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Linear and cyclic dipeptides with antimalarial activity

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ABSTRACT

Several natural and synthetic polypeptides possess important antimalarial activity. Shorter peptides with potent antimalarial activity have also been described, among them linear di-, tri-, tetra- and pentapeptides and their cyclic analogs. In an attempt to find dipeptides with antimalarial activities we show that linear and cyclic dipeptides, the latter known as diketopiperazines, still retain the fundamental core to preserve antimalarial activity. Thirteen linear dipeptides and ten diketopiperazines were investigated. Eight linear dipeptides showed IC_{50} values between 2.78 and 7.07 μM, while eight diketopiperazines were also active with IC_{50} values between 2.26 and 4.26 μM on *Plasmodium berghei* schizont cultures.

Malaria is a parasitic disease expanding quickly around the world. An estimated 3.3 billion people were at risk of malaria in 2010 and about 216 million episodes of malaria occurred that year. About 655 thousand malaria deaths occurred in 2010, with 86% of them being children under five years of age.[1] In humans, malaria is caused by protozoan parasites belonging to five *Plasmodium* species: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*. The most serious infections among these species are caused by *P. falciparum*.[2,3] Uncomplicated *P. falciparum* malaria should be treated with an artemisinin-based combination therapy. *P. vivax* malaria should be treated with chloroquine or with artemisinin-based combination therapy when *P. vivax* resistance to chloroquine has been documented.[1] In recent years, the social impact of malaria has increased due to the lower abundance and high cost of artemisinin and related products, and also because of the emergence of resistant strains *of P. falciparum* and *P. vivax* to chloroquine, mefloquine and pyrimethamine.[4] The number of artemisinin-based combination treatment courses procured by the public sector increased greatly from 11.2 million in 2005 to 76 million in 2006, and reached from 181 million in 2010 to 287 million treatment courses in 2011. International funding for malaria control has continued to rise, to a peak of $2 billion USD in 2011.[1] For these reasons, there is an increasing need for new chemical pharmacophores which may prove effective therapeutic antimalarial agents.

Several antimalarial peptides have been isolated from natural sources and also obtained synthetically.[3,5–15] Linear antimalarial peptides with fifteen or more amino acid residues, such as leukinostatin A, efrapeptin, zervamicin and antiamoebin have been isolated. These fungal peptides kill *P. falciparum* in culture with IC_{50} in the micromolar range.[16] Also shorter peptides with similar range of potency have been described, among them linear di-, tri-, tetra- and pentapeptides and their cyclic analogs.[17–22]

Diketopiperazines are a class of cyclic dipeptides found in nature showing all advantages of cyclic peptides.[23] These compounds show reduction in conformational freedom and extra conformational restrictions, resulting in a wide spectrum of therapeutic activities such as antibacterial and antifungal,[24] antitumoral,[25] antiviral,[26] etc. Structurally, diketopiperazines have two acceptor–donator hydrogen bridge places, making it possible to have higher receptor binding affinities, showing high potential as specific targets to inhibit or activate binding sites of enzymes and proteins.[27] Some diketopiperazines have been described as antimalarial agent, such as 1-demethylhyalodendrin tetrasulfide (IC_{50} 2.5 μg/ml)[28] and brevicompanine B (IC_{50} 35 mg/ml) (Fig. 1).[29]

Two strategies for linear and cyclic peptides syntheses have been developed: solution and solid phase peptide syntheses. Both strategies needed protection, activation, coupling and deprotection reactions as key steps. Today, solid phase peptide synthesis is the best strategy for production of long peptides; however, linear

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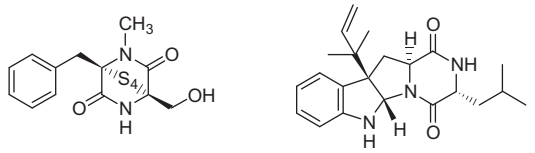


Figure 1. Antimalarial diketopiperazines 1-demethylhyalodendrin tetrasulfide and brevicompanine B.

dipeptides can be obtained using solution synthesis with excellent yields. On the other hand, the use of suitable reagents to carry out an effective activation of the C-terminus of the protected peptide and of suitable nucleophilic amines make the solution peptide synthesis also more convenient and quicker than solid phase peptide synthesis for production of cyclic peptides.[30]

Cyclization of dipeptides to diketopiperazines implies a *trans-cis* isomerization of the linear precursor, followed by nucleophilic attack of an amino group on the C-terminal carbonyl group.[31,32] Specifically, 2,5-diketopiperazine synthesis in solution could be carry out using five main strategies: intramolecular formation of N_1–C_2, intramolecular formation of N_1–C_6, tandem formation of N_1–C_2/C_3–N_4, tandem formation of N_1–C_2/N_4–C_5 and tandem formation of C_2–N_1–C_6, being the first one the most commonly employed method.[33]

In an attempt to find ultra-short peptides with antimalarial activities, we have previously shown that tetrapeptides with the sequence Xdd-Xcc-Phe-Orn have antiplasmodial activities against *Plasmodium berguei* (IC_{50} ranging between 2.5 and 3.3 μM).[34] In this Letter, we report the antiplasmodial activities of protected linear dipeptides **1**–**13** and their cyclic analogs, 2,5-diketopiperazines **14**–**23**, which were synthesized using intramolecular formation of N_1–C_2 strategy (Fig. 2).

We have previously reported the syntheses of dipeptides **1**–**3** and **5**–**13** and of diketopiperazines **14**–**16** and **18**–**23** (Fig. 3) and unambiguously assigned their NMR data.[35] Dipeptide **4** and diketopiperazine **17** were synthesized in aqueous solution using microwave-assisted methodology.[35]

The chemical structures for the newly synthesized dipeptide **4** and diketopiperazine **17** were confirmed on the basis of their spectral IR and NMR data.[36,37] Dipeptide **4** showed IR absorptions at 3337 (NH-amide stretching), 1709 (CO carbamate and ester) and 1675 (CO amide) cm^{-1}; ester and amide carbonyls and Cbz and Boc carbamate groups showed ^{13}C NMR resonance signals at δ 172.48, 172.33, 156.82 and 155.88, respectively. Valine residue was evidenced by methine and methyl groups signals at δH 4.49 (Hα), 2.13 (Hβ) and 0.92 and 0.88 (Hγ) at ^1H NMR, while ornithine fragment showed signals at δH 4.29 (Hα), 3.19 (Hδ) and 1.91–1.50 (Hβ and Hγ). Diketopiperazine **17** showed IR absorptions at 3347 (NH stretching), 1676 (CO amide), 1530 (*trans*-NH in plane), 1454 (*cis*-NH in plane) and 1344 (CN stretching amide) cm^{-1}. Carbonyl amides and Nδ-Cbz-carbamate groups showed ^{13}C NMR resonance signals at δ 170.89, 170.60 and 159.80. The ^1H NMR spectrum for compound **17** showed Hα-valine signal at δH 3.82 and Hα-lysine at δH 3.96. The structure of cyclic peptide **20** previously

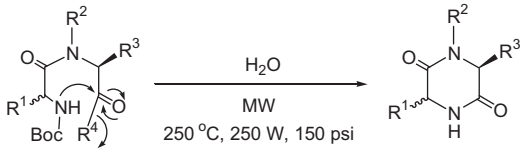
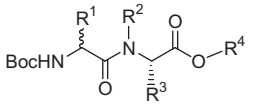
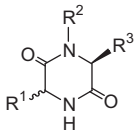


Figure 2. Intramolecular N_1–C_2 strategy for 2,5-diketopiperazines synthesis.



1 R^1=(*S*)-Pr-NHCbz; R^2=H; R^3=Bn; R^4=*t*Bu
2 R^1=(*S*)-Pr-NHCbz; R^2=H; R^3=Bn; R^4=Me
3 R^1=(*S*)-Pr-NHCbz; R^2=H; R^3=*i*Pr; R^4=*t*Bu
4 R^1=(*R*)-Pr-NHCbz; R^2=H; R^3=*i*Pr; R^4=Me
5 R^1=(*R*)-Pr-NHCbz; R^2=H; R^3=*i*Pr; R^4=*t*Bu
6 R^1=R^3=(*S*)-Bn; R^2=H; R^4=*t*Bu
7 R^1=(*S*)-Bn; R^2=H; R^3=*i*Pr; R^4=*t*Bu
8 R^1=(*S*)-Bn; R^2=CH_3; R^3=H; R^4=Me
9 R^1=(*S*)-Bn; R^2=CH_3; R^3=H; R^4=*t*Bu
10 R^1=R^3=(*S*)-*i*Pr; R^2=H; R^4=*t*Bu
11 R^1=(*S*)-*i*Pr; R^2=H; R^3=Bn; R^4=*t*Bu
12 R^1=R^2=H; R^3=*i*Pr; R^4=*t*Bu
13 R^1=R^2=H; R^3=Bn; R^4=*t*Bu



14 R^1=(*S*)-Pr-NHCbz; R^2=H; R^3=Bn
15 R^1=(*S*)-Pr-NHCbz; R^2=H; R^3=*i*Pr
16 R^1=(*R*)-Pr-NHCbz; R^2=H; R^3=*i*Pr
17 R^1=(*S*)-Bu-NHCbz; R^2=H; R^3=*i*Pr
18 R^1=R^3=(*S*)-Bn; R^2=H
19 R^1=(*S*)-*i*Pr; R^2=H; R^3=Bn
20 R^1=(*S*)-Bn; R^2=CH_3; R^3=H
21 R^1=R^3=(*S*)-*i*Pr; R^2=H
22 R^1=R^2=H; R^3=Bn
23 R^1=R^2=H; R^3=*i*Pr

Figure 3. Linear dipeptides **1**–**13** and diketopiperazines **14**–**23**.

synthesized[35] was proved unambiguously using a single crystal X-ray structure for the first time (colorless crystals, Fig. 4).[38]

Plasmodium berghei is a unicellular parasite that does not affect humans, therefore is commonly used as a model to look for antimalarial activity.[39] We used sarcosine (Sar) and ornithine (Orn) to investigate the effect of non-proteinogenic residues, phenylalanine and glycine for the aromatic effects, and lysine and valine for the aliphatic/hydrophobic effects of linear dipeptides and diketopiperazines on antimalarial activity. The chloroquine-sensitive strain ANKA 2.34 of *P. berghei* was used to test the antimalarial activity of dipeptides **1**–**23**. Table 1 shows IC_{50} values of peptides **1**–**13** and diketopiperazines **14**–**23** on *P. berghei* schizont cultures.[40]

The linear dipeptides possessing the non-proteinogenic Boc-Orn(*Z*) fragment at the N-terminal residue (compounds **1** and **2**) were found inactive to *P. berghei* (IC_{50} >200 μM). Compound **3** showed moderate antimalarial activity with an IC_{50} = 33.88 μM, however its analogues Boc-D-Orn(Z) compounds **4** and **5** were the most active dipeptides of this series with an IC_{50} = 3.05 and 2.78 μM, respectively. On the other hand, those series possessing the non-proteinogenic residue sarcosine, dipeptides **8** and **9** and diketopiperazine **20** showed also very good antimalarial activity with an IC_{50} = 3.63, 3.38 and 2.26 μM, respectively.

Four dipeptides with one aromatic amino acid (Phe derivatives) at the N-terminal residue were evaluated (compounds **6**–**9**). The first dipeptide corresponds to the replacement of Boc-Orn(Z) fragment on compound **1** by Boc-Phe residue, leading to the inactive dipeptide **6**. The second dipeptide corresponds to the same exchange in compound **3** to yield dipeptide **7**, which shows very good activity against *P. berghei* with an IC_{50} = 3.23 μM. In same series, introduction of Sar amino acid as initial residue yielded active dipeptides **8** and **9** (vide supra).

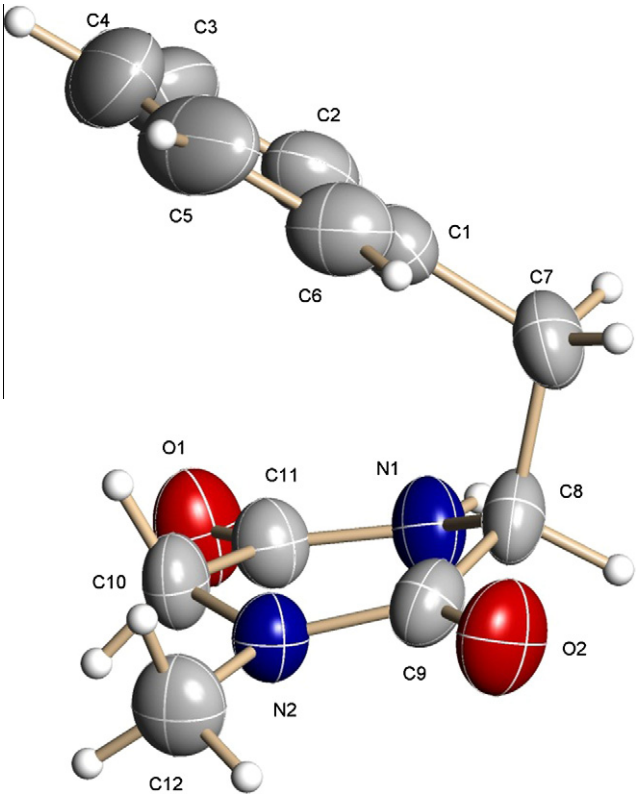


Figure 4. ORTEP drawing of diketopiperazine **20**.

Table 1
IC_{50} (μM) values of dipeptides **1**–**23** on *P. berghei* schizonts

Compound (200 μM)	IC_{50} (μM)
Boc-Orn(*Z*)-Phe-O^{t}Bu (**1**)[35]	>200
Boc-Orn(*Z*)-Phe-OMe (**2**)[35]	>200
Boc-Orn(*Z*)-Val-O^{t}Bu (**3**)[35]	33.88
Boc-D-Orn(*Z*)-Val-OMe (**4**)[36]	3.05
Boc-D-Orn(*Z*)-Val-O^{t}Bu (**5**)[35]	2.78
Boc-Phe–Phe-O^{t}Bu (**6**)[41]	112.20
Boc-Phe-Val-O^{t}Bu (**7**)[35]	3.23
Boc-Phe-Sar-OMe (**8**)[35]	3.63
Boc-Phe-Sar-O^{t}Bu (**9**)[35]	3.38
Boc-Val-Val-O^{t}Bu (**10**)[35]	2.81
Boc-Val-Phe-O^{t}Bu (**11**)[35]	7.07
Boc-Gly-Val-O^{t}Bu (**12**)[35]	3.16
Boc-Gly-Phe-O^{t}Bu (**13**)[35]	164.05
Cyclo[Phe-Orn(*Z*)] (**14**)[35]	123.02
Cyclo[Val-Orn(*Z*)] (**15**)[35]	66.02
Cyclo[Val-D-Orn(*Z*)] (**16**)[35]	3.09
Cyclo[Val-Lys(*Z*)] (**17**)[37]	3.12
Cyclo(Phe–Phe) (**18**)[42]	2.54
Cyclo(Val-Phe) (**19**)[43,44]	3.89
Cyclo(Sar-Phe) (**20**)[35]	2.26
Cyclo(Val-Val) (**21**)[35]	2.45
Cyclo(Phe-Gly) (**22**)[45]	4.26
Cyclo(Val-Gly) (**23**)[46,47]	3.23
Chloroquine	0.06

Linear aliphatic dipeptides including Boc-Val and Val-O^{t}Bu residues were active (compounds **10**–**12**, IC_{50} = 2.81, 7.07 and 3.16 μM, respectively); however, compound **13**, possessing Boc-Gly amino acid at the N-terminal residue and Phe-O^{t}Bu was inactive against the *P. berghei* schizont cultures.

According to IC_{50} values shown in Table 1, linear protected dipeptides possessing Phe as the initial amino acid residue are inactive (compounds **1**, **2**, **6** and **13**) or only moderately active (compound **11**). Hydrophobic amino acid such as Val, and non-proteinogenic amino acids such as Orn and Sar, are excellent residues to ensure antimalarial activity (compounds **4**, **5**, **7**–**10** and **12**). Dipeptide **7** showed excellent activity with an IC_{50} = 3.23 μM, however the inverse sequence dipeptide **11** has approximately half the activity of compound **7** with an IC_{50} = 7.07 μM. Finally, the nature of the protecting group at the initial amino acid residue (O^{t}Bu or OMe) seems not to be significant on the activity.

Diketopiperazines **14**–**23** were efficiently prepared in aqueous solution using microwave-assisted methodology as previously described.[35] In this manner, cyclization of dipeptides **1** (or **2**) and **3** afforded the inactive diketopiperazines **14** and **15**, respectively. On the other hand, dipeptides **4** (or **5**), **7** (or **11**), **8** (or **9**), **10** and **12** yielded diketopiperazines **16**, **19**, **20**, **21** and **23**, respectively, all of them showing practically the same activity than their respective open chain dipeptides with an IC_{50} = 3.09, 3.89, 2.26, 2.45 and 3.23 μM, respectively. Additionally, dipeptides **6** and **13** rendered diketopiperazines **18** and **22**, both increasing the activity largely with an IC_{50} = 2.54 and 4.26 μM, respectively. Finally, diketopiperazine **17** also showed important activity against *P. berghei* schizont with an IC_{50} = 3.12 μM (Table 1).

In both cases, linear dipeptides and diketopiperazines, the change of configuration of the Orn residue (L to D, compounds **3**–**5** and **15**–**16**) dramatically increased antimalarial activity.

The ability to suppress the development of *P. berghei* schizonts in culture of dipeptides **1**–**23** (chemosuppression) after 16 h of exposure are expressed as percentages in Table S1.[48] These data is in agreement with the IC_{50} value obtained for each compound, being **8**, **9**, **17**, **19**, **20** and **23** the most effective compounds reducing the schizont number.

In conclusion, twenty three compounds were screened to assess their in vitro antiprotozoal activity. Linear dipeptides **4**–**5** and **7**–**12** and their corresponding cyclic derivatives diketopiperazines **16**–**23** are active compounds against *P. berghei* schizont cultures. To our knowledge, these dipeptides are the simplest diketopiperazines described until today with only two stereogenic centers including the pharmacophore possessing antimalarial activity. These peptides are candidates for subsequent in vitro assays against *P. falciparum* and *P. vivax* and for antimalarial in vivo assays.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.09.094. These data include MOL files and InChiKeys of the most important compounds described in this article.

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36. Boc-D-Orn(Cbz)-Val-OMe (4). 88% yield; Colorless oil. IR (KBr, cm^{-1}): 3337, 2969, 2881, 1709, 1529, 1456, 1369, 1255, 1167, 1020, 865, 743, 699, 608. ^1H NMR (400 MHz, $CDCl_3$): δ 7.32–7.29 (5H, m, H_{Ar}), 7.16 (1H, br s, NH-Val), 5.51 (1H, br s, NH–Boc), 5.43 (1H, br s, NH-Cbz), 5.10 and 5.09 (1H each, d, J = 14.2 Hz, CH_2-Cbz), 4.49 (1H, dd, J = 8.1,5.1 Hz, Hα-Val), 4.29 (1H, m, Hα-Orn), 3.69 (3H, s, OMe), 3.19 (2H, m, Hδ-Orn), 2.13 (1H, m, Hβ-Val), 1.91–1.50 (4H, m, Hβ,Hγ-Orn), 1.42 (9H, s, CH_3-Boc), 0.92 (3H, d, J = 7.2 Hz, Hγ-Val), 0.88 (3H, d, J = 7.2 Hz, Hγ′-Val). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.48 (s, CO-Val), 172.33 (s, CO-Orn), 156.82 (s, CO-Cbz), 155.88 (s, CO-Boc), 136.69 (s, C_{Ar}), 128.49 (d, C_{Ar}), 128.06 (d, C_{Ar}), 80.09 (s, Boc), 66.55 (t, CH_2-Cbz), 57.21 (d, Cα-Val), 53.75 (d, Cα-Orn), 52.15 (q, OMe), 40.23 (t, Cδ-Orn), 31.08 (d, Cβ-Val), 29.96 (t, Cγ-Orn), 28.35 (q, CH_3-Boc), 26.19 (t, Cβ-Orn), 19.04 (q, Cγ-Val), 17.82 (q, Cγ-Val). FAB^+MS m/z: 480 (15) $[M+H]^+$, 380 (56), 227 (12), 132 (14), 91 (100) $[C_7H_8]^+$, 72 (22), 57 (26). HRMS (FAB^+): m/z 480.2759 (Calcd for $C_{24}H_{38}N_3O_7$, 480.2710).
37. 37. Cyclo[Val-Lys(Cbz)] (17). 50% yield; white powder; IR (KBr, cm^{-1}): 3347, 3206, 3093, 3058, 2962, 2936, 2874, 1676, 1530, 1454, 1344, 1251, 1139, 1045, 836, 739, 697. ^1H NMR (400 MHz, CD_3OD): δ 7.47–7.28 (5H, m, H_{Ar}), 5.06 (2H, s, CH_2-Cbz), 3.96 (1H, m, Hα-Lys), 3.82 (1H, dd, J = 4.0,1.2 Hz, Hα-Val), 3.13 (2H, t, J = 6.8 Hz, Hε-Lys), 2.26 (1H, m, Hβ-Val), 1.83 (2H, m, Hβ-Lys), 1.53 (2H, m, Hδ-Lys), 1.44 (2H, m, Hγ-Lys), 1.04 (3H, d, J = 7.2 Hz, Hγ-Val), 0.94 (3H, d, J = 7.2 Hz, Hγ′-Val). ^{13}C NMR (100 MHz, CD_3OD): δ 170.89 (s, CO-Lys), 170.6 (s, CO-Val), 159.8 (s, CO-Cbz), 138.8 (s, C_{Ar}), 129.62 (d, C_{Ar}), 129.10 (d, C_{Ar}), 128.94 (d, C_{Ar}), 67.48 (t, CH_2-Cbz), 61.51 (d, Cα-Val), 55.97 (d, Cα-Lys), 41.60 (t, Cε-Lys), 35.43 (t, Cβ-Lys), 33.51 (d, Cβ-Val), 30.65 (t, Cδ-Lys), 23.54 (Cγ-Lys), 19.40 (q, Cγ-Val), 17.78 (q, Cγ′-Val). FAB^+MS m/z: 384 (78) $[M+Na]^+$, 362 (100) $[M+H]^+$, 338 (21), 318 (42), 254 (55). HRMS (FAB^+): m/z 362.2106 $[M+H]^+$ (Calcd for $C_{19}H_{27}N_3O_4$ 362.2080).
38. CCDC 863172 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via http://www.ccdc.cam.ac.uk/data_request/cif. The X-ray crystal structure for compound **20** shows the aromatic ring folded on diketopiperazine ring, in a boat conformation, with an important interaction of diketopiperazine and aromatic rings, probably due to a dipole-dipole interaction between both amide groups and the π electrons of the aromatic ring. Evidence of this folding was observed also in solution at ^1H NMR, where only one of the Hα sarcosine methylene protons was observed upfield [δ 3.48 (Hα) vs 2.79 (Hα′)].
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40. Chloroquine-sensitive cultured plasmodium berghei schizonts (ANKA 2.34) were used to assess the antimalarial activity of compounds **1**–**23**. Peptides **1**–**23** were dissolved in PBS containing DMSO 0.1% and 5% ethanol. Parasites were incubated for 16 h at 37 °C. Chloroquine (C6628 sigma chloroquine diphosphate salt solid, ⩾98% purity) was used as positive control. Samples of 0.5 mL of each culture were taken to prepare smears and used to count numbers of schizonts in 2000 erythrocytes. The IC_{50} values of each compound were determined using concentrations of 5, 50, 100, and 200 μM and by extrapolation from the concentration response curve. The IC50 value represents the drug concentration producing a 50% reduction in the number of P. berghei schizonts (compared to drug-free control cultures). For each assay, each drug dilution was analyzed in duplicate, and the results were averaged in each case.
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48. see Supplementary data.