Synthesis and Profiling of Benzylmorpholine 1,2,4,5-tetraoxane Analogue N205: Towards Tetraoxane Scaffolds with Potential for Single Dose Cure of Malaria

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**Abbreviations**

AUC – area under the curve, BA – bioavailability, CLplasma – plasma clearance, CLint – intrinsic clearance, CYP – cytochrome P450, DCM – dichloromethane, DMF – dimethyl formamide, ED – effective dose, FaSSIF - fasted state simulated intestinal fluid, FeSSIF - fed state simulated intestinal fluid, IC – inhibitory concentration, IV – intravenous, LAH – lithium aluminium hydride, PD – pharmacodynamic, PK – pharmacokinetic, PO – oral, SCID – severe combined immunodeficiency, THF – tetrahydrofuran, Vdss – volume of distribution at steady state.

**Abstract**

A series of aryl carboxamide and benzylamino dispiro 1,2,4,5-tetraoxane analogues have been designed and synthesized in a short synthetic sequence from readily available starting materials. From this series of endoperoxides, molecules with in vitro IC50s versus *Plasmodium falciparum* (3D7) as low as 0.84 nM were identified. Based on an assessment of blood stability and *in vitro* microsomal stability, N205 (**10a**) was selected for rodent pharmacokinetic and *in vivo* antimalarial efficacy studies in the mouse *Plasmodium berghei* and *Plasmodium falciparum Pf*3D70087/N9 severe combined immunodeficiency (SCID) mouse models. The results indicate that the 4-benzylamino derivatives have excellent profiles with a representative of this series, N205, an excellent starting point for further lead optimization studies.

**1. Introduction**

The emergence of malaria parasite resistance to most available drugs, [1] including the semi-synthetic artemisinin derivatives artemether and artesunate, [2-4] has led to efforts to create new synthetic peroxides as potential antimalarial agents. Leading examples of synthetic endoperoxides include OZ277 (arterolane) (**1**), [5] a molecule deployed in combination with piperaquine (known as Synriam), [6] and OZ439 (**2**) a second generation derivative with improved pharmacokinetics and enhanced *in vivo* antimalarial activity. [7-9] 1,2,4,5-Tetraoxanes are another class of peroxide with excellent antimalarial profiles against both chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum* and oral activity in murine models of the disease.[10-15] Previously in our group, RKA182 (**3**) (Figure 1) was selected as a candidate for full preclinical development from a series of synthetic tetraoxane derivatives; this compound shows superior *in vitro* and *in vivo* activity compared to artemether and artesunate, has good oral bioavailability in rodent models and is more stable than arterolane in malaria infected human red blood cells.[16, 17] Although the PK profile for RKA182 is compatible with that of a 3-day dosing regimen, there is now a drive for the development of endoperoxides with PK/PD properties predicted to allow single dose cure in humans. Three distinct tetraoxane templates were simultaneously investigated; a representative described here, alongside the series which led to the discovery of E209 (**4**) [17, 18], both of which have PK/PD characteristics that are compatible with a single-dose cure.



Figure 1. Structures of synthetic peroxides OZ277, OZ439, RKA182 and E209.

**2. Results and Discussion**

Herein we describe the design and synthesis of a new series of tetraoxanes (Templates 1 and 2, Figure 2) and present data on their *in vitro* and *in vivo* antimalarial activity profiles. The new series were designed to increase the lipophilicity (CLogP/LogD) compared with RKA182 (by inclusion of an aromatic ring in the side-chain) and enhance blood stability (rodent and human) in addition to enhancing PK/PD properties in appropriate animal models. For the benzyl series, we focused on the use of morpholine and fluoropiperidine to enable direct comparisons with OZ439 and E209.

**2.1. Chemistry**



**Scheme 1** (a) Tf2O, NEt3, -25 oC- rt, 12 hr, (b) Pd(OAc)2, CO, atm, DIPEA, dppp, DMF/MeOH, 70 oC, 5hr, rt, o/n, (c) oxalyl chloride, AlCl3, DCM, 0 oC- rt o/n, 0 oC, MeOH, pyridine, rt, 3 hr, (d) Formic acid/HCl, 50% H2O2, acetonitrile, 0 oC, 30-60 min (e) 2-adamantanone, Re2O7, DCM, rt, 1 hr (f) MeOH, KOH, 70 oC (g) NEt3, 0 oC, CH3COCl, 1 hr, NHR1R2, 0 oC, 30 min then rt, 1.5 hr (h) THF, LAH, 0 oC, 30 min (i) NEt3, THF, 0 °C, methane sulfonyl chloride, 1.5 hrs (j) NEt3, DCM, 0 oC, 10 min, then amine, rt 3.5 hr

Two routes were explored for the synthesis of key ester **4c**. Initially, we examined the conversion of commercially available 4-(4-hydroxyphenyl)cyclohexanone **4a** into the corresponding triflate **4b**. This was then subjected to a palladium mediated carbonylation reaction in the presence of methanol to provide methyl ester **4c**. This key intemediate could be converted into the tetraoxane ester **6** according to the procedure developed by Dussault et al.[19] An alternative approach to **4c** involves the use of a modified Friedel-Crafts procedure on 4-phenyl cyclohexanone; this latter route has advanatages in terms of scale up of chemistry for production of multi-gram quantities. For the synthesis of template 1 analogues the ester **6** was first hydrolysed to the carboxylic acid **6b** and converted to target amides via a mixed anhydride intermediate. For template 2 analogues, the ester **6** was reduced with LAH to the alcohol **8** and treated with methane sulfonyl chloride to form the mesylate **9** which was then allowed to react with morpholine (or 4-fluoro cyclohexanone for N214, **10b**).

**2.2. Biological Assessment**

All compounds synthesized were tested *in vitro* against the 3D7 strain of *Plasmodium falciparum*.[20] With the exception of the amino cyclobutane **7d** and thiomorpholine analogue **7e**, all of the tetraoxane analogues displayed potent single digit nanomolar activity with several compounds more potent than OZ439 positive control. No correlation was seen with calculated physicochemical parameters such as CLogP, LogD or calculated solubility (Table 1) consistent with previous reports for trioxolane derivatives. [5,8]. Due to the importance of blood stability for enhancing overall drug exposure in this class, compounds depicted in Table 1 were screened for stability in human and rat blood and it was shown that the amide series was unstable particularly in rat blood (half-life < 4 h, data not shown). Amides **7h**, and **7i** (all of which have higher ClogP values than RKA182) were selected for *in vivo* analysis along with the benzylamino analogues **10a** and **10b** (the latter compounds demonstrated comparatively better rodent and human blood stability).

**Table 1**: *In vitro* 3D7 IC50, CLogD, calculated solubility, CLog P

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Compound** | **Structure** | **IC50 3D7 (nM)** | **CLogD\* 7.4** | **Calculated\***  **Solubility (mg/mL)** | **ClogP\*** |
| **7a** |  | 7.1 ± 0.4 | 3.96 | 0.02 | 3.22 ± 0.81 |
| **7b** |  | 5.3 ± 0.6 | 1.31 | 0.029 | 4.66 ± 0.80 |
| **7c** |  | 0.84 ± 0.03 | 1.36 | 0.083 | 3.85 ± 0.80 |
| **7d** |  | 33.0 ± 1.0 | 1.11 | 0.013 | 2.59 ± 0.80 |
| **7e** |  | 15.0 ± 1.0 | 4.58 | 0.01 | 4.10 ± 0.90 |
| **7f** |  | 6.4 ± 0.6 | 3.93 | 0.10 | 3.68 ±0.84 |
| **7g** |  | 0.82 ± 0.11 | 1.70 | 0.034 | 3.61 ± 0.84 |
| **7h** |  | 3.0 ± 0.4 | 2.76 | 0.13 | 3.18 ± 0.86 |
| **7i** |  | 4.2 ± 0.45 | 1.11 | 0.027 | 3.37 ± 0.79 |
| **7j** |  | 1.10 ± 0.1 | 1.07 | 0.69 | 3.39 ± 0.90 |
| **10a** ( N205) |  | 1.3 ± 0.1 | 4.42 | 0.076 | 4.50 ± 0.82 |
| **10b** ( N214) |  | 1.8 ± 0.7 | 5.02 | 0.025 | 5.36 ± 0.86 |
| OZ439 |  | 8.0 ± 0.3 | 4.83 | 0.55 | 4.63 ± 0.70 |

\*log D, log P and Solubility values were calculated using the Virtual Computational Chemistry Laboratory (VCCLAB); [http://www.vcclab.org](http://www.vcclab.org/).

Data presented in Table 2 summarises the results in terms of cure and mean survival time following a single 30 mg/kg oral dose treatment of *Plasmodium berghei* (*P. berghei*) infected mice. The performance of the amides, although superior to artesunate and equivalent to RKA182 and OZ277, was comparatively poor relative to OZ439. In contrast, the benzylamino analogues performed better than the amides with a 26day mean survival for N205 (**10a**) and 13 day average survival for fluoropiperdine analogue **10b**. For the benzyl morpholine analogue N205, 2/3 mice were cured; use of the mesylate salt of N205 in a standard suspension vehicle (SSV) was next examined to see if a better performance could be obtained with the salt form. A similar result was obtained with a 66% single dose cure rate and average survival of 25 days. Snapshot PK data for this latter study revealed significant levels of N205 in the plasma at the 24 h timepoint (>100 ng/ml). These results are superior data recorded for the tetraoxane version of OZ439 tested previously in the same animal model where the mean survival was 15 days with no cures [21]. Based on these results, N205 was selected for more extensive profiling to determine if comparative studies should be performed in the humanized mouse model of malaria. OZ439 was used as the benchmark compound throughout these studies.

**Table 2:** Percentage activity and mean mouse survival time following 30 mg/kg single oral dose in the *P. berghei* model

|  |  |  |
| --- | --- | --- |
| **Compound Number** | **% Activity** | **Mean survival time (days)**  **following 30mg/kg oral dose** |
| **7h** | 99.0 | 10.0 (9, 10, 11) |
| **7i** | 99.0 | 8.0 (8, 8, 8) |
| **10a** ( N205) | 99.42 | 26.3 (16, 30, 30) |
| **10a** ( N205 mesylate) | 99.30 | 25.0 (15, 30, 30) |
| **10b** ( N214 mesylate) | 99.98 | 13 (12, 13, 14) |
| OZ277 | 99.98 | 10.2 (8, 10, 8, 10, 15) |
| RKA182 | 99.98 | 11.4(14, 15, 7, 7, 14) |
| OZ439 | 99.40 | 30 (30, 30, 30) |
| Artesunate | 99.09 | 6.8 (6, 7, 7, 7, 7) |
| Untreated Control | - | 4.0 (4, 4, 4) |

Table 3 shows data for the measured solubility of the mesylate salt of N205. Compound **10a** is more soluble in water than OZ439 mesylate but is less soluble in 0.01 and 0.1M HCl, FaSSIF and FeSSIF media. Overall, the profile points towards lower overall solubility than OZ439 (it should be noted that the final salt form and levels of crystallinity will influence solubility data in these assays).

**Table 3:** Overall solubility results for N205 (**10a**) mesylate at 37°C after 4 hours. All

solubility values refer to the free base equivalent.

|  |  |  |
| --- | --- | --- |
| **Medium** | **Solubility (µg/mL) after 4 h at 37°C** | |
| **OZ439 mesylate** | **N205 mesylate** |
| Water | 6800 | >9000 |
| pH 2.0 buffer | >8000 | >9000 |
| 0.1 N HCl pH 1.0 | 34 | 17 |
| 0.01 N HCl pH 2.0 | 240 | 172 |
| pH 7.4 PBS | not meas. | <0.1 |
| FaSSIF pH 6.5 | 120 | 18 |
| FeSSIF pH 5.0 | >1500 | 760 |

Both plasma and microsomal protein binding of N205 and OZ439 were found to be very high (>99.9% bound for both compounds in each matrix)..

N205 (**10a**) exhibited degradation in human, rat and mouse liver microsomes with rates generally being fastest in rat and slowest in mouse (Table 4). The rates of degradation were similar in human and rat liver microsomes at substrate concentrations of 1 and 5 µM, however the rate of degradation in mouse liver microsomes appeared to be somewhat lower at the higher substrate concentration suggesting a possible concentration dependency.

**Table 4**: *In vitro* Metabolism in Liver Microsomes

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Compound Details** | **Species** | **Substrate Concentration**  **(M)** | **Degradation half-life**  (min) | ***In vitro* CLint**  (µL/min/mg protein) | **Microsome-Predicted EH** |
| **N205**  (CDCO\_01) | Human | 1 | 34  (33, 35) | 51  (53, 50) | 0.67  (0.68, 0.66) |
| 5 | 43  (44, 41) | 41  (39, 42) | 0.62  (0.61, 0.63) |
| Rat | 1 | 15  (13, 16) | 118  (130, 105) | 0.75  (0.77, 0.73) |
| 5 | 20  (17, 23) | 89  (101, 76) | 0.69  (0.72, 0.66) |
| Mouse | 1 | 48  (42, 54) | 37  (41, 32) | 0.44  (0.47, 0.41) |
| 5 | 87  (93, 82) | 20  (19, 21) | 0.30  (0.29, 0.31) |
| **OZ439**  (OZ-439/PC-276/02) | Human | 1 | 25  (26, 23) | 71  (66, 75) | 0.74  (0.72, 0.75) |
| 5 | 66  (73, 59) | 27  (24, 29) | 0.51  (0.49, 0.54) |
| Rat | 1 | 95  (86, 103) | 18  (20, 17) | 0.32  (0.34, 0.30) |
| 5 | 141  (75, 207) | 16  (23, 8) | 0.27  (0.37, 0.18) |
| Mouse | 1a | 126 | 14 | 0.23 |
| 5 | 197 | 9 | 0.16 |

Data represent the mean values of two technical replicates (individual values in parenthesis), except for OZ439 in mouse liver microsomes where only one value is available. a Data reported previously

Metabolite identification (see Supplementary Material) for N205 revealed that the major route of metabolism for N205 is hydroxylation of the adamantane ring accounting for almost 50% of the turnover observed in human liver microsomes (based on peak area only). Minor metabolites observed included products stemming from tetraoxane ring cleavage (M-182) and morpholine ring cleavage (M-26). Thus the metabolic weak spot in these structures is the lipophilic admantane ring system and improvement in the DMPK profile may be possible through chemical substitution within the adamantylidene portion of the molecule.

As noted previously, an important feature of OZ439 is its enhanced blood stability [7] thought to be due at least in part to reduced degradation in the presence of Fe(II). Table 5 shows data on the stability in human and rat blood, predicted plasma clearance and rat pharmacokinetic data for N205 and OZ439. N205 has similar human blood stability compared to OZ439 but is less stable in rat blood with a measured half-life of approximately 8 h. The high plasma protein binding for both N205 and OZ439 in human plasma may have some impact on the observed blood stability and further studies are in progress to explore this. Predicted human clearance values for OZ439 and N205 are similar with higher clearance predicted in rats for the tetraoxane analogue.[21] In head to head comparisons of tetraoxanes with 1,2,4-trioxolanes in our laboratory, it is generally observed that rodent microsomal clearance is faster for the adamantylidene tetraoxane scaffold. The higher predicted rat hepatic clearance and lower rat blood stability translates into a comparatively worse performance for N205 in terms of rat pharmacokinetics (Table 5 and Figure 2). N205 exhibited higher clearance and lower oral bioavailability of 52% compared with approximately 100% for OZ439. Both IV and PO half-lives of OZ439 were superior to N205, although the 17 h oral half-life of N205 demonstrates a large improvement over all other endoperoxides examined in this class (e.g. PO half-life of RKA182 at same dose was 3.5 h).[16]

**Table 5:** Human/rat blood stability, predicted plasma clearance and rat pharmacokinetic parameters of N205 versus OZ439. PK parameters for OZ439 are from [7].

|  |  |  |
| --- | --- | --- |
| **Property** | **N205 Mesylate** | **OZ439 mesylate** |
| *In vitro* blood stability (37ºC, 4 h)  T1/2 (h) in rat blood  T1/2 (h) in human blood | ~8  ~10% loss | >15  No degradation detected |
| Pred CLplasma (mL/min/mg)\*  human  rat  mouse | 14  50  53 | 15  21  28 |
| Rat PK  CLplasma (mL/min/kg)  Vdss (L/kg)  estimated IV T1/2 (h)BA (%) | 77  11  6.352 | 40  18  32  ~100 |

**Figure 2**; Plasma concentrations in male Sprague Dawley rats following IV and PO administration of 1.6 and 8.1 mg/kg, respectively. Data represent the mean of n=2 rats except for the point marked with \* where only one measurement was available.



Additional profiling in human liver microsomes to determine CYP450 inhibition (Table 6) revealed no concerns (IC50 >20 µM for each isoform).

**Table 6** IC50 values against five drug-metabolising CYP isoforms using a substrate specific approach in human liver microsomes.

|  |  |  |
| --- | --- | --- |
| **CYP isoform** | **IC50 (μM)** | |
| **N205** | **Reference Inhibitor** |
| **CYP1A2** | >20 | 3.6  (Furafylline) |
| **CYP2C9** | >20 | 0.72  (Sulfaphenazole) |
| **CYP2C19** | >20 | 0.48  (Ticlopidine) |
| **CYP2D6** | >20 | 0.025  (Quinidine) |
| **CYP3A4**  (Midazolam 1’-hydroxylation) | >20 | 0.022  (Ketoconazole) |
| **CYP3A4**  (Testosterone 6β-hydroxylation) | >20 | 0.013  (Ketoconazole |

In order to provide an assessment of the therapeutic efficacy of N205 against *P. falciparum Pf*3D70087/N9, potency was assessed by administering a single oral dose (2.5, 5, 15, 30, 50 and 100 mg·kg-1) at day 3 after infection and measuring the effect on blood parasitemia by flow cytometry (Figure 3A-C and Table 7)*.*[22, 23]The parameters of efficacy estimated in the study were a) the dose of N205 that reduces parasitemia at day 7 after infection by 90% with respect to vehicle-treated mice (parameter denoted as ED90) and b) the estimated average daily exposure in whole blood necessary to reduce *P. falciparum* parasitemia in peripheral blood at day 7 after infection by 90% with respect to vehicle-treated mice (parameter used to measure the potency of the compound and denoted as AUCED90). In the experimental conditions used in the assay N205 is efficacious against *P. falciparum*, with ED90 = 8.6 mg·kg-1 and AUCED90 < 0.75 μg·h·ml-1· following single oral dose administration. In contrast to studies in *P. berghei*, the dose levels administered were not able to cure mice even at the top dose of 100 mg/kg. The *in vivo* data confirms that N205 has outstanding antimalarial activity within the same region as OZ439 and E209. [7,18] In this model, an ED90 of 10 mg/kg was obtained for artesunate after four daily doses indicating that a single dose of N205 has similar oral potency to multiple doses of artesunate.

**Figure 3**; **A**) Parasitemia in peripheral blood of mice infected with *P. falciparum Pf*3D70087/N9. Data shown correspond to individual parasitemia values for mice treated with N205 or vehicle (n=2). **B**) Dose-response relationship for N205;data are presented as log10 [percentage of parasitemia at day 7 after infection] of individual mice *versus* the dose in mg·kg-1. Parasitemias lower than the limit of detection of flow cytometry (0.01%) are computed and plotted as 0.01% for the dose-response curve fitting. **C**) Upper panels show peripheral blood smears stained with Giemsa and lower panels show flow cytometry dot plots from samples of peripheral blood stained with TER-119-Phycoerythrine and SYTO-16. Dots inside the polygonal region represent *P. falciparum*-infected human erythrocytes.

**Figure 2.tif**

**Table 7** PK Parameters for N205 in the humanised *Pf* SCID mouse model at five different doses

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Blood PK Parameters in humanized mouse** | | | | | | |
|  | | | | | | |
| **Dose (mg/Kg)** | 5 | 15 | | 30 | 50 | 100 |
| **Cmax (µg/mL)** | 0.1090 | 0.1480 | | 0.1480 | 0.6070 | 0.2500 |
| **Cmax /Dose (µg/mL per mg/kg** | 0.0218 | 0.0099 | | 0.0049 | 0.0121 | 0.0025 |
| **tmax (h)** | 1 | 1 | | 1 | 2 | 0.5 |
| **AUC(0-t) (µg.h/mL)** | 1.2801a | 0.7489b | | 0.9399b | 2.9973b | 2.3211c |
| **DNAUCd(0-t) (µg·h/mL per mg/kg)** | 0.2560 | 0.0499 | | 0.0313 | 0.0599 | 0.0232 |
| **Efficacy Parameters in humanized mouse** | | | | | | | |
|  | | | | | | | |
| **ED90 mg·kg-1**  **AUCED90 µg·h·ml-1**  **AUCPCC µg·h·ml-**1 | | | 8.6 / 6.2\*  <0.75 / 0.68\*  - / -\* | | | | |

a t = 4h; b t = 8 h; ct = 23h; dDNAUC, dose normalized AUC0-t \* data for OZ439

**3. Experimental Methods**

**3.1. Biological Assessment**

Please see Supplementary Information for solubility, plasma protein binding and blood stability experimental methods.

**3.1.1 *In Vitro* Sensitivity Assays**

Drug susceptibilities were assessed at the Liverpool School of Tropical Medicine by the measurement of fluorescence after the addition of SYBR Green I as previously described by Smilkstein et al. [20] Drug IC50s were calculated from the log of the dose/response relationship as fitted with Grafit software (Erithacus Software, Kent, United Kingdom). Results are given as the mean of at least three separate experiments.

For the fluorescence assay, after 48 h of growth, 100 μl of SYBR Green I in lysis buffer (0.2 μl of SYBR Green I/ml of lysis buffer) was added to each well, and the contents were mixed until no visible erythrocyte sediment remained. After 1 hr of incubation in the dark at room temperature, fluorescence was measured with a Varioskan fluorescence multiwell plate reader from Thermo Electron Corporation with excitation and emission wavelengths of 485 and 530 nm, respectively.

**3.1.2. *In vitro* physicochemical and ADME studies and *in vivo* animal experiments**

***3.1.2.1* *In vitro* ADME and *in vivo* PK**

The *in vitro* ADME studies and *in vivo* PK studies were conducted at the Centre for Drug Candidate Optimisation, Monash University (Australia). Methods for solubility, metabolite identification, plasma and microsomal protein binding, and plasma analysis are described in the Supplementary Information. Microsomal stability and CYP inhibition were conducted as described previously [24] using liver microsomes from Sekisui XenoTech (Kansas City, KS). Rat PK and rat blood stability studies were performed as described previously [7] in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and the study protocols were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee. The intravenous formulation for N205 was prepared in 5% w/v glucose solution, whilst the oral formulation was prepared as a solution in aqueous vehicle containing 0.5% w/v hydroxypropylmethyl cellulose, 0.5% v/v benzyl alcohol and 0.4% v/v Tween 80.

***3.1.2.2.* *In vivo* antimalarial screening (*Plasmodium berghei*)**

*In vivo* efficacy studies in *P. berghei*-infected mice were conducted at the Swiss Tropical and Public Health Institute (Basel, Switzerland), adhering to local and national regulations of laboratory animal welfare in Switzerland (permission no. 1731). The tetraoxanes and OZ439 were dissolved or suspended in a vehicle consisting of 0.5% w/v hydroxypropylmethyl cellulose, 0.5% v/v benzyl alcohol, 0.4% v/v Tween 80, and 0.9% w/v sodium chloride in water, and administered orally on day 1 post infection. Antimalarial activity was measured as a percent reduction in parasitemia on day 3 post infection. Animals were considered cured if there were no detectable parasites on day 30 post infection. The onset of action was determined after a single oral dose of compounds to mice (n = 3) on day 3 post infection, resulting in a high initial parasitemia to allow the onset of action to be assessed. The reduction in parasitemia was initially monitored at 12 h post treatment, and the time of recrudescence was assessed by daily blood smears for 14 d followed by intermittent assessment up to 30 days. All groups, including an untreated control group, were infected simultaneously with *P. berghei*. Parasitemia was determined on day 3 post infection, and compared with values in control animals.

***3.1.2.3 In vivo* efficacy (*Pf* SCID mice)**

Humanised mouse efficacy and pharmacokinetic studies in *Pf* SCID mice were conducted at GSK Tres Cantos, Madrid. Studies of murine *P. falciparum* infection were ethically reviewed and carried out in accordance with European Directive 2010/63/EU and the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals. *In vivo* efficacy against *P. falciparum* was conducted [22] in age-matched female immunodeficient NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ mice (8-10 weeks of age; 22-24 gm) supplied by Charles River, UK, under license of The Jackson Laboratory, Bar Harbor.  Mice were engrafted with human erythrocytes (Red Cross Transfusion Blood Bank in Madrid, Spain) by daily intraperitoneal injection with 1 mL of a 50% hematocrit erythrocyte suspension (RPMI 1640 (Invitrogen), 25 mM HEPES (Sigma), 25% decomplemented AB+ human serum (Sigma) and 3.1 mM hypoxanthine (Sigma)). Mice with ~40% circulating human erythrocytes were intravenously infected with 2×107 *P. falciparum* Pf3D70087/N9-infected erythrocytes (day 0). Efficacy was assessed by administering one oral dose of N205 (2.5, 5, 15, 30, 50 and 100 mg.Kg-1) at day 3 after infection. Treatment group assignments were allocated randomly. Parasitemia was measured by flow cytometry in samples of peripheral blood stained with the fluorescent nucleic acid dye SYTO-16 (Molecular Probes) and anti-murine erythrocyte TER119 monoclonal antibody (Becton Dickinson) in serial 2 μL blood samples taken every 24 hours until assay completion. The ED90 was estimated by fitting a four parameter logistic equation using GraphPad 6.0 Software

## Systemic exposure in infected *Pf* SCID mice

The levels of N205 were evaluated in whole blood in order to determine standard pharmacokinetic parameters in the individual animals used in the efficacy study. Peripheral blood samples (25 ml) were taken at different times (0.25, 0.5, 1, 2, 4, 6, 8 and 23 h) after drug administration, mixed with 25 µl of Milli-Q water and immediately frozen on dry ice. The frozen samples were stored at -80°C until analysis. Vehicle-treated mice experienced the same blood-sampling regimen. Blood samples were processed by liquid–liquid extraction. Quantitative analysis by Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed using a Waters UPLC system and Sciex API4000 mass spectrometer. The lower limit of quantification in this assay was 0.005 micrograms/ml (5 ng/ml). Blood concentration vs time was analyzed by non-compartmental analysis (NCA) using Phoenix ver.6.3 (from Pharsight), from which exposure-related values (Cmax and AUC0-23, AUC0-t) and tmax were estimated.

**3.2. Chemistry**

With exception of those stated all reagents were obtained from commercial suppliers. Dichloromethane, triethylamine and THF were freshly distilled before use. Analytical thin layer chromatography was performed on pre-coated silica gel (0.25mm layer of silica gel F254) aluminium sheets. UV light (254nm) was used for all visualizations and flash column chromatography was performed using Merck 938S Kieselgel 60 Silica gel. IR spectra were run using a Perkin-Elmer 298 infrared spectrophotometer. Solid samples were dissolved in CHCl3 and liquids/oils applied neat on to sodium chloride discs.

1H NMR spectra were recorded using a Bruker 400 MHz NMR spectrophotometer. Spectra were referenced to the residual solvent peak and chemical shifts expressed in ppm from TMS as the internal reference peak. All NMR experiments were performed at room temperature. The following annotations are used to describe multiplicity; s, singlet, bs, broad-singlet, d, doublet, t, triplet, q, quartet, m, multiplet and coupling constants are expressed in Hertz.

Mass spectra were recorded between 20-70eV using a VG7070E and/or Micromass LCT mass spectrometers. The molecular ion M+ with intensities in parenthesis is given followed by peaks corresponding to major fragment losses. Melting points are expressed in degree Celsius (°C) and performed using the Gallemkamp melting point apparatus and capillary tubes.

**Preparation of 4-(4-oxocyclohexyl)phenyl trifluoromethanesulfonate 4b [25].** To a stirred solution of **4a** (10g, 52 mmol)) in dry DCM (75 ml) at -78 0C was added triethylamine (10 ml). To this mixture was added triflic anhydride (10.6 ml (density = 1.67g/ml, 63 mmol)) drop-wise over 30 minutes. After this time the solution was allowed to warm to room temperature and stirred overnight. The reaction mixture was washed with water (30 ml), dried over MgSO4 and concentrated. Purification by flash column chromatography using ethyl acetate/hexane (20/80) afforded the pure triflate **4b** in 91% yield as off-white foam. 1H NMR (400MHz, CDCl3) δH, 1.81-1.99 (m, 2H, CH2), 2.18-2.28 (m, 2H, CH2), 2.48-2.58 (m, 4H, CH2), 3.02-3.15 (m, 1H, CH), 7.22 (d, 2H, J = 7.2 Hz, Ar), 7.32 (d, 2H, J = 7.2Hz, Ar). 13CNMR (100MHz, CDCl3), δC 34.2, 41.5, 42.6, 117.6, 121.9, 128.9, 145.7, 148.5, 210.6 MS (ES+), [M + Na] (100), 345.0 HRMS calculated 345.0384 for C13H13O4Na found 345.0392.

**Preparation of methyl 4-(4-oxocyclohexyl)benzoate 4c [26].** To a solution of the triflate **4b** (2.41g, 0.007 mol)) in DMF (25 ml) and MeOH (12 ml) was added di-isopropylethylamine (2.7 ml) followed by Pd(OAc)2 (84 mg, 0.376 mmol) and dppp (155 mg, 0.376 mmol). A stream of carbon monoxide (CO) gas was bubbled into the solution for 5 min then a balloon filled with CO was added to the top of the reflux condenser. After allowing to stir for 16 hrs at 80-90 oC, the reaction mixture was allowed to cool to room temperature, diluted with ethyl acetate (250 mL), washed with saturated bicarbonate solution (50 ml), water (50ml), brine (50ml) and dried over sodium sulphate. Purification by flash chromatography (EtOAc/Hex, 20/80) gave **4c** in 72% mp= 94°C lit mp = 93-94°C 1HNMR (400MHz, CDCl3) δH, 1.81-2.04 (m, 2H, CH2), 2.18-2.28(m, 2H, CH2), 2.48-2.58 (m, 4H, CH2), 3.02-3.15 (m, 1H, CH), 3.90 (s, 3H, CH3), 7.34 (d, 2H, J = 7.2 Hz, Ar), 8.04 (d, 2H, J = 7.2 z, Ar). 13CNMR (100MHz, CDCl3), δC 34.06, 41.62, 43.2, 52.51, 127.37, 128.97, 130.38, 150.48, 167.24, 211.11, MS (ES+), [M + Na] (100), 255.1 HRMS calculated 255.0997 for C14H16O3Na found 255.0396.

**Alternative Preparation of methyl 4-(4-oxocyclohexyl)benzoate 4c.** A solution of oxalyl chloride 4.51 mL (53.3 mmol) in DCM (50 mL) was added to a suspension of 4-phenylcyclohexanone (7g, 40 mmol) and AlCl3 (16.07g, 120 mmol) in DCM (150mL) at 0oC. The reaction mixture was stirred at 0 oC for 1 hour then at room temperature for 2 hours. A mixture of methanol (10 mL) and pyridine (8.1 mL) was added drop wise to the reaction mixture and left to stand overnight. The reaction mixture was then washed with water, 3N HCl, NaHCO3, dried over NaSO4, filtered and concentrated. Purification by flash column chromatography gave **4c** in 65 % yields with identical spectroscopic and physical properties to that described above.

**Preparation of methyl 4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]benzoate 6a.** To a solution of the ketone **4c** (4g, 17 mmol) in acetonitrile (75 mL) at 0 oC was added formic acid (8 mL) and 50% H2O2 (16 mL). The resulting reaction mixture was allowed to stir for 30 min at 0oC, then allowed to warm to room temperature and diluted with water (30 mL). The resulting mixture was extracted in DCM (3 x 50 mL), dried over MgSO4 and concentrated to give the crude *gem*-bishydroperoxide **5** which was used without further purification. The *gem*-bishydroperoxide **5** was dissolved in CH2Cl2 (50mL) and added to a stirring solution of the required adamantanone (1.5 equiv.) and rhenium (VII) oxide (0.02 eqv) in CH2Cl2 (50 ml) at room temperature. The reaction mixture was stirred for 1 hour, filtered through a plug of silica and concentrated. Purification by flash column chromatography gave **6a** in 48% as a white foam. 1H NMR (400 MHz, CDCl3-d6) δH 7.89 (d, 2H, J = 8.3 Hz, Ar), 7.22 (d, 2H, J = 8.3 Hz, Ar), 3.83 (s, 3H, CH3), 2.60 (tt, 1H, J = 11.5 Hz, 3.9 Hz, CH), 2.06-1.44 (m, 23H, CH2/CH) 13C NMR (100 MHz, CDCl3-d6) δc 167.4, 151.6, 130.2, 128.7, 127.3, 111.0, 107.7, 52.5, 44.1, 37.4, 34.7, 33.6, 29.9, 27.5. MS (ES+), [M + Na] + (100) 437.2

**Preparation of 4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]benzoic acid 6b.** A solution of **6a** (3.86 mmol) in 10% w/v potassium hydroxide/methanol (12.6 ml) was stirred at reflux for 90 min. The solution was allowed to cool to room temperature and concentrated under reduced pressure. The resulting residue was taken up in water (15 ml) and washed with diethyl ether (3 × 12 ml). The aqueous layer was acidified with concentrated hydrochloric acid and a white precipitate formed. Diethyl ether (18 ml) was added to dissolve the precipitate and the aqueous phase extracted with diethyl ether (2 × 12 ml). The combined organic phases were washed with brine (10 ml), dried over Na2SO4, filtered and concentrated under reduced pressure to give a white solid. Recrystallization from ethanol gave the carboxylid acid **6b** as a white solid in 91% yield. 1H NMR (400 MHz, CDCl3-d6) δH 8.04 (d, 2H, J = 8.4 Hz, Ar), 7.34 (d, 2H, J = 8.4 Hz, Ar), 2.75-2.66 (m, 1H, CH), 2.12-1.45 (m, 22H, CH/CH2) 171.3, 155.3, 132.8, 131.0, 129.1, 127.3, 126.8, 114.4, 44.5, 36.7, 33.8, 32.9, 27.9, 25.8 MS (ES+), [M - H] - (100) 399.2 HRMS calculated for 399.1808 C23H27O6, found 399.1808.

**General procedure for the amide formation (7a-j).** To a solution of the acid **6b** **(**2.33 mmol) in dry DCM (30 mL) was added triethylamine (0.7 mmol, 1.5 eq) and ethylchloroformate (2.33 mmol, 1.0 eq). The reaction was allowed to stir for 60 minutes at 0 oC. (2.33mmol, 1.0 eq) of the required amine was added, and after stirring for 30 minutes, the reaction mixture was allowed to warm to room temperature and and then allowed to stir for a further 90 minutes. The reaction mixture was then diluted with water and extracted with DCM (3 x 30mL). The combined organic extracts were washed with brine, dried over anhydrous Na2SO4 and concentrated. Purification by flash column chromatography afforded the required amide.

**Preparation of** **{4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]phenyl](morpholin-4-yl)methanone 7a**. White solid (Yield 87%) Mpt = 123-124 oC 1H NMR (400MHz, CDCl3) δH, 7.38 (d, 2H, J = 8 Hz, Ar), 7.29 (d, 2H, J = 8 Hz, Ar), 3.4-3.9 (m, 4H, CH2), 3.14-3.40 (m, 2H, CH), 2.6-2.7 (m, 1H, CH), 1.60-2.10 (m, 20H, CH2/CH), 13CNMR (100MHz, CDCl3), δC 170.9, 169.5, 148.3, 127.8, 127.4, 117.7, 110.9, 107.8, 67.3, 43.9, 39.6, 37.6, 34.7, 33.6, 32.3, 30.6, 29.9, 27.8MS (ES+), [M + Na] 492.2 (100), HRMS calculated for 292.2362 C27H35NO6Na found, 492.2372. % C, H, N calculated; C= 69.06, H=7.51, N=2.98; found C= 69.40, H=7.85, N=3.21

**Preparation of** **4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]-*N*-(piperidin-4-ylmethyl)benzamide 7b.** Off white powder (Yield 77 %) Mpt = 132-134 oC 1H NMR (400 MHz, CDCl3-d6) δH 8.41 (t, 1H, J = 5.9 Hz, NH), 7.77 (d, 2H, J = 8.3 Hz, Ar), 7.32 (d, 2H, J = 8.3 Hz, Ar), 3.11 (t, 2H, J – 5.9 Hz, NCH2), 2.98 (t, 4H, J = 10.6 Hz, CH2N), 2.83 (t, 2H, J = 6.3 Hz, CH2), 2.78-2.70 (m, 1H, CH), 2.55-2.45 (m, 1H, CH), 1.95-0.94 (m, 26 H, CH/CH2) 13C NMR (100 MHz, CDCl3-d6) δc 177.5, 149.2, 133.0, 127.7, 126.9, 110.1, 107.6, 51.5, 46.5, 45.3, 42.5, 40.5, 38.8, 36.6, 32.9, 29.9, 27.2, 26.8 MS (ES+), [M + H] + (100) 497.3 HRMS calculated for 497.3015 C29H41N2O5, found 497.3017; % C, H, N calculated; C= 70.13, H=8.12, N=5.64; found C= 70.05, H=7.95, N=5.42

**Preparation of 4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]-*N*-[2-(methylamino)ethyl]benzamide 7c**. White foam (Yield, 64 %) Mpt 60-62 oC 1H NMR (400 MHz, CDCl3-d6) δH 8.81 (t, 1H, J = 5.5 Hz, NH), 7.88 (d, 2H, J = 8.2 Hz, Ar), 7.40 bs, 1H, NH), 7.34 (d, 2H, J = 8.2 Hz, Ar), 3.60 (q, 2H, J = 5.9 Hz, NCH2), 3.07 (t, 2H, J = 5.9 Hz, CH2N), 2.79-2.72 (m, 1H, CH), 2.56 (s, 3H, NCH3), 1.99-1.57 (m, 22H, CH/CH2) MS (ES+), [M + H] + (100) 457.3 HRMS calculated for 457.2702 C26H37N2O5, found 457.2701. % C, H, N Calculated; C= 68.40, H=7.95, N=6.14; found C= 68.10, H=7.62, N=5.92

**Preparation of** ***N*-(3-aminocyclobutyl)-4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]benzamide 7d.** White foam (Yield 82 %) Mpt = 116-118 oC 1H NMR (400 MHz, CDCl3-d6) δH 8.74 (s, 1H, NH), 7.88 (bs, 2H, NH2), 7.56 (d 2H, J = 8.3 Hz, Ar), 7.34 (d, 2H, J = 8.3 Hz, Ar), 3.55-3.50 (m,1H, CH), 3.45-3.24 (m, 5H, CH/CH2), 3.20-3.18 (m, 1H, CH), 1.99-1.52 (m, CH/CH2) 13C NMR (100 MHz, CDCl3-d6) δc 169.5, 149.4, 128.2, 127.0,l 110.1, 107.5, 42.5, 40.7, 36.5, 32.9, 31.5, 26.8 MS (ES+), [M + H] + (100) 455.3 HRMS calculated for 455.2546 C26H35N2O5, found 455.2532. % C, H, N Calculated; C= 69.21, H=7.74, N=5.98; found C= 69.01, H=7.38, N=5.63

**Preparation of** **{4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]phenyl}(thiomorpholin-4-yl)methanone 7e.** White solid (Yield 76%) 1HNMR (400MHz, CDCl3) δH, 7.40 (d, 2H, J = 8 Hz, Ar), 7.27 (d, 2H, J = 8 Hz, Ar), 3.4-3.9 (m, 4H, CH2), 3.14-3.40 (m, 2H, CH), 2.58-2.7 (m, 1H, CH), 1.60-2.10 (m, 20H, CH2/CH), 13CNMR (100MHz, CDCl3), δC 170.8, 169.5, 148.3, 133.6, 127.8, 127.4, 110.9, 107.7, 43.9, 42.9, 39.6, 37.3, 34.6, 33.6, 32.2, 30.5, 27.8, 27.5 MS (ES+), [M + Na] 508.2 (100), HRMS calculated for 508.2134 C27H35NO5NaS found, 508.2138. % C, H, N Calculated; C= 66.78, H=7.26, N=2.88; found C= 66.39, H=6.98, N=2.47

**Preparation of** **{4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]phenyl}(4-methylpiperazin-1-yl)methanone 7f**. White solid (Yield 81%) mpt 108-110 oC 1HNMR (400MHz, CDCl3) δH, 7.4 (d, 2H, J = 9 Hz, Ar), 7.27 (d, 2H, J = 9 Hz, Ar), 3.4-3.9 (m, 4H, CH2), 3.14-3.34 (m, 2H, CH), 2.6-2.8 (m, 1H, CH), 2.42 (s, 3H, CH3), 1.50-2.10 (m, 20H, CH2/CH) 13CNMR (100MHz, CDCl3), δC, 170.4,150.6, 147.9, 133.2, 129.9, 127.3, 127.0, 126.7, 110.5, 107.3, 45.0, 43.6, 43.4, 41.2, 34.2, 33.1, 31.7, 29.5, 27.0 MS (ES+), [M + H] 483.3 (100), HRMS calculated for 483.2859 C28H39N2O5 found, 483.2858. % C, H, N Calculated; C= 69.68, H=7.94, N=5.80; found C= 69.41, H=7.42, N=5.49

**Preparation of** **1,4-diazepan-1-yl{4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]phenyl}methanone 7g.** White powder (Yield 58 %) 1HNMR (400MHz, CDCl3) δH, 7.24-7.4 (m, 4H, Ar), 3.5-3.8 (m, 6H, CH/CH2), 3.1-3.4 (m, 4H, CH), 2.65-2.57 (m, 1H, CH), 1.48-2.11 (m, 22H, CH2/CH), 13CNMR (100MHz, CDCl3), δC 172.2, 147.8, 134.9, 127.4, 127.1, 126.8, 110.9, 107.8, 80.2, 45.2, 43.8, 29.9, 28.8, 27.5, 27.0 MS (ES+), [M + H] 483.3 (100), HRMS calculated for 483.2859 C28H39N2O5 found, 483.2856. % C, H, N calculated; C= 69.68, H=7.94, N=5.80; found C= 69.41, H=7.65, N=5.51

**Preparation of** **{4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]phenyl}(piperazin-1-yl)methanone 7h**. White solid (Yield 78%) 1HNMR (400MHz, CDCl3) δH, 7.24-7.41 (m, 4H, Ar), 3.8-4.2 (m, 4H, CH2), 3.1-3.4 (m, 2H, CH), 2.6-2.8 (m, 1H, CH), 1.50-2.2 (m, 20H, CH2/CH), 13CNMR (100MHz, CDCl3), δC, 171.4, 156.1, 148.9, 132.9, 128.0, 127.7, 110.9, 107.7, 66.2, 53.3, 43.9, 42.5, 37.3, 33.6, 30.2, 27.4 MS (ES+), [M + H] 469.3 (100), HRMS calculated for 468.2624 C27H37N2O5 found, 468.2631. % C, H, N calculated; C= 69.21, H=7.74, N=5.98; found C= 69.36, H=7.85, N=5.61

**Preparation of** **(4-aminopiperidin-1-yl){4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]phenyl}methanone 7i.** White powder (Yield 86 %) Mpt = 116-118 oC. 1HNMR (400MHz, CDCl3) δH, 7.24-7.4 (m, 4H, Ar), 3.7-4.1 (m, 7H, CH/CH2), 3.1-3.4 ( m, 4H, CH), 2.6 (m, 1H, CH), 1.60-2.1 (m, 20H, CH2/CH), 13CNMR (100MHz, CDCl3), δC 172.0, 155.6, 149.3, 132.2, 128.0, 127.9, 126.7, 110.9, 107.7, 53.7, 43.9, 43.8, 41.1, 37.3, 33.5, 27.4 MS (ES+), [M + H] 483.3 (100), HRMS calculated for 483.2814 C28H39N2O5 found, 483.2805. % C, H, N Calculated; C= 69.68, H=7.94, N=5.80; found C= 69.41, H=7.81, N=5.71

**Preparation of** ***N*-(2-aminoethyl)-4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]benzamide 7j**. White powder (Yield, 76 %). 1H NMR (400 MHz, CDCl3-d6) δH 8.74 (t, 1H, J = 5.5 Hz, NH), 8.12 (bs, 2H, NH2), 7.86 (d, 2H, J = 8.3 Hz, Ar), 7.34 (d, 2H, J = 8.3 Hz, Ar), 3.52 (q, 2H, J = 5.8 Hz, NCH2), 2.97 (t, 2H, J = 5.8 Hz, CH2N),2.80-2.71 (m, 1H, CH), 1.99-1.52 (m, 22H, CH/CH2) 13C NMR (100 MHz, CDCl3-d6) δc 167.3, 157.1, 149.8, 132.0, 128.4, 110.3, 107.8, 52.4, 42.5, 41.1, 38.9, 37.5, 35.6, 33.5, 32.8, 28.9, 27.1, 26.8 MS (ES+), [M + H ] + (100) 443.3 HRMS calculated for 443.2546 C25H35N2O5, found 443.2545. % C, H, N calculated; C= 67.85, H=7.74, N=6.33; found C= 67.40, H=7.34, N=6.21

**Preparation of {4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]phenyl}methanol 8.** To a stirred solution at 0 ºC of methyl benzoate **6** (1.5 g, 3.65 mmol) in THF (50 mL) was added LiAlH4 (0.28 g, 7.29 mmol)). The suspension was allowed to stir at 0 ºC and was monitored by TLC to determine the consumption of the benzoate. The reaction mixture was quenched with 1N HCl and was then extracted with ethyl acetate (3 x 50 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. Purification by flash column chromatography afforded the required alcohol **8** as a white powder (1.36g, 97%). 1H NMR (400 MHz, CDCl3-d6) δH 7.27 (d, 2H, J = 8.2 Hz, Ar), 7.21 (d, 2H, J = 8.2 Hz, Ar), 4.62 (s, 2H, ArCH2OH), 2.62 (tt, 1H, J = 11.4 Hz, 4.0 Hz, Ar), 2.08-1.57 (m, 22H, CH2/CH) 13C NMR (100 MHz, CDCl3-d6) δc 145.9, 139.3, 127.6, 110.9, 107.9, 65.6, 60.8, 43.8, 37.4, 36.2, 34.7, 33.6, 30.1, 27.5 MS (ES+), [M + Na] + (100) 409.2 HRMS 409.1991 calculated for C23H30NO5Na found 409.1990.

**Preparation of 4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]benzyl methanesulfonate 9.** Methanesulfonyl chloride (3.36 mmol) and triethylamine (3.62 mmol) were added at 0 oC under nitrogen atmosphere to a solution of **8** (1.81mmol) in dry DCM (50 mL). The mixture was allowed to stir for 60 min at the same temperature, washed with aqueous 5% NaHCO3 and water, and dried over Na2SO4. Evaporation of the solvent gave the mesylate 9 as colourless oil (0.8, 95%). 1H NMR (400 MHz, CDCl3-d6) δH 7.36(d, 2H, 8.1 Hz, Ar), 7.27 (d, 2H, J = 8.1 Hz, Ar), 5.21 (s, 2H, CH2O), 2.92 (s, 3H, SO2CH3), 2.64 (tt, 1H, J = 11.4 Hz, 3.8 Hz, CH), 2.09-1.56 (m, 22H, CH2/CH) MS (ES+), [M + Na] + (100) 487.3 HRMS calculated for 487.1766 C24H32NO7Na, found 487.1765.

**General procedure for the amine formation (10a-b).** To a solution of mesylate **9** (1.08 mmol 1 eq) in dichloromethane (25 mL) were added triethylamine (2.15 mmol, 2 eq) followed by the amine (2.15 mmol 2, eq) at 0 oC temperature. The reaction mixture was stirred at room temperature over a period of 12 h. The resulting reaction mixture was diluted with dichloromethane (50 mL), washed with water (3 x 20 mL), brine (10 mL) and dried over sodium sulphate. The crude product obtained was purified by flash column chromatography to the required amine.

**Preparation of** **4-{4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]benzyl}morpholine 10a (N205).** White powder (Yield, 62%). Mtp 138-140 oC. 1H NMR (400 MHz, CDCl3-d6) δH 7.24 (d, 2H, J = 8.1 Hz, Ar), 7.17 (d, 2H, J = 8.1 Hz, Ar), 3.73-3.68 (m, 4H, NCH2), 3.46 (s, 2H, ArCH2N), 2.67-2.54 (m, 1H, CH), 2.43 (bs, 4H, CH2O), 2.09-1.58 (m, 22H, CH/CH2) 13C NMR (100 MHz, CDCl3-d6) δc 145.2, 136.0, 129.7, 127.1, 110.9, 107.9, 67.4, 63.6, 54.0, 43.8, 37.4, 34.8, 33.6, 33.4, 32.3, 30.1, 27.5 MS (ES+), [M + H] + (100) 456.3 HRMS calculated for 456.2750 C27H38NO5, found 456.2762; C28H39NO4F, found 472.2859. % C, H, N Calculated; C= 71.18, H=8.18, N=3.07; found C= 70.92, H=7.99, N=3.10

**Preparation of** **1-{4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]benzyl}-4-fluoropiperidine 10b**. White powder (Yield, 60 %), Mtp 122-124 oC 1H NMR (400 MHz, CDCl3-d6) δH 7.23 (d, 2H, J = 8.1 Hz, Ar),7.17 (d, 2H, J = 8.1 Hz, Ar), 4.79-4.53 (m, 1H, CHF), 3.46 (s, 2H, CH2N), 2.64-2.53 (m, 5H, NCH2/CH), 2.40-2.31 (m, 4H, CH2), 2.06-1.54 (m, 22H, CH2/CH) 13C NMR (100 MHz, CDCl3-d6) δc 145.1, 136.6, 129.6, 127.1, 110.9, 107.9, 90.0, 88.3, 63.1, 50.0, 43.8, 37.4, 34.7, 33.6, 32.3, 31.9, 30.6, 27.5 MS (ES+), [M + H] + (100) 472.3 HRMS calculated for 472.2863 C28H39NO4F, found 472.2859. % C, H, N Calculated; C= 71.31, H=8.12, N=4.03; found C= 70.97, H=7.91, N=3.80

**Preparation of 4-{4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo [3.3.1.13,7]decan]-4-yl]benzyl}morpholine** **Mesylate.** 0.3 mmol of **10a** was dissolved in 2 ml of anhydrous diethyl ether and 1.5 mmol of 100 mM methane sulfonic acid stock solution was added. The precipitate formed was collected, washed with diethyl ether and air dried to give the titled salt. White powder (Yield 70 %). Mpt 158-160 oC 1H NMR (400 MHz, DMSO-d6) δH 9.76 (s, 1H, NH), 7.44 (d, 2H, J = 8.2 Hz, Ar), 7.37 (d, 2H, J = 8.1 Hz, Ar), 4.31 (d, 2H, J = 5.0 Hz, CH2), 3.96 (d, 2H, J = 10.3 Hz, CH2), 3.62 (t, 2H, J = 11.6 Hz, CH2), 3.26 (d, 2H, J = 11.9 Hz, CH2), 3.18-3.03 (m, 4H, CH2), 2.78-2.69 (m, 1H, CH), 2.35 (s, 3H, SO2CH3), 1.96-1.51 (m, 22H, CH/CH2) MS (ES+), [M + H] + (100) 456.3 HRMS calculated for 456.2750 C27H38NO5, found 456.2762. % C, H, N calculated; C= 60.96, H=7.49, N=2.54; found C= 60.88, H=7.40, N=2.21

**Preparation of 1-{4-[(1''*r*,3''*r*,5''*R*,7''*R*)-dispiro[cyclohexane-1,3'-[1,2,4,5]tetroxane-6',2''-tricyclo[3.3.1.13,7]decan]-4-yl]benzyl}-4-fluoropiperidine** **mesylate.** 0.3 mmol **10b** was dissolved in 2 ml of anhydrous diethyl ether and and 1.5 mmol of methane sulfonic acid stock solution was added. The precipitate formed was collected, washed with diethyl ether and air dried to give the titled salt. White powder (Yield 68 %). Mpt 108-110 oC 1H NMR (400 MHz, DMSO-d6) δH 9.41 (s, 1H, NH), 7.44 (d, 2H, J = 8.1 Hz, Ar), 7.36 (d, 2H, J = 8.1 Hz, Ar), 5.07-4.70 (m, 1H, CHF), 4.32 (d, 2H, J = 5.1 Hz, CH2), 3.27 (d, 2H, J = 11.1 Hz, CH2), 3.19-2.96 (m, 4H, CH2), 2.79-2.66 (m, 1H,CH), 2.38 (a, 3H, SO2CH3), 2.15-1.96 (m, 2H, CH2), 1.95-1.50 (m, 22H, CH/CH2) MS (ES+), [M + H] + (100) 472.3 HRMS calculated for 472.2863 C28H39NO4F, found 472.2859. % C, H, N Calculated; C= 71.31, H=8.12, N=2.97; found C= 71.02, H=8.01, N=2.68

**4. Conclusion**

N205 represents a molecule with a vastly improved overall profile compared to the first generation tetraoxane RKA182 with comparable antimalarial potency compared to OZ439. Whilst data with human liver microsomes and human blood indicate similar levels of stability compared to OZ439, the latter has superior in rodent *in vitro* microsomal and blood stability and *in vivo* rat PK profiles. Data obtained in the *Pf* SCID mouse model for N205 were extremely encouraging and suggest that the benzylamino tetraoxane template **2** should be explored further to enhance the solubility, metabolic and blood stability even further. A recent paper by Vennerstom *et al.* has examined a series of analogues of OZ439 to determine the key features that impart *in vivo* potency in the mouse model of malaria; the conclusion from this work is that whilst prolonged plasma exposure is important for curative activity, there are other factors involved in imparting high efficacy in rodent models.[8] This observation provides an additional key challenge in understanding the dynamics of this class of drug.

**5. Acknowledgements**

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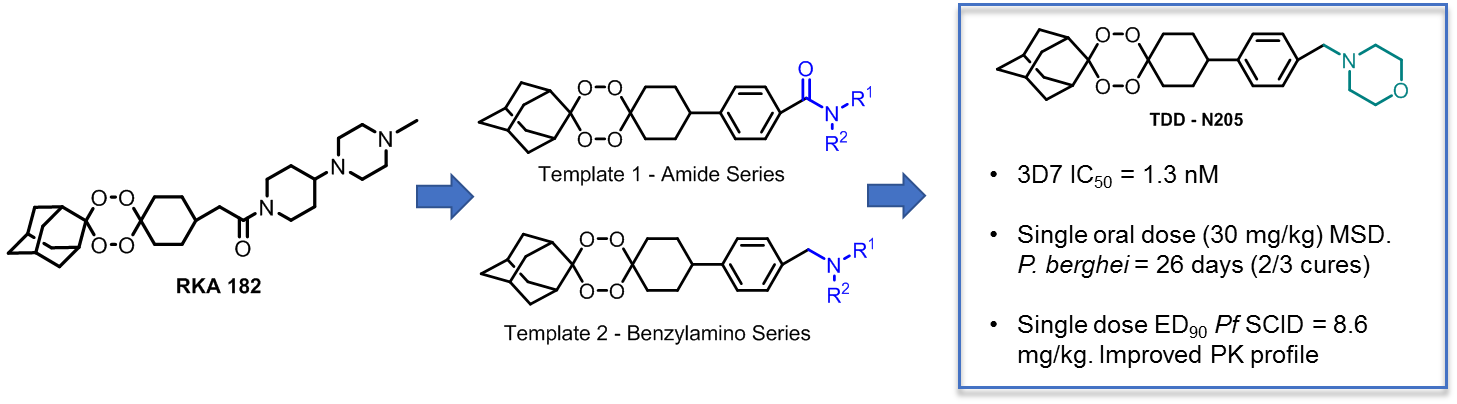
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**7. Graphical Abstract**

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**Supporting Information**

***Solubility methods:*** Solubility studies were conducted at 37°C in water, acidic media (0.1 and 0.01 N HCl), 100 mM citric acid monophosphate pH 2.0, isotonic phosphate buffer (ionic strength of 154 mM) 7.4, and fasted (FaSSIF) and fed (FeSSIF) state simulated intestinal fluids prepared as described previously [1] (M. Marques, Dissolution Technologies, 11:16, 2004).

Approximately 1.0 to 5.0 mg of each compound was weighed into individual screw cap polypropylene tubes and aqueous buffer or simulated intestinal fluid was added to provide a compound concentration of between 500 and 5000 μg/mL. The resulting samples were vortexed, placed in a 37°C incubator and mixed on an orbital shaker (IKA® VXR basic Vibrax® orbital shaker) set at 600 rpm for the duration of the study. Over the incubation period, samples were regularly examined to ensure excess solid was present. Sampling was conducted after 4 hours by centrifuging each sample at 10000 rpm for 3 minutes, transferring 200 µL aliquots into fresh Eppendorf tubes and centrifuging again at 10000 rpm for 3 minutes. Triplicate aliquots of the final supernatant were then removed and diluted to an appropriate analytical concentration in 50% aqueous methanol prior to analysis by HPLC.

LCMS analysis was conducted on a Waters 2795 HPLC system coupled to a Waters Micromass LCT mass spectrometer operating under positive ion electrospray conditions, with a cone voltage of 30V. Analysis was conducted using a Phenomenex Luna C8(2) column (5 µm, 150 x 4.6 mm i.d.) maintained at 40°C. HPLC analysis was performed using gradient conditions from 55% to 95% methanol in water with both phases containing 1% formic acid. Separations were conducted using a flow rate of 1.0 mL/min and an injection volume of 4 µL. Processed samples were maintained in the autosampler at a temperature of 10°C. Compounds were quantified by comparison to calibration curves prepared over the concentration range of 0.05 - 10 µg/mL in 50% aqueous methanol.

***Plasma and microsomal protein binding methods:*** Plasma protein binding was assessed in human plasma separated from whole blood obtained from the Volunteer Blood Donor Registry (Water and Eliza Hall Institute, Melbourne, Australia). Given the expected high protein binding for both compounds, studies were conducted using diluted plasma (1 in 10 dilution pH 7.4, 0.1 M phosphate buffered saline) to facilitate detection of the unbound concentration with correction of the data for dilution as described previously (Kalvass and Maurer, Biopharm Drug Dispos, 23:327-338, 2002). Compounds were spiked into diluted plasma at a total nominal concentration of 2000 ng/mL. Samples were briefly vortex mixed and aliquots (n=3) were transferred to rapid equilibrium dialysis (RED, ThermoFisher) units maintained at 37°C under ambient atmosphere on a plate shaker (ThermoMixer C, Eppendorf). Samples were dialysed against 0.1 M phosphate buffered saline (pH 7.4) for a period of 6 h. At the end of the dialysis period, samples were removed from both the donor and dialysate chambers of the RED units. Samples were matrix matched with the opposite medium (i.e. blank plasma added to the dialysate samples or blank buffer added to the total plasma samples) and stored at -80°C until analysis. Analysis was conducted by LC/MS as described below with concentrations determined by comparison to calibration standards prepared in the same matrix (i.e. 50% buffer, 50% plasma).

Microsomal binding was assessed via ultracentrifugation as described previously (Coteron et al., J Med Chem, 54:5540-5561, 2011. The suspension of human liver microsomes was prepared in 0.1 M phosphate buffer (pH 7.4) at a protein concentration of 0.4 mg/mL to mimic the conditions used for the microsomal stability studies, and compounds were spiked into the matrix at a total nominal concentration of 1 µM.

***LC/MS method for the analysis of plasma samples:*** Plasma samples were analysed for N205 following protein precipitation with acetonitrile. Chromatography was conducted using a Waters Acquity UPLC and Waters Micromass Xevo TQ mass spectrometer in positive electrospray ionisation mode with multiple-reaction monitoring. The column was a Supelco Ascentis Express RP Amide column (50 x 2.1 mm, 2.7 µm) maintained at 40ºC. The mobile phase consisted of a mixture of water and acetonitrile (each containing 0.05% formic acid) eluted under gradient conditions with a flow rate of 0.4 mL/min and an injection volume of 3 µL. Samples were maintained in the autosampler at 10ºC prior to analysis. Concentrations were determined by comparison of the response to that of calibration standards prepared in blank rat plasma and processed in the same manner as the samples. The calibration range was 1 to 5000 ng/mL and the lower limit of quantitation was 1 ng/mL. The accuracy and precision were within ±10% and < 10%, respectively.

***Methods for metabolite identification in liver microsomes:*** Incubations in liver microsomes were conducted as described previously [Younis et al., J Med Chem, 55:3479-3487, 2012] using liver microsomes from Sekisui XenoTech (Kansas City, KS) at a substrate concentration of 5 µM. Samples were analysed by UPLC-MS (Waters/Micromass Xevo G2 QTOF) under positive electrospray ionisation and MS spectral data were acquired in a mass range of 80 to 1200 Daltons. A full metabolite search was conducted for the metabolic transformations listed below.

Table S1: List of N205 metabolites monitored for in human (H), rat (R) and mouse (M) liver microsomes incubations under ESI positive ionisation mode. D=detected; ND=not detected

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Metabolite description** | **∆Mass (Daltons)** | **[MH+]** | **D/ND** | **tR**  **(min)** | **Metabolite**  **Code** |
| Parent | - | 456 | D | 3.68 | TDD-N205 |
| Mono-oxygenation | +16 | 472 | D | 1.59 | M+16 (I) |
| 1.93 | M+16 (II) |
| 2.28 | M+16 (III)  (M only) |
| 3.63 | M+16 (IV)  (R & M only) |
| 3.73 | M+16 (V) |
| Ring dehydrogenation | -2 | 454 | ND | - | - |
| Morpholine ring cleavage | -26 | 430 | D | 3.54 | M-26 |
| Morpholine ring opening (alcohol) | +18 | 474 | ND | - | - |
| Tetroxane cleavage (ketone) | -182 | 274 | D | 0.84 | M-182 |
| Tetroxane cleavage (alcohol) | -180 | 276 | D | 0.86 | M-180 |
| Morpholine dealkylation | -70 | 386 | ND | - | - |
| Morpholine deamination (alcohol) | -69 | 387 | ND | - | - |
| Morpholine ring cleavage (acid) | -12 | 444 | ND | - | - |
| Morpholine ring opening (acid) | +32 | 488 | ND | - | - |
| Morpholine deamination (acid) | -55 | 401 | ND | - | - |
| Bis-oxygenation | +32 | 488 | D | 1.10 | M+32 (I) |
| 1.18 | M+32 (II)  (M only) |
| 1.43 | M+32 (III)  (R only) |
| 1.76 | M+32 (IV) |
| 2.12 | M+32 (V)  (H & R only |
| Mono-oxygenation & Morpholine ring cleavage | -10 | 446 | D | 1.33 | M-10 (I) |
| Mono-oxygenation & Morpholine ring cleavage | -10 | 446 | D | 1.64 | M-10 (II) |
| Bis-Oxygenation & dehydrogenation | +30 | 486 | ND | - | - |
| Mono-oxygenation & dehydrogenation | +14 | 470 | ND | - | - |
| Morpholine ring opening (2 acids) | +45 | 502 | ND | - | - |
| Ring dehydrogenations (x2) | -4 | 452 | ND | - | - |

A metabolite with a molecular ion 26 Daltons ([MH+] 430, M-26) less than the parent with an accurate mass and MS/MS spectrum consistent with a morpholine ring cleavage metabolite (Figure 1) was detected in all three species. Five putative mono-oxygenation metabolites at [MH+] 472 (M+16 (I) to (V)) were detected. MS/MS spectra for M+16 (I), (II) and (III, detected in mouse only) indicate hydroxylation on the adamantane ring (Figure S1) and considering retention times, are probably metabolites hydroxylated at the distal and bridgehead adamantane positions. MS/MS spectra for M+16 (IV, detected in rodent only) and (V) suggest oxygenation on the non-adamantane side of the molecule (Figure S1). Five putative bis- oxygenation metabolites at [MH+] 488 (M+32 (I), (II, detected in mouse only), (III, detected in rat only), (IV) and (V, detected in human and rat only) were observed. MS/MS spectra for M+32 (I) indicate bis-hydroxylation on the adamantane ring (Figure S1). MS/MS spectra for the remaining M+32 metabolites were too weak to confirm the site of metabolism but are most likely formed via combinations of the M+16 metabolites described above. Two cleavage metabolites (M-182 and M-180) were detected in all three species and their structures were confirmed by MS/MS spectra (Figure S1). Two putative secondary metabolites with molecular ions [MH+] 446 consistent with combined morpholine cleavage and mono-oxygenation (M-10) were detected however their MS signals were too weak to enable structure confirmation. M-182 was detected in controls (non-cofactor) for human, rat and mouse incubations and M-180 was detected in control human incubations only, suggesting a contribution of non-NADPH dependent metabolic pathways to the formation of these metabolites. Comparing the metabolite profile across the three species (Table S2), hydroxylation at the adamantane ring to M+16 (I) and/or (II) are likely to represent important metabolic pathways in human, rat and mouse microsomes.

Table S2: Metabolite profiles for N205 observed in human, rat and mouse liver microsome incubations supplemented with NADPH.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Species | % Substrate Consumed | **Relative Peak Area**  (expressed as % total metabolite peak area generated over 60 minutes) | | | | | | | | | | | | | | |
| M+16 (I) | M+16 (II) | M+16  (III) | M+16 (IV) | M+16 (V) | M+32 (I) | M+32  (II) | M+32  (III) | M+32 (IV) | M+32 (V) | M-182\* | M-180\* | M-26 | M-10 (I) | M-10 (II) |
| **Human** | 63 | **46** | **26** | ND | ND | Trace | 9 | ND | ND | 1 | 1 | 2 | 8 | 1 | 3 | 2 |
| **Rat** | 82 | 14 | **44** | ND | 3 | 2 | 12 | ND | 9 | Trace | 2 | 2 | Trace | 5 | 1 | 5 |
| **Mouse** | 39 | **67** | 6 | 5 | Trace | 4 | 1 | 2 | ND | Trace | ND | 1 | 4 | 8 | 1 | Trace |

ND: not detected; Trace: <1% total metabolite peak area

\* M-182 was detected in control samples (without cofactor) for human, rat and mouse incubations; M-180 was detected in control samples for human only, suggesting a contribution of non-NADPH dependent metabolic pathways to the formation of these metabolites.

Refer to Table S1 for metabolite descriptions

|  |  |  |
| --- | --- | --- |
|  |  |  |
| M+16 (I), (II) & (III), M+32 (I) | M+16 (IV) & (V) |  |

|  |  |  |
| --- | --- | --- |
|  |  |  |
| M-182 | M-180 | M-26 |

Figure S1. Structures of putative metabolites for N205. Dotted line indicates proposed site of hydroxylation.