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# Prolonged Survival of a Murine Model of Cerebral Malaria by Kynurenine Pathway Inhibition

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**C57BL/6J mice infected with *Plasmodium berghei* ANKA develop neurological dysfunction and die within 7 days of infection. We show that treatment of infected mice with a kynurenine-3-hydroxylase inhibitor prevents them from developing neurological symptoms and extends their life span threefold until severe anemia develops.**

One possible cause of death associated with cerebral malaria is an imbalance in the production of neurotoxic and neuroprotective factors brought about by parasite-triggered cerebral inflammation (7, 13, 14). A candidate mechanism in this process is the kynurenine pathway from tryptophan, which is activated in macrophages and microglia by inflammatory stimuli and which generates excitotoxic and neuroprotectant metabolites (1, 8). The compound 3,4-dimethoxy-*N*-[4-(3-nitrophenyl)thiazol-2-yl]benzenesulfonamide (Ro-61-8048; synthesized by F. Hoffmann-La Roche Ltd.) has been developed as a high-affinity inhibitor of kynurenine-3-hydroxylase (16) (Fig. 1). It has been shown to reduce the formation of quinolinic acid (5), a known excitant and neurotoxin acting through *N*-methyl-D-aspartate (NMDA) receptors (19), and increase the formation of the neuroprotective glutamate antagonist kynurenic acid (5, 19) in the brains of mice immune stimulated with pokeweed mitogen. In order to assess the importance of kynurenines in the morbidity and mortality associated with cerebral malaria, we tested the effect of Ro-61-8048 treatment in a well-characterized murine model of this condition. It has been commented that in mice, mononuclear leukocytes are the predominant cell type sequestered in the cerebral microvasculature, and in humans, parasitized red blood cells are prominent (10). However, recent work has shown that leukocytes are seen clearly in brain vessels in human cerebral malaria in adults and parasitized red blood cells are sequestered in the brain in the murine disease (10). Also, cerebral malaria is a syndrome in which the same altered cell (the endothelial cell) plays a pivotal role in both human and experimental lesions (10).

C57BL/6J mice infected with asexual blood stages of *Plasmodium berghei* ANKA have been shown to develop neurological behavioral changes such as ataxia, convulsions, and coma at approximately day 5 after infection and usually die between days 6 and 8 postinfection (15). Similarly, in our study, all 10 mice infected with *P. berghei* ANKA (6) and receiving only the solvent in which Ro-61-8048 was dissolved (vehicle, 0.9% NaCl–60 mM NaOH, pH adjusted to 7.5) exhibited signs of cerebral involvement on day 6 postinfection, with reduced locomotion and marked ataxia. All the mice in this group died or were euthanized to avoid unnecessary suffering between days 7 and 9 postinfection. In contrast, none of nine infected mice treated intraperitoneally with 200 mg/kg Ro-61-8048 (days 1, 2, 3, 4, 6, 8, 10, and 12 postinfection) showed any signs of cerebral dysfunction throughout the duration of the experiment, and all were surviving on day 21 postinfection ($P = 0.0002$, Fisher's exact test), when the experiment was terminated. This significantly prolonged survival was not due to any change in the number of *P. berghei* ANKA parasites, as mice treated with Ro-61-8048 exhibited parasitemias (6) (1.4% ± 0.3%, 5.4% ± 0.6%, 10.6% ± 1.3%, and 15.8% ± 3.2%) that were not significantly different from vehicle-treated mice (2.1% ± 0.4%, 6.6% ± 0.5%, 12.2% ± 1.0%, and 19.5% ± 1.1%) on days 4, 5, 6, and 7 postinfection, respectively ($n = 5$; unpaired $t$ test [two tailed] with Welch correction where required). On day 20 postinfection, the Ro-61-8048-treated mice displayed severe anemia and overwhelming parasitemia (54.5% ± 5.3%; $n = 6$), which led us to terminate the experiment. These mice were found to exhibit pronounced macroscopic signs of anemia, such as pale mucous membranes, relatively scanty red blood cells on thin blood smears, and reticulocytosis well above normal.

We then compared the concentrations of several kynurenines on day 6 postinfection in the brains of *P. berghei* ANKA-infected and control mice receiving Ro-61-8048 or vehicle. Isocratic reversed-phase high-performance liquid chromatography was performed with fluorescence detection (20). Anthranilic acid was determined at an excitation wavelength of 313 nm and an emission wavelength of 420 nm. Kynurenic acid was detected by changing the wavelengths after 20 min to excitation at 344 nm and emission at 390 nm. The electron capture negative-ion gas chromatography-mass spectrometry method was used as previously described (18). We found that *P. berghei* ANKA infection resulted in a significantly increased level of picolinic acid compared with control mice (Table 1).

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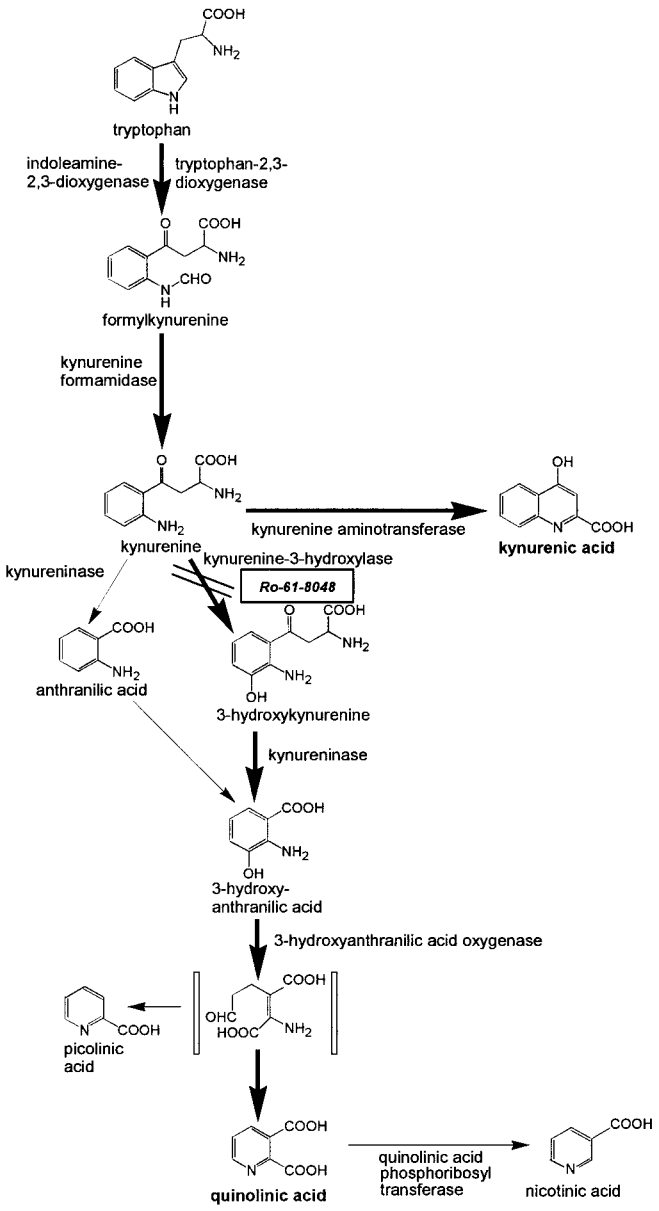


FIG. 1. Schematic diagram of the kynurenine pathway illustrating the site of action of Ro-61-8048. The conversion from tryptophan to formylkynurenine is performed by tryptophan-2,3-dioxygenase in the liver and by indoleamine-2,3-dioxygenase in most other tissues.

However, infected mice treated with Ro-61-8048 exhibited a level of picolinic acid similar to control mice (Table 1). Ro-61-8048 treatment of infected mice significantly increased their levels of kynurenic acid and anthranilic acid (Table 1). Indeed, the kynurenic acid to quinolinic acid ratio (i.e., of the neuroprotective kynurenic acid in relation to the excitotoxin) was approximately 10-fold higher in infected mice treated with Ro-61-8048 ($0.230 \pm 0.066$; $n = 4$; $P < 0.005$, unpaired $t$ test [two tailed]) compared with vehicle-treated infected mice ($0.021 \pm 0.005$; $n = 6$). Anthranilic acid has also been found to produce dose-related antagonism of the neurotoxicity associated with quinolinic acid (12). Although we did not observe an increased brain level of quinolinic acid in *P. berghei* ANKA-infected mice compared with controls as reported by Sanni et al. (17), which may be due to methodological differences, we detected a highly significant rise in the levels of its antagonists in infected mice treated with Ro-61-8048. It is well established that inflammation, either peripheral or central, is associated with an increased sensitivity of NMDA receptors (9). As murine cerebral malaria is an encephalitis (11), there will be increased activation of NMDA receptors, potentially leading to neuronal damage (19) and contributing to the neurological symptoms and death associated with the disease. The increased levels of kynurenic acid and anthranilic acid in Ro-61-8048-treated infected mice would protect against the activation of NMDA receptors and could play a significant role in the reduced symptomatology and mortality.

As shown in Table 1, we observed a 3.5-fold increase in the brain level of picolinic acid in *P. berghei* ANKA-infected mice on day 6 postinfection compared with controls, and treatment of infected mice with Ro-61-8048 resulted in a level of picolinic acid similar to controls. Picolinic acid has been shown to induce macrophage inflammatory protein 1α (MIP-1α) and MIP-1β mRNA expression selectively in mouse macrophages and not a range of other chemokines (2). It has recently been reported that deletion of MIP-1α expression in Sandhoff disease mice resulted in a substantial decrease in monocyte infiltration and macrophage- or microglia-associated pathology together with neuronal apoptosis, and these mice showed improved neurologic status and a longer life span (21). These results raise the possibility that MIP-1α has a key role in the pathogenesis of diseases associated with macrophage- or microglia-mediated inflammation, such as cerebral malaria. MIP-1α mRNA expression has been reported in peritoneal exudate neutrophils and macrophages from *P. berghei* ANKA-infected mice, while no expression was found in control mice

TABLE 1. Quantification of the levels of kynurenines and MIP-1α on day 6 postinfection in the brains of *P. berghei* ANKA-infected and control mice receiving Ro-61-8048 or vehicle[a]

| Treatment | Picolinic acid | Kynurenic acid | Anthranilic acid | Quinolinic acid | MIP-1α |
|---|---|---|---|---|---|
| Control + vehicle | 309 ± 49 | 1.3 ± 0.5 | 2.0 ± 0.3 | 144 ± 34 | 0.58 ± 0.06 |
| Control + Ro-61-8048 | 265 ± 32 | 10.2 ± 1.5 | 76.9 ± 23.3 | 150 ± 32 | 0.57 ± 0.09 |
| *P. berghei* ANKA + vehicle | 1,077 ± 265[b] | 3.5 ± 0.9 | 2.7 ± 0.6 | 186 ± 28 | 4.11 ± 0.18[c] |
| *P. berghei* ANKA + Ro-61-8048 | 300 ± 72 | 35.6 ± 6.0[d] | 487 ± 96[d] | 170 ± 29 | 2.26 ± 0.28[d] |

[a] The kynurenine values are expressed in fmol/mg (wet weight) and are mean ± SEM of five or six mice in each group. The MIP-1α values are expressed in pg/mg protein and are mean ± SEM of between six and nine mice in each group. Statistical comparisons were made using one-way analysis of variance with the Tukey-Kramer multiple-comparison post test.

[b] $P < 0.01$ versus control plus vehicle, control plus Ro-61-8048, and *P. berghei* ANKA plus Ro-61-8048.

[c] $P < 0.001$ versus control plus vehicle and control plus Ro-61-8048.

[d] $P < 0.001$ versus control plus vehicle, control plus Ro-61-8048, and *P. berghei* ANKA plus vehicle.

(4). We hypothesized that the high brain level of picolinic acid, via its induction of MIP-1α (2), may play a significant role in the neurological symptoms and death associated with *P. berghei* ANKA infection in mice. We tested this hypothesis by measuring the level of MIP-1α on day 6 postinfection in the brains of *P. berghei* ANKA-infected and control mice receiving Ro-61-8048 or vehicle using a mouse-specific sandwich enzyme immunoassay (R&D Systems Europe Ltd.). Total protein content was analyzed by the Bio-Rad protein assay, based on the method of Bradford (3). We found *P. berghei* ANKA infection resulted in a significantly increased level of MIP-1α compared with control mice (Table 1). Infected mice treated with Ro-61-8048 also exhibited a significantly increased level of MIP-1α compared with control mice, but this level was significantly lower than the level in vehicle-treated infected mice (Table 1).

In conclusion, our findings suggest that the reduced symptomatology and mortality associated with Ro-61-8048-treated *P. berghei* ANKA-infected mice may be a consequence of increased brain levels of kynurenic acid and anthranilic acid and/or lower brain levels of picolinic acid and MIP-1α. As the level of picolinic acid has been reported to be raised in the cerebrospinal fluid of humans with cerebral malaria (13, 14), our findings suggest that compounds which inhibit the kynurenine pathway may potentially be effective in the treatment and prevention of human cerebral malaria and may also be useful in other neurodegenerative diseases which involve an inflammatory component.

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