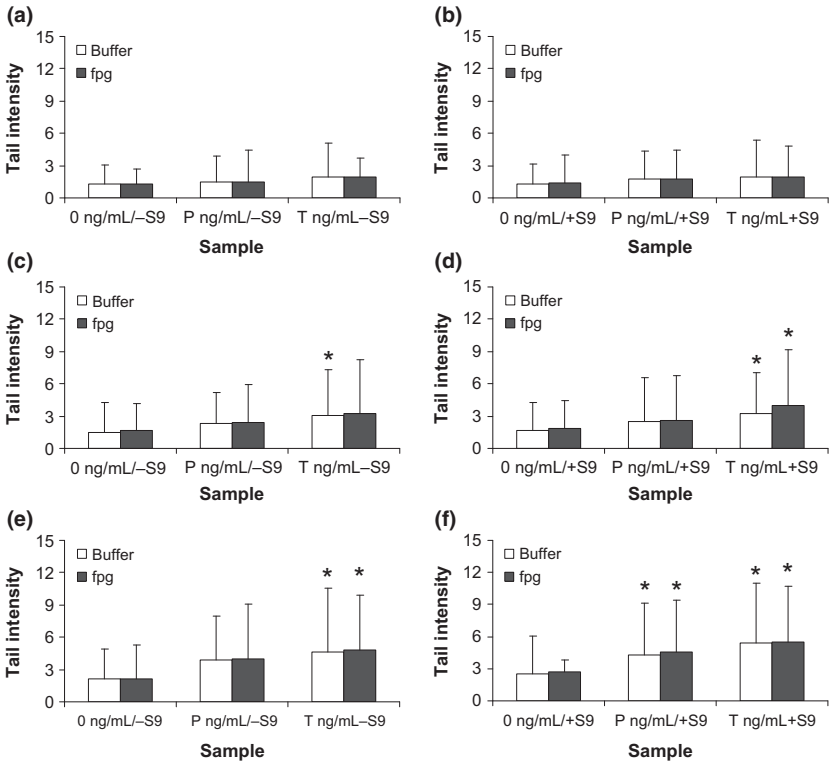

Figure 5 Results of the formamidopyrimidine-DNA glycosylase (Fpg)-modified comet assay parameter (tail intensity) in human peripheral blood lymphocytes after joint treatment with atovaquone and proguanil hydrochloride in concentrations of 2950/130 ng/mL (P – prophylactic treatment) and 11 800/520 ng/mL (T – treatment of malaria) without (a, c, e) and with (b, d, f) S9 metabolic activation in different time periods. Data are presented as mean values ± SD (standard deviation of the mean). *Statistically significant increase compared to the corresponding control ($P < 0.05$). Note: there was no statistically significant differences between Fpg-digested and undigested samples ($P < 0.05$).

and in vitro [36,42–45]. Therefore, the in vitro comet assay is proposed as an alternative to standard cytogenetic assays in early genotoxicity screening of drug candidates [46,47].

For ATO, the oral median lethal dose (LD_{50}) in rats was >1825 mg/kg. The intravenous LD_{50} in rats was 36 mg/kg and in mice was 26 mg/kg. Responses included ataxia, decreased activity, prostration and laboured breathing. For PROG, the oral LD_{50} in rats was estimated to be between 58 and 200 mg/kg. Responses to acute administration of PROG included ataxia and laboured breathing. In standard laboratory studies with ATO, oral doses up to 500 mg/kg/day over 28 days in rats caused no remarkable adverse effects except for slight decreases in erythrocyte parameters. Repeated oral administration of PROG in standard laboratory studies on laboratory rodents has been causally linked to tubular necrosis in the kidney, thymic and lymph node atrophy, hypocellular bone marrow and gastrointestinal effects. Following 28 days of oral dosing with PROG and ATO, a no observable effect level of 8 mg/kg/day PROG (in combination with 20 mg/kg ATO) was identified [48,49].

Although ATO was not carcinogenic in rats, studies in mice showed treatment-related increases in the incidence of hepatocellular adenoma and hepatocellular carcinoma. On the contrary, there are no data available on the carcinogenic potential of PROG. ATO was not mutagenic or DNA damaging when evaluated in the Ames *Salmonella* mutagenicity assay, the L5178Y TK+/− mouse lymphoma cell mutagenesis assay and the cultured human lymphocyte cytogenetic assay. No evidence of genotoxicity was observed in the in vivo mouse micronucleus (MN) assay. PROG was not mutagenic or DNA damaging with or without metabolic activation when evaluated in the Ames assay and the mouse lymphoma cell mutagenesis assay. No evidence of genotoxicity was observed in the in vivo mouse MN assay as well. CYC, the active metabolite of PROG, was also negative in the Ames test, but positive in the mouse lymphoma assay and the mouse MN assay. These positive effects with CYC were significantly reduced or abolished with folinic acid supplementation [48,49].

In our previous studies, we have shown that ATO did not cause any changes in the viability of treated cells, regardless of dose and exposure time. Samples

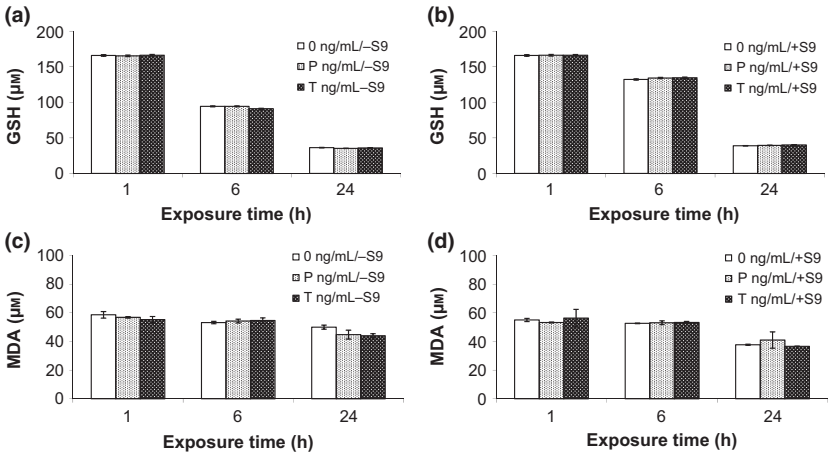

Figure 6 The level of oxidative stress markers glutathione (GSH) and malondialdehyde (MDA) in whole-blood samples after joint treatment with atovaquone and proguanil hydrochloride in concentrations of 2950/130 ng/mL (P – prophylactic treatment) and 11 800/520 ng/mL (T – treatment of malaria) without and with S9 metabolic activation in different time periods. GSH level (a, b) was assessed spectrophotometrically, whereas MDA level (c, d) was assessed using high-performance liquid chromatography (HPLC). Data are presented as mean values ± SD (standard deviation of the mean). Note: there was no statistically significant difference between treated samples compared to corresponding controls ($P < 0.05$).

with S9 metabolic activation also did not show any significant difference in percentage of viable cells, which was expected as no active metabolite of ATO has been identified so far [50,51]. Comet assay results also showed that ATO did not cause any DNA-damaging effect in HPBLs after treatment with both concentrations, regardless of exposure time or addition of S9 metabolic activation. Based on these results, we concluded that ATO at the tested concentrations is not cyto/genotoxic [24].

On the contrary, PROG treatment of HPBLs decreased the percentage of viable cells in a dose- and time-dependent manner, with significant decrease noticed only for 130 ng/mL treatments with S9 metabolic activation, whereas 520 ng/mL treatments decreased viability with and without S9 metabolic activation. Comet assay also showed that PROG is capable of inducing certain amount of DNA damage in HPBLs after treatment with higher concentrations of PROG in lower exposure periods, whereas after long exposure, even concentrations measured for prophylaxis induced genetic alterations. DNA damage was more prominent with the addition of S9 metabolic activation. In the same study, sister chromatid exchange (SCE) results failed to show significant deviation from the control samples, even though mean SCE per cell in all of the exposed samples was slightly higher and was more prominent with the addition of S9 metabolic activation. These results lead us to the conclusion that PROG and its metabolites have an impact on cell viability and DNA integrity. Given that a greater part of DNA-damaging effect was induced after S9 metabolic activation, it is to presume that principal metabolite CYC has the greatest impact on DNA molecule [23].

In the present study, we explored the joint effect of ATO and PROG on HPBLs in vitro. According to our results, joint treatment decreased the percentage of viable cells in a dose- and time-dependent manner. Moreover, combined treatment with ATO and PROG induced DNA damage at higher concentrations and lengths of time based on the alkaline comet assay results. The effect was more prominent with the addition of S9 metabolic activation. On the contrary, Fpg-modified comet assay failed to show a significant difference between undigested and Fpg-digested samples, indicating that the DNA damage induced was unlikely to be due to oxidative stress. In relation to Fpg-modified comet assay results, we also examined the levels of MDA and GSH, which are well-known biomarkers of LPO and oxidative stress, respectively [52–54]. Results showed that the levels of both GSH and MDA were not significantly affected by ATO/PROG treatment regardless of concentration, exposure time or addition of S9 metabolic activation, suggesting that oxidative stress is not

implicated in their mechanism of action against human lymphocytes.

In the study carried out by Bygbjerg [55] regarding the effect of both PROG and CYC on HPBLs in vitro, PROG had no effect on ^{14}C-thymidine incorporation or on the number of cells. On the contrary, CYC in concentrations corresponding to the plasma levels found in clinical practice blocked the endogenous synthesis of thymidine and decreased the number of mitogen- and antigen-stimulated cells. Also, the effect on phytohaemagglutinin-stimulated cells was temporary. Higher concentrations of CYC, corresponding to intralymphocytic levels in clinical practice, permanently suppressed the growth of lymphocytes. Also, the effect of CYC could be reversed by low doses of folinic acid and high doses of folic acid, suggesting that the mechanism of action of CYC is competitive blocking of dihydrofolate reductase. Moreover, the shortage of thymidine can lead to uracil misincorporation into DNA, its subsequent excision and resultant strand breakage [56–58]. Another study investigating the effect of oral PROG on human lymphocyte proliferation found that prophylactic dosage of PROG did not alter the number of mononuclear cells in peripheral blood, but the number of neutrophils was slightly reduced [59]. Additionally, Kharazmi et al. [60] have shown that a therapeutic level of PROG in serum can inhibit chemotaxis by neutrophils.

To the best of our knowledge, this study is the first to report a cyto/genotoxicity assessment of clinically relevant concentrations of ATO and PROG on HPBLs in vitro. According to our results, the ATO/PROG combination displayed only weak cyto/genotoxicity towards human lymphocytes with no impact on oxidative stress parameters, suggesting that oxidative stress is not implicated in their mechanism of action. Moreover, the used methods proved to be useful in detecting cyto/genotoxicity of this type of drugs and could be used as an alternative to standard cytogenetic assays in early cyto/genotoxicity screening of drug candidates. Given that the greater part of DNA-damaging effect is induced after S9 metabolic activation, it is to presume that principal PROG metabolite CYC has the major impact on DNA molecule. Overall, the obtained results indicate that the ATO/PROG combination is relatively safe for the consumption from the aspect of cyto/genotoxicity, especially if used for prophylactic treatment. Nevertheless, the present study has also confirmed the need for further cytogenetic research and regular patient monitoring to minimize the risk of any adverse event especially among frequent travellers.

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CONFLICTS OF INTEREST

No conflict of interests, financial or otherwise, are declared by the authors.

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5. RASPRAVA

Malarija je zarazna bolest uzrokovana krvnim parazitom roda *Plasmodium*. Širi se ubodom zaražene ženke komarca vrste *Anopheles*, ali i transfuzijom zaražene krvi te iglom injekcije koju je prethodno upotrijebila zaražena osoba, a iznimno rijetko se prenosi sa zaraženog na zdravog pojedinca. Unijet u tijelo parazit se umnožava u jetri, a zatim inficira crvene krvne stanice. Ako se pravovremeno ne liječi, bolest može uzrokovati ozbiljne komplikacije pa čak i smrt. U većini razvijenih zemalja zbog mjera prevencije, upotrebe insekticida i lijekova, malarija je danas rijetka bolest, ali je zato endemična u tropskim zemljama. Posjetitelji iz tropskih zemalja ili turisti i poslovni ljudi koji se vraćaju iz tih područja mogu prouzročiti manju epidemiju u zemljama u kojima ta bolest više nije česta (1,2,5,9,13,15–17,20,35–38,40,199).

Jedan od najzastupljenijih i najučinkovitijih lijekova koji se koristi u profilaksi i liječenju malarije je fiksna kombinacija atovakvona i progvanil hidroklorida, trgovačkog naziva Malarone (84,87,88,114,129,200-203). Za odrasle tableta sadrži 250 mg atovakvona u kombinaciji s 100 mg progvanil hidroklorida. Za djecu fiksna kombinacija sadrži 62,5 mg atovakvona u kombinaciji s 25 mg progvanil hidroklorida (114,129-131).

Iako atovakvon i progvanil hidroklorid ne uzrokuju veliki broj nuspojava (142,143), prilikom njihove primjene ipak su zabilježeni i određeni neželjeni učinci (201,204-206). Najčešći neželjeni učinci koji se javljaju pri uzimanju atovakvona i progvanil hidroklorida su bolovi u trbuhu, glavobolja, mučnina, povraćanje, proljev i kašalj. U pacijenata liječenih atovakvonom može doći do poremećaja razine serumskih transaminaza i amilaza. Dugoročni učinci atovakvona i progvanil hidroklorida na rast, pubertet i opći razvoj nisu u potpunosti istraženi (102,115,132,141). Ipak, preporuka je da atovakvon i progvanil hidroklorid ne koriste dojenčad, trudnice, dojilje ili bolesnici s teškim oštećenjem bubrega. Prilikom akutnog tretmana atovakvonom i progvanil hidrokloridom nisu zabilježeni neželjeni učinci, no potrebno je više kliničkih istraživanja kako bi se utvrdili mogući rijetki, neuobičajeni ili dugotrajni toksični učinci atovakvona i progvanil hidroklorida (102,114,132,144,145).

Cilj ovog istraživanja bio je procijeniti toksikološku sigurnost te mehanizme djelovanja atovakvona i progvanil hidroklorida svakoga pojedinačno, ali i u kombinaciji (engl. *toxicology of mixtures*) na ljudskim ne-ciljnim stanicama u uvjetima *in vitro*. U tu svrhu istraživanja su provedena na ljudskim limfocitima periferne krvi kao osjetljivim pokazateljima stupnja izloženosti fizikalnim i kemijskim agensima. Kako bi se prevladala moguća interindividualna varijabilnost u staničnom odgovoru na tretman, u svim pokusima su

korišteni uzorci periferne krvi od jednog mladog (28 godina, nepušač), zdravog muškog darivatelja (engl. *single donor approach*) te su pokusi ponovljeni tri puta. Kod takvog pristupa uvelike se smanjuje razlika u pozadinskim vrijednostima genomskih oštećenja koje se razlikuju kod različitih donora te je takav pristup uobičajen u istraživanjima procjene citogenotoksičnosti kemijskih i fizikalnih agenasa u uvjetima *in vitro* (207-215). Nadalje, darivatelj krvi nije bio izložen ionizirajućem ili ne-ionizirajućem zračenju u dijagnostičke svrhe u proteklih dvanaest mjeseci. Također, darivatelj krvi nije bio cijepljen, primao antibiotike i ostale lijekove u terapijske svrhe niti je bio izložen kemijskim mutagenima koji bi mogli utjecati na rezultate istraživanja. Venska krv je izvađena u heparinizirane spremnike nakon čega su uzorci venske krvi podijeljeni i korišteni u daljnjim istraživanjima. Tijekom eksperimentalnog procesa, puna krv je tretirana i kultivirana na 37 °C i 5% CO_2 u atmosferi. U istraživanju su primijenjene klinički relevantne koncentracije atovakvona i progvanil hidroklorida. To su koncentracije koje su utvrđene u ljudskoj plazmi kada je korištena fiksna doza kombinacije atovakvona i progvanil hidroklorida: 2950, odnosno 130 ng/mL nakon profilaktičkog liječenja te 11800, odnosno 520 ng/mL nakon liječenja malarije. U istraživanju je korišten i izolat jetre štakora (tzv. S9 frakcija) koji sadrži enzime potrebne za biotransformaciju ispitivane supstance te se kao takav rutinski koristi u citogenetičkim i molekularno-biološki istraživanjima (149,150).

Dosadašnja toksikološka ispitivanja atovakvona na štakorima pokazala su da je doza koja uzrokuje 50% smrtnosti, tzv. letalna doza (LD_{50}) oralno primijenjenog atovakvona veća od 1825 mg/kg. Kada su štakori tretirani intravenozno LD_{50} je bila 36 mg/kg. U miševa koji su tretirani intravenozno atovakvonom LD_{50} je bila 26 mg/kg. U tim ispitivanjima u životinja su zabilježene ataksija, smanjenje aktivnost i otežano disanje. Standardni laboratorijski testovi pokazali su da oralne doze atovakvona do 500 mg/kg/dan tijekom 28 dana u štakora nisu izazivale značajne štetne učinke osim neznatnog sniženja eritrocitnih parametara (216,217). Iako atovakvon nije imao karcinogeni učinak u štakora, istraživanja na miševima pokazala su povećanu učestalost hepatocelularnog adenoma i hepatocelularnog karcinoma. Amesov test na soju bakterije *Salmonella typhimurium*, mutacijski test na stanicama mišjeg limfoma L5178Y TK +/- te standardni citogenetički testovi na ljudskim limfocitima pokazali su da atovakvon nije mutagen niti genotoksičan. Genotoksični učinci nisu zabilježeni niti u *in vivo* mikronukleus testu na miševima (216,217).

U ispitivanjima provedenim u ovom istraživanju pojedinačni tretman s atovakvonom nije imao citotoksičan učinak na ljudske limfocite periferne krvi nakon tretmana klinički relevantnim koncentracijama (2950 i 11800 ng/mL, koncentracije koje su nađene u plazmi

nakon primjene atovakvona u profilaksi i liječenju), neovisno o vremenu tretiranja stanica. Uzorci koji su tretirani s atovakvonom i S9 frakcijom također nisu pokazali značajne razlike u postotku preživljenja stanica. Ovaj rezultat je bio očekivan jer do sada nije utvrđen aktivni metabolit atovakvona u ljudima koji bi mogao uzrokovati neželjene učinke (218-220). Novija studija koja je provedena na gljivici *Cunninghamella echinulata var. elegans* pokazala je da ta gljivica učinkovito metabolizira atovakvon. U ispitivanju koje je provedeno na ljudskim stanicama karcinoma dojke SKBR-3 te na ljudskim fibroblastima GM07492-A nastali metabolit, trans-3-4[4'-(4"-klorofenil)cikloheksil)-1,2.dioko-dihidro-1H-inden-3-karboksilna kiselina, bio je toksičniji od samog atovakvona (221). Iako se gljivice koriste kao surogat za ljudski metabolizam nije uvijek dokazana mogućnost da će takav metabolit nastati i u ljudskom organizmu. Također, i rezultati komet testa u ovome istraživanju pokazali su da atovakvon nije genotoksičan za ljudske limfocite periferne krvi u primijenjenim klinički relevantnim koncentracijama, neovisno o vremenu tretmana ili prisustvu S9 frakcije.

Toksikološka istraživanja progvanil hidroklorida pokazuju da je u štakora tretiranih oralno progvanil hidrokloridom LD_{50} u rasponu od 58 do 200 mg/kg. Kao odgovor na oralnu akutnu primjenu progvanil hidroklorida u laboratorijskih glodavaca je zabilježena ataksija i otežano disanje. Višekratni oralni tretman laboratorijskih glodavaca progvanil hidrokloridom povezuje se s pojavom nekroze bubrežnih tubula, atrofijom timusa i limfnih čvorova, gastrointestinalnim poremećajima te promjenama u koštanoj srži. No, podataka o kancerogenom potencijalu progvanil hidroklorida nema (216,217). Amesov test na soju bakterije *Salmonella typhimurium* i mutacijski test na stanicama mišjeg limfoma L5178Y TK +/- pokazali su da progvanil hidroklorid nije mutagen niti genotoksičan bez i u prisutnosti S9 frakcije. Genotoksični učinci progvanil hidroklorida nisu zabilježeni niti u *in vivo* mikronukleus testu na miševima. Ciklogvanil, aktivni metabolit progvanil hidroklorida, također se pokazao negativnim u Amesovom testu. Međutim, mutacijskim testom na stanicama mišjeg limfoma L5178Y TK +/- i u *in vivo* mikronukleus testu na miševima zabilježen je mutageni i genotoksični učinak ciklogvanila (216,217).

U ovom je istraživanju pojedinačni tretman s klinički relevantnim koncentracijama progvanil hidroklorida uzrokovao citotoksičan učinak na ljudske limfocite periferne krvi te se postotak vijabilnih stanica smanjivao ovisno o primijenjenoj koncentraciji, vremenu tretmana i primjeni S9 frakcije. Tretman s progvanil hidrokloridom u koncentraciji 130 ng/mL (koncentraciji koja je nađena u ljudskoj plazmi nakon primjene progvanil hidroklorida za profilaksu) uzrokovao je značajno smanjenje preživljenja stanica samo uz dodatak S9 frakcije. Međutim, tretman s koncentracijom progvanil hidroklorida od 520 ng/mL (koncentracija koja

je nađena u ljudskoj plazmi nakon primjene progvanil hidroklorida u terapijske svrhe) značajno je smanjio staničnu vijabilnost bez i u prisutnosti S9 frakcije. Dobiveni rezultati pokazuju da je ciklogvanil, metabolit progvanil hidroklorida potencijalno citotoksičan, kao i sam progvanil hidroklorid.

Rezultati komet testa pokazali su da je progvanil hidroklorid genotoksičan za ljudske limfocite periferne krvi u klinički relevantnim koncentracijama (u onima nakon tretmana za profilaksu i onima nakon terapijske upotrebe progvanil hidroklorida). Kao i prilikom ispitivanja citotoksičnosti, oštećenje molekule DNK bilo je izraženije u prisutnosti S9 frakcije. S obzirom da je genotoksični učinak bio izraženiji nakon dodatka S9 frakcije može se pretpostaviti da genotoksično djelovanje većim djelom pripada upravo aktivnom metabolitu ciklogvanilu.

U studiji u kojoj je uzeta krv pacijenata koji su koristili progvanil hidroklorid oralno u svrhu profilakse zabilježen je pad mononuklearnih stanica periferne krvi, ali i smanjen broj neutrofila (222). Također Kharazmi i sur. (223) pokazali su da terapijska razina progvanil hidroklorida u serumu može inhibirati kemotaksiju neutrofilima. Ti rezultati potvrđuju mogući citotoksični učinak progvanil hidroklorida na krvne stanice te su u sukladnosti s rezultatima dobivenim u ovom istraživanju.

U istraživanju koje je proveo Bygbjerg (224) ispitujući učinke progvanil hidroklorida i njegovog metabolita cikogvanila na ljudskim limfocitima u uvjetima *in vitro* pokazano je da progvanil hidroklorid ne djeluje na ugradnju ^{14}C-timidina u DNK niti na brojnost stanica. Naprotiv, u drugome istraživanju ciklogvanil u koncentracijama koje odgovaraju koncentracijama u plazmi koje su pronađene u kliničkoj praksi zaustavile su endogenu sintezu timidina i smanjile broj stanica. Sam nedostatak timidina na taj način može dovesti do pogrešne ugradnje uracila u molekulu DNK, njegovo posljedično izrezivanje iz DNK lanca što dovodi do oštećenja u samoj molekuli (139,225,226). Više koncentracije ciklogvanila koje odgovaraju intralimfocitnim razinama u kliničkoj praksi, trajno su zaustavile rast limfocita. Navedena istraživanja uključujući i istraživanje opisano u ovome radu, potvrđuju da progvanil hidroklorid i njegov metabolit imaju genotoksični učinak i to u klinički relevantnim koncentracijama. Istraživanja su pokazala da se učinak ciklogvanila može spriječiti kada se u pokuse uključe niske koncentracije folatne kiseline ili visoke koncentracije folne kiseline što pokazuje da je mehanizam djelovanja ciklogvanila kompetitivno blokiranje dihidrofolat reduktaze (216,217).

Iako se atovakvon i progvanil hidroklorid u profilaksi i liječenju malarije koriste zajedno, provedeno je vrlo malo toksikoloških ispitivanja njihovog zajedničkog učinka.

Dostupni su podaci samo za jedan njihov kombinirani toksikološki profil proveden u uvjetima *in vivo*. Nakon 28 dana oralne primjene kombinacije atovakvona i progvanil hidroklorida u dozi od 8 mg/kg/dan progvanil hidroklorida u kombinaciji s 20 mg/kg/dan atovakvona na laboratorijskim glodavcima nisu uočeni značajniji učinci (216,217).

Upravo stoga što nedostaju toksikološki podaci o zajedničkom učinku atovakvona i progvanil hidroklorida jedan od ciljeva ovog istraživanja bio je ispitati i njihov zajednički učinak na ljudskim limfocitima periferne krvi u uvjetima *in vitro* u klinički relevantnim koncentracijama. Dobiveni rezultati su pokazali da zajednički tretman ovom kombinacijom lijekova je citotoksičan za ljudske limfocite periferne krvi u ovisnosti o dozi, vremenu tretmana i primjeni S9 frakcije. Rezultati dobiveni alkalnim komet testom pokazuju da zajednički tretman atovakvonom i progvanil hidrokloridom u klinički relevantnim koncentracijama uzrokuje oštećenje molekule DNK, a taj učinak je bio izraženiji u prisutnosti S9 frakcije. Dobiveni rezultati potvrđuju važnu ulogu aktivnog metabolita progvanil hidroklorida, ciklogvanila na citogenotoksični učinak prilikom zajedničkog tretmana.

U ovom dijelu istraživanja korištena je i Fpg-modificirana verzija komet testa koja ukazuje na stupanj oštećenja molekule DNK uzrokovan oksidacijskim stresom (166,170). Rezultati Fpg-modificiranog komet testa pokazali su da tretman s atovakvonom i progvanil hidrokloridom nije uzrokovao značajna oštećenja molekule DNK. Ti rezultati govore da nastala genomska oštećenja (detektirana alkalnim komet testom prilikom tretmana s atovakvonom i progvanil hidrokloridom) nisu oksidativne prirode, odnosno nisu posljedica oksidacijskog stresa već su vjerojatno posljedica direktnog učinka ovih lijekova na molekulu DNK. Kako bi se dodatno ispitao mehanizam toksičnog djelovanja kombinacije lijekova atovakvona i progvanil hidroklorida u uzorcima pune krvi nakon tretmana ovim lijekovima izmjerene su koncentracije glutationa i malondialdehida, biomarkera oksidacijskog stresa (179,182,186). Dobiveni rezultati nisu ukazali na značajnije promjene u razinama glutationa i malondialdehida nakon tretmana bez obzira na koncentraciju, vrijeme tretmana ili prisutnost S9 frakcije. Ti rezultati potvrdili su da oksidacijski stres nije uključen u mehanizam djelovanja ove kombinacije antimalarijskih lijekova na ljudskim krvnim stanicama, ali ni njihovih metabolita.

U posljednjih nekoliko godina u toksikološkim istraživanjima sve je veći naglasak na ispitivanju kombinacija određenih agensa pa tako i lijekova koji se u vidu terapije, ali i u samom okolišu mogu naći u određenim kombinacijama i kao takvi uzrokovati neželjene učinke koji nisu jasno izraženi ako se ispituje samo pojedinačna supstanca. Takav tip istraživanja može ukazati da kombinacija može predstavljati veći rizik za integritet stanice i

genoma od pojedinačnih supstanci te da podaci o toksičnosti pojedinačnih spojeva nisu uvijek dovoljni za predviđanje toksičnosti u složenom okruženju koje predstavlja kombinirana terapija određenim lijekovima (engl. *real life exposure*). Prisutnost lijekova u različitim količinama i s različitim mehanizmima djelovanja na taj način ukazuje na potrebu proučavanja odnosa između potencijalno citogenotoksičnih komponenata koje se nalaze u određenoj kombinaciji i rezultirajućih učinaka, uzimajući u obzir različiti mehanizam djelovanja svake pojedine komponente (227-234). Stoga, ova studija daje nove podatke potrebne za znanstveno utemeljenu procjenu rizika antimalarijskih lijekova koji se koriste u terapijske svrhe u navedenoj kombinaciji.

Rezultati dobiveni u ovom istraživanju ukazuju da klinički relevantne koncentracije atovakvona i progvanil hidroklorida u kombinaciji mogu uzrokovati citogenotoksične učinke na ljudskim limfocitima periferne krvi. S obzirom da je najveći dio štetnih učinaka bio induciran dodatkom S9 frakcije, pretpostavlja se da je glavni metabolit progvanil hidroklorida, ciklogvanil, imao najveći utjecaj na citogenotoksičnost. Nadalje, rezultati su pokazali da nije došlo do promjena niti u jednom od mjerenih parametara oksidacijskih oštećenja što ukazuje da oksidacijski stres nije uključen u njihov mehanizam djelovanja. U konačnici, dobiveni rezultati ukazuju da je kombinacija atovakvona i progvanil hidroklorida relativno sigurna sa stanovišta citogenotoksičnosti, naročito ako se koristi za profilaksu. Unatoč tome, ova studija je potvrdila potrebu za daljnjim citogenetičkim ispitivanjima te redovitim praćenjem pacijenata kako bi se smanjili rizici od neželjenih učinaka, osobito među onima koji često putuju u malarična područja (engl. *frequent travellers*). Nadalje, metode korištene u ovom istraživanju pokazale su se korisnima za otkrivanje citotoksičnosti i genotoksičnosti ovog tipa lijekova te bi se mogle koristiti kao alternativa standardnim citogenetičkim testovima u ranom otkrivanju citogenotoksičnosti kandidata za potencijalne lijekove.

6. ZAKLJUČCI

1. Pojedinačni tretman atovakvonom nije pokazao značajni citogenotoksični učinak bez obzira na koncentraciju, vrijeme tretmana ili dodatak S9 frakcije. Na temelju ovih rezultata zaključeno je da atovakvon u ispitivanim klinički relevantnim koncentracijama nije citogenotoksičan te da je siguran sa stanovišta toksikološke sigurnosti.

2. Pojedinačni tretman progvanil hidrokloridom pokazao je značajni citogenotoksični učinak u ovisnosti o koncentraciji, vremenu tretmana i dodatku S9 frakcije. Ovi rezultati ukazuju na potencijalno citogenotoksično djelovanje progvanil hidroklorida, ali i njegovog metabolita u klinički relevantnim koncentracijama. S obzirom da je citogenotoksični učinak bio jače izražen nakon dodatka S9 frakcije, pretpostavlja se da toksično djelovanje većim djelom pripada aktivnom metabolitu ciklogvanilu.

3. Kombinacija atovakvona i progvanil hidroklorida pokazala je značajni citogenotoksični učinak u ovisnosti o koncentraciji, vremenu tretmana i dodatku S9 frakcije. Ovi rezultati ukazuju na potencijalno citogenotoksično djelovanje atovakvona i progvanil hidroklorida u kombinaciji u klinički relevantnim koncentracijama. S obzirom da je veći dio štetnog učinka na citotoksičnost i molekulu DNK induciran dodatkom S9 frakcije, pretpostavlja se da glavni metabolit progvanil hidroklorida, ciklogvanil ima najveći utjecaj na citogenotoksičnost.

4. Kombinacija atovakvona i progvanil hidroklorida nije pokazala utjecaj na parametre oksidacijskog stresa, ukazujući da oksidacijski stres nije uključen u njihov mehanizam djelovanja.

5. Iako su klinički relevantne koncentracije atovakvona i progvanil hidroklorida u kombinaciji koje se koriste u liječenju uzrokovale citogenotoksične učinke na ljudskim limfocitima periferne krvi, dobiveni rezultati pokazuju da je kombinacija ovih dvaju lijekova relativno sigurna sa stanovišta citogenotoksičnosti ako se ona koristi za profilaksu. Ove rezultate bi bilo vrijedno analizirati u novim studijama u kulturama limfocita nekolicine ispitanika. Također, ovo istraživanje je potvrdilo

potrebu za daljnjim citogenetičkim ispitivanjima te redovitim praćenjem pacijenata kako bi se smanjio rizik od neželjenih učinaka, osobito među onima koji često putuju u malarična područja (engl. *frequent travellers*).

6. Korištene metode, a posebice komet test, pokazale su se učinkovitima u otkrivanju citogenotoksičnosti ove vrste lijekova i mogle bi se koristiti kao alternativa standardnim citogenetičkim testovima u ranom otkrivanju citogenotoksičnosti kandidata za potencijalne lijekove.

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8. POPIS KRATICA I NAZIVA

Kratica	Puni naziv
ACT	engl. *artemisinin-based combination therapy*
AO	engl. *acridine orange*, akridin-oranž
ATO	engl. *atovaquone*, atovakvon
CYC	engl. *cycloguanil*, ciklogvanil
DDT	diklor-difenil-trikloretan
DNK	deoksiribonukleinska kiselina
DTNB	5,5'-ditiobis-2-nitrobenzoat
EDTA	etilendiamin tetraoctena kiselina
EtBr	engl. *edthidium bromide*, etidij-bromid
FPG	engl. *formamidopyrimidine DNA glycosylase*, formamidopirimidin DNK glikozilaza
G6PD	glukoza-6-fosfat dehidrogenaza
GSH	engl. *glutathione*, glutation
HPBL	engl. *human peripheral blood lymphocytes*, ljudski limfociti periferne krvi
IPTp	engl. *intermittent preventive treatment in pregnancy*
IRS	engl. *indoor residual spraying*
ITN	engl. *insecticide-treated net*
LD	engl. *lethal dose*, letalna doza
LLIN	engl. *long lasting insecticidal net*
LMP	engl. *low melting point agarose*, agaroza niskog tališta
LPO	engl. *lipid peroxidation*, lipidna peroksidacija lipid peroxidation
MDA	engl. *malondialdehyde*, malondialdehid
MDA	engl. *mass drug administration*
MPA	engl. *metaphosphoric acid*
NMP	engl. *normal melting point agarose*, agaraoza normalnog tališta
OECD	engl. *Organisation for Economic Co-operation and Development*
PBS	engl. *phosphate saline buffer*, fosfatni pufer
PfSPZ	engl. *Plasmodium falciparum sporozite Vaccine*
PRI	engl. *proliferation rate index*
PROG	engl. *proguanil hydrochloride*, progvanil hidroklorid
ROS	engl. *reactive oxygen species*, reaktivni kisikovi spojevi
SCE	engl. *sister chromatid exchanges*
SCGE	engl. *single cell gel electrophoresis*
SBET	engl. *stand by emergency treatment*
SMC	engl. *seasonal malaria chemoprevention*
TMP	engl. 1,1,3,3-tetramethoxy propane
WHO	engl. *World Health Organization*

9. ŽIVOTOPIS

Domagoj Dinter rođen je 06.11.1981. godine u Rijeci. Osnovnu školu završio je 1996. godine, a Prvu riječku hrvatsku Gimnaziju 2000. godine u Rijeci s Nagradom za učenika generacije koju dodjeljuje talijanska Zaklada Ileana (*Fondazione Ileana*) i Rotary Clube Este, te kao državni prvak iz kemije 1996. i 2000. godine. Iste godine upisao je Farmaceutsko-biokemijski fakultet, Sveučilišta u Zagrebu, smjer Farmacija. Diplomirao je 2006. godine sa diplomskim radom pod naslovom „Utvrđivanje intenziteta miorelaksirajućeg djelovanja baklofena“. Diplomski rad je izradio na Zavodu za farmakologiju, Farmaceutsko-biokemijskog fakulteta, Sveučilišta u Zagrebu pod vodstvom prof. dr. sc. Ite Samaržije. Tijekom srednjoškolskog obrazovanja bio je stipendist Grada Rijeke, a tijekom studija je primao državnu stipendiju koju dodjeljuje Ministarstvo znanosti, obrazovanja i sporta. 2005. godine upisuje dodatni studij Japanologije na Filozofskom fakultetu, Sveučilišta u Zagrebu, a radni staž započinje 2006. godine u Plivi u odjelu proizvodnje suhih oralnih oblika kao tehnolog u proizvodnji. 2008. godine završava studij Japanologije te 2010. godine upisuje poslijediplomski studij Farmacije na Farmaceutsko-biokemijskom fakultetu, Sveučilišta u Zagrebu. 2011. godine postaje koordinator odjela u Plivi, a krajem 2013. do sredine 2015. godine odlazi na privremeni rad u Japan kao savjetnik za proizvodnju suhih oralnih oblika unutar farmaceutske kompanije Teve koja je trenutni vlasnik Plive. Nakon povratka u Hrvatsku promoviran je u rukovoditelja odjela tabletiranja unutar proizvodnje suhih oralnih oblika, a ubrzo nakon toga prelazi na novo radno mjesto Tevinog voditelja globalnog MES (engl. *Manufacturing Execution System*) programa gdje se bavi implementacijom programa za elektronsko vođenje proizvodnje lijekova unutar cijele Tevine grupe. Uz farmaceutsko-tehnološku karijeru objavio je i 3 znanstvena rada u časopisima indeksiranima u bazi *Current Contents*, 4 kongresna priopćenja te je sudjelovao na nekoliko međunarodnih znanstvenih skupova sa posterskim prezentacijama iz područja doktorskog rada. Za svoj profesionalni rad nagrađen je Plivinom godišnjom nagradom 2013. godine.

Znanstveni radovi u časopisima indeksiranima u bazi *Current Content*:

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Kongresna priopćenja:

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Durgo K, Stančić A, Belščak-Cvitanović A, Komes D, Curcic M, Antonijević B, Dinter D, Franekić J (2011) Compositional analysis, cytotoxic and cytoprotective activity of red raspberry leaves extract. 71st World Congress of Pharmacy and Pharmaceutical Sciences, International Pharmaceutical Federation (FIP), Hyderabad, Indija.

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Sudjelovanja na znanstvenim skupovima:

6th BBBB International Conference on Pharmaceutical Sciences: Strategies to Improve the Quality and Performance of Modern Drug Delivery Systems, Helsinki, Finska, 2015.

24th Congress of Federation of Asian Pharmaceutical Associations (FAPA), Bali, Indonezija, 2012.

71st World Congress of Pharmacy and Pharmaceutical Sciences, International Pharmaceutical Federation (FIP), Hyderabad, Indija, 2011.

16th World Congress of Basic and Clinical Pharmacology, International Union of Basic and Clinical Pharmacology (IUHPAR), Kopenhagen, Danska, 2010.

43rd Annual Scientific Meeting "The right of Medicines", Australasian Society of Clinical and Experimental Pharmacologists and Toxicologist (ASCEPT) Sydney, New South Wales, Australija, 2009.

3rd Pan-European PAT Science Conference (EuPAT): Scientific Progress Underpinning Innovative Manufacturing Control and Quality by Design, Göteborg, Švedska, 2008.

Profesionalno usavršavanje:

11th Annual Forum on Manufacturing Execution System (MES), CBI Research, Philadelphia, PA, SAD, 2016.

Razvojni razgovori u procesu upravljanja učinkom, Pliva, Zagreb, Hrvatska, 2016.

Vještine intervjuiranja, Creativa, Zagreb, Hrvatska, 2016.

Vođenje godišnjih razgovora, Pliva, Zagreb, Hrvatska, 2016.

Financije za nefinancijaše, Pliva, Zagreb, Hrvatska, 2015.

Proces upravljanja učinkom, Pliva, Zagreb, Hrvatska, 2015.

8th Annual Forum on Manufacturing Execution System (MES), CBI Research, Philadelphia, PA, SAD, 2013.

ITMS-Operator Training-Kaye Validator, Stuttgart, Njemačka, 2013.

Right First Time, Pliva, Zagreb, Hrvatska, 2012.

Vještine Vođenja, Creativa, Zagreb, Hrvatska, 2011.

Building Intercultural Competences, Pliva, Zagreb, Hrvatska, 2009.

Efikasno upravljanje vremenom i stresom, Creativa, Zagreb, Hrvatska, 2008.

Pharmaceutical Tablet Technology, Natoli Engineering Company, St. Louis Missouri, SAD, 2008.

10th Infotehna Annual Pharmaceutical International Conference, Bol, Hrvatska, 2008.

Edukacija za stroj za tabletiranje, Fette Compacting GmbH, Schwarzenbek, Njemačka, 2007.

Upravljanje konfliktima, Pliva, Zagreb, Hrvatska, 2007.

9th Interphex International Pharmaceutical, R&D and Manufacturing Conference, Tokyo, Japan, 2007.

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Plivina godišnja nagrada (2013)

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Farmaceutsko-biokemijski fakultet

Doktorska disertacija

CITOGENETIČKI UČINCI ATOVAKVONA I PROGVANIL HIDROKLORIDA NA LJUDSKIM LIMFOCITIMA *IN VITRO*

DOMAGOJ DINTER

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Malarija je oduvijek bila glavni uzrok smrti u tropskim krajevima, a antimalarijski lijekovi odigrali su ključnu ulogu u kontroli njezina širenja primjenom u liječenju pacijenata zaraženih plazmodijskim parazitima i prilikom kontrole njihovog prijenosa. S druge strane, antimalarijski lijekovi mogu imati i nepoželjne učinke i nuspojave koje ponekad mogu biti ozbiljne. Atovakvon (ATO) i progvanil hidroklorid (PROG) fiksna je kombinacija lijekova koja se koristi za prevenciju i liječenje malarije uzrokovane *Plasmodium falciparum*. Kako su sigurni i učinkoviti antimalarijski lijekovi potrebni u liječenju i profilaksi malarije, ovo istraživanje je provedeno kako bi se istražio njihov mogući citogenotoksični učinak te mehanizam odgovoran za njihovo djelovanje na ne-ciljnim ljudskim stanicama, limfocitima iz periferne krvi. Dvije različite koncentracije ATO i PROG, bilo zasebno ili u kombinaciji, korištene su sa i bez S9 frakcije. Korištene koncentracije bile su one koje su pronađene u ljudskoj plazmi kada su ATO i PROG primijenjeni u profilaksi (2950/130 ng/mL) i u liječenju malarije (11800/520 ng/mL). Mogući učinci na staničnoj razini i na molekulu DNK procijenjeni su testovima stanične vijabilnosti i alkalnim komet testom, dok je mogući mehanizam djelovanja procijenjen formamidopirimidin-DNK glikozilazom (Fpg)-modificiranim komet testom, uz mjerenje razine malondialdehida i glutationa kao biomarkera oksidacijskog stresa. Prema dobivenim rezultatima pojedinačni tretman ATO nije pokazao citogenotoksični učinak, dok je pojedinačni tretman PROG pokazao statistički značajan citogenotoksični potencijal. Kombinacija ATO/PROG također je pokazala statistički značajan citogenotoksični potencijal prema ljudskim limfocitima bez utjecaja na parametre oksidacijskog stresa, što pokazuje da oksidacijski stres nije uključen u njihov mehanizam djelovanja. S obzirom da je najveći dio štetnih učinaka bio induciran dodatkom S9 frakcije, pretpostavlja se da je glavni metabolit PROG, ciklogvanil, imao najveći utjecaj na citogenotoksičnost. U konačnici, dobiveni rezultati pokazuju da je kombinacija ATO/PROG relativno sigurna sa stanovišta citogenotoksičnosti, naročito ako se koristi za profilaksu. Ipak, potrebna su daljnja citogenetička istraživanja i redovito praćenje pacijenata kako bi se smanjio rizik od nuspojava, osobito među čestim putnicima.

(86 stranica, 7 slika, 1 tablica, 234 literaturnih navoda, jezik izvornika-hrvatski)

Rad je pohranjen u Centralnoj knjižnici Farmaceutsko-biokemijskog fakulteta, 10000 Zagreb, Hrvatska

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University of Zagreb
Faculty of Pharmacy and Biochemistry

Doctoral Thesis

CYTOGENETIC EFFECTS OF ATOVAQUONE AND PROGUANIL HYDROCHLORIDE ON HUMAN LYMPHOCYTES *IN VITRO*

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Malaria has always been a major cause of death in the tropics, and antimalarial drugs have played a key role in controlling its spread through the treatment of patients infected with plasmodial parasites and the control of its transmissibility. On the other hand, antimalarial drugs may exert adverse effects and side-effects that can sometimes be serious. Atovaquone (ATO) and proguanil hydrochloride (PROG) is the fixed combination for the prevention and treatment of *Plasmodium falciparum* malaria. As safe and effective antimalarial drugs are needed in both the treatment and the prophylaxis of malaria, this study was performed in order to investigate their possible cytogenotoxic potential towards human lymphocytes, as non-target cells, and the possible mechanism responsible for it. Two different concentrations of ATO and PROG, either alone or in combination, were used with and without S9 fraction. The concentrations used were those found in human plasma when a fixed-dose combination of ATO and PROG was used: 2950/130 ng/mL after prophylactic treatment and 11800/520 ng/mL after treatment of malaria, respectively. Possible cellular and DNA-damaging effects were evaluated by cell viability and alkaline comet assays, while oxidative stress potential was evaluated by formamidopyrimidine-DNA glycosylase (Fpg)-modified comet assay, in addition to assessing malondialdehyde and glutathione levels as biomarkers of oxidative stress. Based on the obtained results, single exposure to ATO did not display cytogenotoxic effect while PROG showed week but significant cytogenotoxic potential. The ATO/PROG combination also displayed weak but significant cytogenotoxic potential towards human lymphocytes with no impact on oxidative stress parameters, suggesting that oxidative stress is not implicated in their mechanism of action. Given that the key portion of the damaging effects was induced after S9 metabolic activation, it is to presume that the principal metabolite of PROG, cycloguanil, had the greatest impact on their cytogenotoxic potential. The obtained results indicate that the ATO/PROG combination is relatively safe for the consumption from the aspect of cytogenotoxicity, especially if used for prophylaxis. Nevertheless, further cytogenetic research and regular patient monitoring are warranted to minimize the risk of adverse events especially among frequent travellers.

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