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# QUINOLINE-2,4-DICARBOXYLIC ACIDS: SYNTHESIS AND EVALUATION AS INHIBITORS OF THE GLUTAMATE VESICULAR TRANSPORT SYSTEM

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**Abstract**: Twenty-six quinoline-2,4-dicarboxylic acids (QDC's) were synthesized by a modified Doebner-von Miller pathway and tested as inhibitors against the glutamate vesicular transport (GVT) protein. The QDC's were active as inhibitors with the most potent QDC's found to contain halogens at the 6-/8-position, a hydroxyl at the 8-position, or a tethered aromatic moiety at the 6- or 7-position of the quinoline. 

## Introduction

Glutamate-mediated excitatory neurotransmission has been intensely studied over the past two decades and significant progress in delineating the participating receptors and transporters has been advanced through a combination of chemical, molecular, and pharmacological approaches.[1] In this regard, the development of conformationally constrained analogues of glutamate has proven to be a particularly effective strategy for identifying the selective agonists or antagonists used to define the five major classes of glutamate receptors (NMDA, KA, AMPA, ACPD, and AP4), as well as selective inhibitors of the cellular transport systems.[2] Surprisingly, however, much less is known about the glutamate vesicular transporter (GVT), the protein(s) responsible for mediating the uptake of L-glutamate into synaptic vesicle.[3] The energetics of vesicular transport has been studied in detail,[3b] yet very few competitive inhibitors have been identified.[3de] The identification of novel GVT inhibitors could provide essential diagnostic tools for defining the structural requirements for binding and translocation by this transport system and to probe a critical step in glutamate-mediated excitatory neurotransmission.

To date, only a very limited number of GVT inhibitors have been identified principally naphthalene disulfonic acid azo-dyes[4] and quinoline-2-carboxylic acids (QCA's),[5] which differ in their resemblance to glutamate (Figure 1), their inhibitory strength (the azo-dyes being 100- to 1000-fold more potent), and cross-reactivity/selectivity. The azo dyes and QCA's both contain a possible embedded glutamate diacid mimic (Figure 1; bold outline) within their structures that may partially explain their activity at the GVT. Alternatively, our prior report suggested[5] that the carbocyclic ring of a QCA may be an isostere for the distal carboxylate of glutamate.

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With these known inhibitors as reference points, we selected quinoline-2,4-dicarboxylic acids (QDC's) as candidates for development as selective inhibitors of the GVT. QDC's were selected owing to: (1) their structural

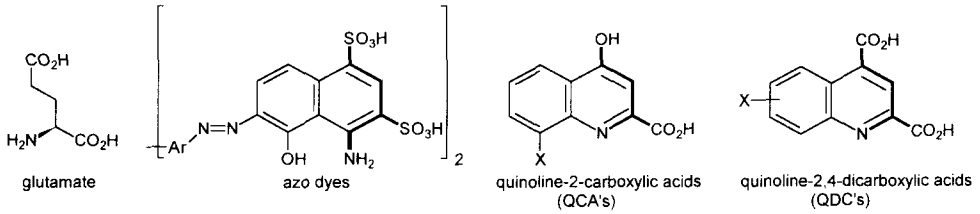
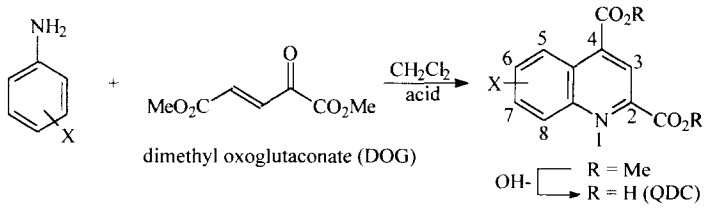



**Figure 1.** Structures of L-glutamate, azo-dye and QCA inhibitors and QDC's (proposed targets).

resemblance to QCA's, (2) an embedded glutamate structure, (3) potential for selectivity at the GVT,[6] and (4) the relative ease of synthesis[7] and installation of various substituent patterns. With respect to point 3, the QDC structure represents a conformationally-restricted, "W-conformation" of glutamate (Figure 1) in which the carboxylates are coplanar (as are the two sulfonic acid groups of certain azo dyes). This conformation is distinct from those proposed for the inhibition of the glutamate ionotropic receptors.[2]

## Results and Discussion

**Synthesis**: Synthesis of the QDC's was accomplished by expanding upon the method reported by Corey and Tramontano.[7] Dimethyl-2-oxoglutaconate (DOG)[7] was reacted with substituted anilines ($CH_2Cl_2$; $H^+$) to afford carbocyclic-ring substituted, dimethyl quinoline-2,4-dicarboxylates (Eq. 1; Table 1) in a variation of the Doebner-von Miller synthesis.[8] The synthetic process includes a Michael addition, cyclization, dehydration and oxidative-aromatization which required careful attenuation of the reaction conditions for each substituent type. Most important, the Michael addition occurs readily but a catalytic amount of acid is needed to promote the cyclization step. Various acids promote the synthesis (HCl, TFA, $H_3PO_4$, TsOH, $BF_3{\bullet}Et_2O$, etc.) and the addition of oxidants ($O_2$, $Cr^{VI}$, etc.) did not increase the QDC diester yield.




(1)

Some trends in the QDC synthesis were noted. As expected, strong electron donor groups (X = OMe, OH) facilitate the reaction (enhancing Michael addition and cyclization), whereas electron withdrawers (X = F) decrease product yield. When the aniline is meta substituted two regioisomers are possible. When $X_{(meta)}$ = halogen, OH, 5-

substituted QDC's result whereas when $X_{(meta)}$ = MeO, BnO predominantly 7-substituted QDC's are isolated. When substitution of the aniline can lead to a 5-substituent (Entries 18 and 19), the reaction is very sluggish, likely the result of steric interaction with the 4-carboxylate group.

**Table 1. Inhibition of the GVT by Quinoline-2,4-Dicarboxylic Acids (Eqn. 1)**

| Entry | X | Uptake of $^3$H-L-Glu (% of Control) | Entry | X | Uptake of $^3$H-L-Glu (% of Control) |
|---|---|---|---|---|---|
| 1 | H | 38 ± 4 (4) | 17 | 5-F, 6-$OCH_3$ | 39 ± 3 (4) |
| 2 | 5-OH | 74 ± 4 (4) | 18 | 5,7-di-$OCH_3$ | 15 ± 2 (6) |
| 3 | 5-F | 54 ± 6 (6) | 19 | 5,8-di-$OCH_3$ | 62 ± 3 (6) |
| 4 | 5-Br | 11 ± 2 (4) | 20 | 6,7-di-$OCH_3$ | 58 ± 4 (8) |
| 5 | 6-$OCH_3$ | 51 ± 2 (6) | 21 | 6,7-[-$OCH_2O$-] | 24 ± 1 (4) |
| 6 | 6-Cl | 1 ± 1 (6) | 22 | 6,7-[-$OCH_2CH_2O$-] | 12 ± 2 (4) |
| 7 | 6-Br | 2 ± 1 (8) | 23 | 6,8-di-$OCH_3$ | 40 ± 4 (8) |
| 8 | 6-I | 10 ± 1 (4) | 24 | 6-$OCH_3$, 8-$NO_2$ | 23 ± 2 (6) |
| 9 | 6-$OCH_2$Ph | 3 ± 1 (6) | 25 | 6,8-di-Br | 2 ± 1 (6) |
| 10 | 6-N=N-Ph | 0 ± 0 (4) | 26 | 7,8-di-Cl | 2 ± 1 (4) |
| 11 | 7-$OCH_3$ | 48 ± 3 (8) | 27 | L-Glutamate | 29 ± 1 (8) |
| 12 | 7-$OCH_2$Ph | 1 ± 1 (5) | 28 | Quinoline | 56 ± 4 (5) |
| 13 | 8-$OCH_3$ | 20 ± 2 (6) | 29 | Quinaldate | 34 ± 3 (4) |
| 14 | 8-OH | 2 ± 1 (6) | 30 | Kynurenate | 11 ± 1 (15) |
| 15 | 8-F | 39 ± 2 (4) | 31 | Xanthurenate | 4 ± 1 (12) |
| 16 | 8-Br | 7 ± 1 (6) | 32 | 2,4-Pyridine dicarboxylate | 92 ± 4 (12) |

GVT activity was determined using 250 $\mu$M [glutamate] in the presence of 5 mM [inhibitor].[9] Values are reported as mean % ± SEM (n) of Control uptake (≈1500 ± 110 pmol/min/mg protein)

The QDC dimethyl esters were hydrolyzed (LiOH or NaOH) and neutralized (HCl) to precipitate the product QDC's in near quantitative yield.[8] As expected, the hydrochloride salt of the QDC's could not be prepared. Twenty-six QDC's were prepared that have tested the breadth of the synthesis and in effect, established preliminary patterns for increased potency at the GVT.

**Biological Results**: The inhibitory activities of the carbocyclic-substituted QDC's at the GVT were determined by quantifying the ability of the analogues to block the uptake of $^3$H-L-glutamate (250 $\mu$M) into synaptic vesicles isolated from rat forebrain.[10] As this was the first screen for such activity, the compounds were included in the assays at a 20-fold excess (i.e., 5 mM). The results of these assays, which are reported as % of Control (uptake in the absence of any inhibitor), are summarized in Table 1. For comparative purposes, glutamate, as well as a few previously characterized quinoline monocarboxylates[5] have also been included in Table 1. All the QDC's displayed at least some inhibitory activity (0% indicates complete inhibition) toward the GVT. The unsubstituted QDC (X = H; entry 1) showed modest activity as an inhibitor and the effect of substituents can be preliminarily assessed relative to this parent compound. In general, the presence of a halogen led to increased inhibition with the lone exception of fluorine. Substitution of a halogen at C-6 or C-8 led to a noteworthy increase in potency (entries 6, 7, 8 and 16). Substitution of methoxy or hydroxy groups did not in general afford greater inhibition, although 8-hydroxy QDC was a very potent inhibitor (entry 14). Correspondingly, since kynurenic acid (entry 30; Figure 1, QCA; X = H) is a less potent inhibitor of the GVT than xanthurenic acid (entry 31; Figure 1, QCA; X = OH), the addition of a hydroxy group at C-8 (entry 14) is clearly important. Two cyclic, electron-donor substituted QDC's were prepared, the 6,7-dioxolane (entry 21) and 6,7-dioxane (entry 22) QDC's. Surprisingly, both of these compounds showed greater inhibition of the GVT than the corresponding 6,7-dimethoxy QDC (entry 20). Moreover, entry 22 (dioxane) is significantly more potent than entry 21, indicating a tolerance for larger groups or perhaps the need or ability to accommodate an additional ring. Although simple electron donors at C-7 tend to reduce activity, C-6 halogens improved inhibition. Since the NMDA receptor (glycine site) prefers quinoline-type inhibitors with substituents in the 5,7- positions (e.g., 5,7-dichloro),[11] our results suggest that further elaboration of a QDC analog panel could possibly afford selectivity between the GVT and NMDA sites.

Certain QDC's were prepared with a tethered aromatic group (entries 9, 10 and 12). Entry 10 was prepared specifically to mimic the linker group of the azo dye inhibitors. All of these structures potently inhibited the GVT. The 7-benzyloxy QDC and 6-azophenyl QDC completely inhibited uptake. In sum, inhibition of glutamate uptake by these three compounds would be consistent with the presence of a lipophilic binding pocket in the GVT or neighboring environment. This premise is supported, in part, because the two most potent GVT inhibitors to date are azo dyes[4] that contain a biphenyl linker group (e.g., Chicago Sky Blue and Evans Blue).

To confirm that the observed inhibitory activity is competitive, a more detailed kinetic analysis was carried out with 6-benzyloxy-QDC (entry 9). A representative Lineweaver–Burk plot and replot of $K_{m,app}$ vs inhibitor concentration (inset) for 6-benzyloxy-QDC are depicted in Figure 2. The pattern of inhibition observed in these analyses, as well as in previous studies with kynurenate, xanthurenate, and quinoline-2-carboxylate,[5] is consistent with competitive inhibition. The $K_m$ and $V_{max}$ values for the transport of L-glutamate were found to be 1.65 ± 0.3 mM and 18.3 ± 2.2 pmol/min/mg protein (n = 7) in agreement with previously reported values.[10a,12] The $K_i$ for 6-benzyloxy-QDC reported as the mean ± sem (n = 3), was 204 ± 66 μM.

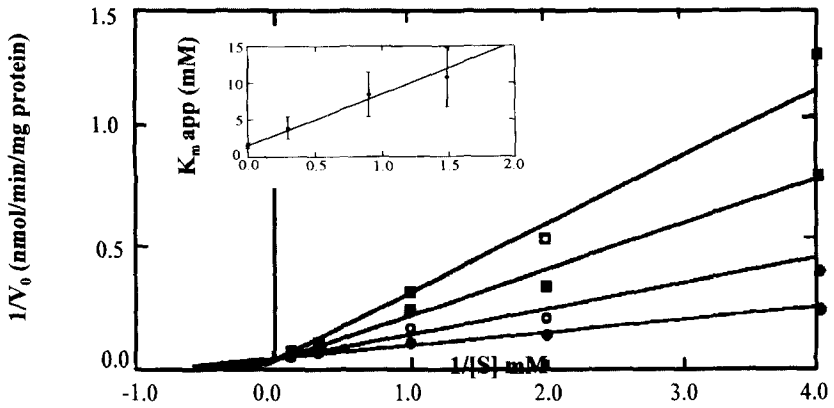



**Figure 2.** Representative Lineweaver–Burk plot of a single experiment demonstrating competitive inhibition by 6-benzyloxy-QDC on the uptake of $^3$H-L-glutamate (0.25–8.0 mM) into rat brain synaptic vesicles. The above plots yielded a control $V_{max}$ =21.6 ± 3.2 nmol/min/mg protein and $K_m$ = 1.5 ± 0.4 mM. Inset shows a replot of $K_m$ apparent vs. 6-benzyloxy-QDC concentrations producing a representative $K_i$ = 213 ± 24 mM (n = 1).

## Summary

Our results indicate that QDC's are an excellent template upon which to build potent inhibitors of the GVT and to aid delineation of the GVT pharmacophore. Highly interesting was the finding that addition of an 8-OH group to the QDC template significantly reduces glutamate uptake (as does the identical addition to kynurenic acid to form the more potent xanthurenic acid). We are also currently investigating in detail the finding that QDC's bearing halogen and lipophilic substituent groups exhibit markedly greater inhibitory activity at the GVT.

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8. A representative synthesis is as follows: a substituted aniline (1.0 mmol) was added to a solution of dimethyl oxoglutaconate (1.2 mmol) in $CH_2Cl_2$ (10 mL). A catalytic amount of acid was added (approx. 0.01–0.1 mmol) and the reaction was stirred at rt for 24 h (reflux is employed with TsOH as the acid), more acid was added (0.3–3.0 mmol) and the reaction monitored by TLC for the production of QDC diester (typically 6–48 h). The $CH_2Cl_2$ was washed with sat $NaHCO_3$ (2 x 10 mL), 0.1 M HCl (1 x 10 mL), water (1 x 10 mL), separated and dried over anhydrous $Na_2SO_4$. The solution was concentrated and the crude product was chromatographed on silica gel using EtOAc/hex. Yields of dimethyl quinoline-2,4-dicarboxylates ranged from 5–50%. QDC diesters (1.0 mmol) were dissolved in THF (10 mL), water added (5 mL) and 0.5 mL of 6 M NaOH (or LiOH) added with stirring. When the reaction was complete (typically 6–48 h), the THF/water was removed by rotary evaporation, the semi-solid redissolved in water (2–3 mL), the solution chilled, HCl (concd) added to precipitate the product, and the QDC isolated by centrifugation (2000 x *g*). All QDC's reported in Table 1 had satisfactory spectral and elemental or mass spectral analyses. Details to be reported elsewhere.
9. Synaptic vesicles were isolated and assayed for transport using the method of Kish and Ueda (ref 10b) in a pH 7.4 solution containing 5 mM HEPES, 2 mM ATP, 4 mM KCl, 4 mM $MgCl_2$ and $^3$H-L-glutamate (0.25–8 mM). Assays were initiated by the addition of $^3$H-L-glutamate ± inhibitors (0.01–5 mM) to the synaptic vesicles (total vol. 100 $\mu$L @ 0.1 mg pro). Uptake proceeded at 30 °C for 1.5 min, after which the vesicles were collected by vacuum filtration through Millipore HAWP filters. The filters were rinsed twice and the retained radioactivity was quantified by liquid scintillation counting. Non-specific uptake was determined in the absence of ATP. Initial assays confirmed uptake was linear with time and protein. Lineweaver–Burk plots and associated kinetic analysis of the transport inhibitors were carried out using $k_{cat}$ kinetic program (BioMetallics Inc.) with weighting based on constant relative error. $K_i$ values were estimated on the basis of a replot of $Km_{app}$ values.
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