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Inhibition of Long Chain Fatty Acyl-CoA Synthetase (ACSL) and Ischemia Reperfusion Injury

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Keywords: Hydroxytriazene, Ischemia reperfusion injury; Long chain fatty acyl-CoA synthetase
(ACSL) inhibitors; Oxadiazolidine dione, Triacsin C.

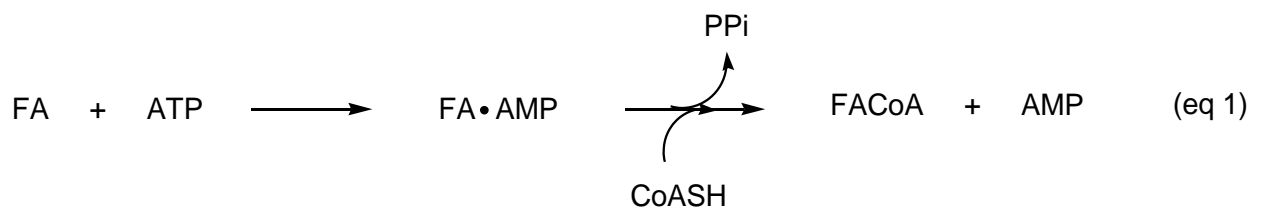
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ABSTRACT

Various triacsin C analogs, containing different alkenyl chains and carboxylic acid
bioisoteres including 4-aminobenzoic acid, isothiazolidine dioxide, hydroxylamine,
hydroxytriazene, and oxadiazolidine dione, were synthesized and their inhibitions of long chain

fatty acyl-CoA synthetase (ACSL) were examined. Two methods, a cell-based assay of ACSL activity and an *in situ* [¹⁴C]-palmitate incorporation into extractable lipids were used to study the inhibition. Using an *in vivo* leukocyte recruitment inhibition protocol, the translocation of one or more cell adhesion molecules from the cytoplasm to the plasma membrane on either the endothelium or leukocyte or both was inhibited by inhibitors **1**, **9**, and triacsin C. The results suggest that inhibition of ACSL may attenuate the vascular inflammatory component associated with ischemia reperfusion injury and lead to a decrease of infarct expansion.

Ischemia reperfusion (I/R) injury is a cascade of various cellular and molecular events, including impaired nitric oxide (NO) release and rapid translocation of cell adhesion molecules from the cytoplasmic compartment to the plasma membrane.¹ Together, these events initiate leukocyte recruitment and subsequent inflammatory injury. Long chain fatty acyl CoA synthetase (ACSL or LC-FACS) catalyzes the formation of a thioester between a coenzyme A (CoA) and a fatty acid (FA) by a two-step process as shown in equation 1.²



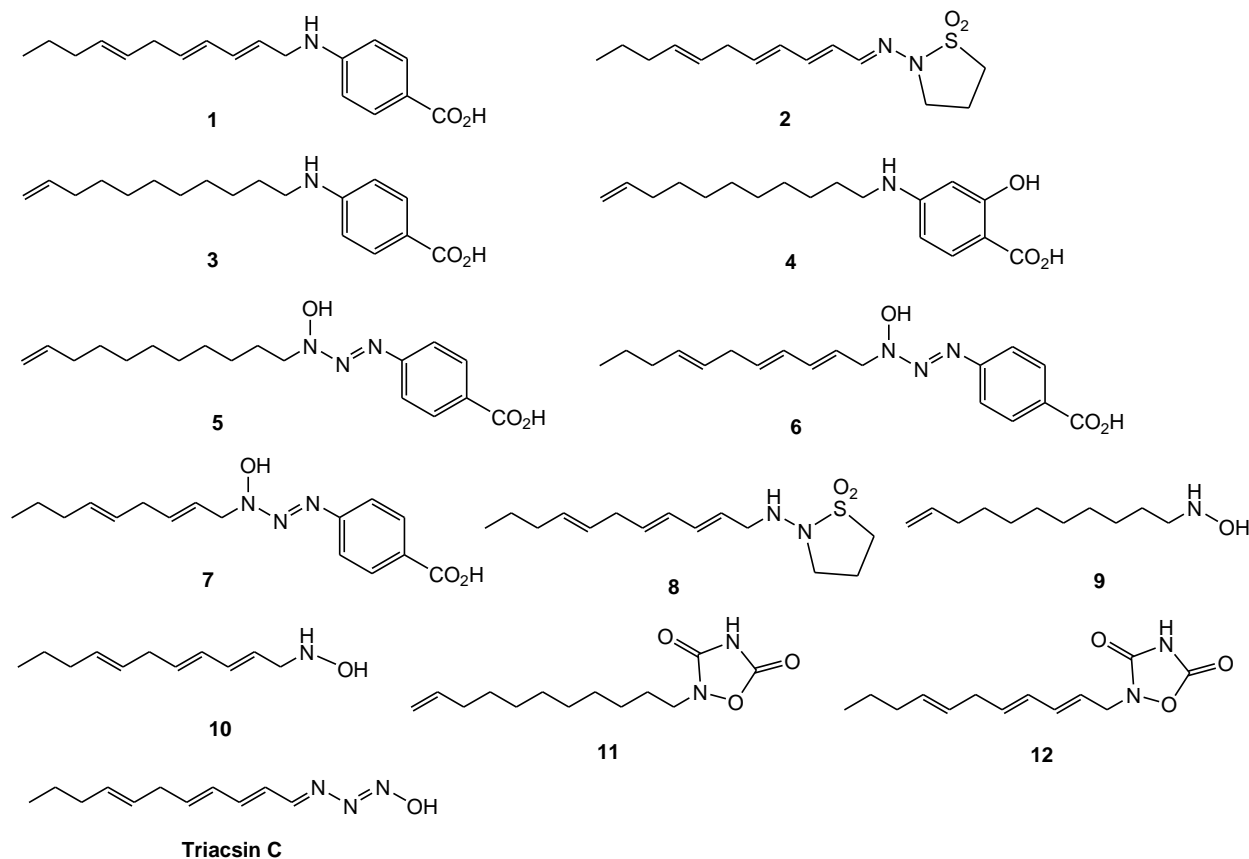
Protein palmitoylation occurs when the fatty acid moiety is transferred to a free SH group on a cysteine residue of CoA. While fatty acyl CoA (FACoA) transferase has been described, the process is also thought to occur spontaneously requiring seconds to complete.³ Endothelial

nitric oxide synthase (eNOS) generates endothelial NO. Its activity is controlled by palmitoylation as well as by protein-protein interaction and phosphorylation.⁴ The function of cell adhesion molecules necessary for neutrophil infiltration of ischemic tissue, such as *p*-selectin, integrins, intercellular adhesion molecule (ICAM), and platelet endothelial cell adhesion molecule (PECAM), is dependent on a rapid depalmitoylation/repalmitoylation cycle,⁵⁻⁸ suggesting that the I/R injury cascade is dependent, at least in part, on protein palmitoylation. Hence, interrupting that cascade would be expected to mitigate I/R injury. Triacsin C (Figure 1) blocks eNOS palmitoylation,⁹ increases post-ischemic intravascular NO generation, and inhibits neutrophil recruitment and infiltration in a model of ischemia.¹⁰ Triacsin C is a natural product isolated from *Streptomyces aureofaciens*,^{11,12} which inhibits ACSL, including those expressed by the vascular endothelium.¹³ Together, the data suggests that these processes are dependent on palmitoyl CoA formation and imply that protein palmitoylation is dependent on palmitoyl CoA availability.

Our earlier work¹⁴ described several triacsin C analogs, including compounds **1** and **2** (Figure 1), with high anti-viral activities. However, the efficacy of these compounds to inhibit ACSL activity was not tested directly. Here, we expand the library of triacsin C analogs, examine their activities as ACSL inhibitors both *in vitro* and *in vivo*, and evaluate their potential as inhibitors of neutrophil infiltration in an *in vivo* model of ischemia. The hydroxytriazene function of triacsin C remains an elusive functionality to be synthesized. In the reported synthesis of triacsin C,¹⁵ the final three steps for the construction of hydroxyazoimine or hydroxytriazene moiety involved the hydrazone formation of (*E,E,E*)-2,4,7-undecatrien-1-al with hydrazine followed by silylation with trimethylsilyl chloride and addition to dinitrogen trioxide, resulting in only 0.4% yield. The scarcity of triacsin C hinders various *in vivo* biological studies.

Moreover, the hydroxyazoimine function of triacsin C is a reactive moiety. Hence, analogs **3** – **12** with varying alkenyl chains, possessing different functionalities, and mimicking hydroxyazoimine moiety of triacsin C were synthesized and bioevaluated (Figure 1).

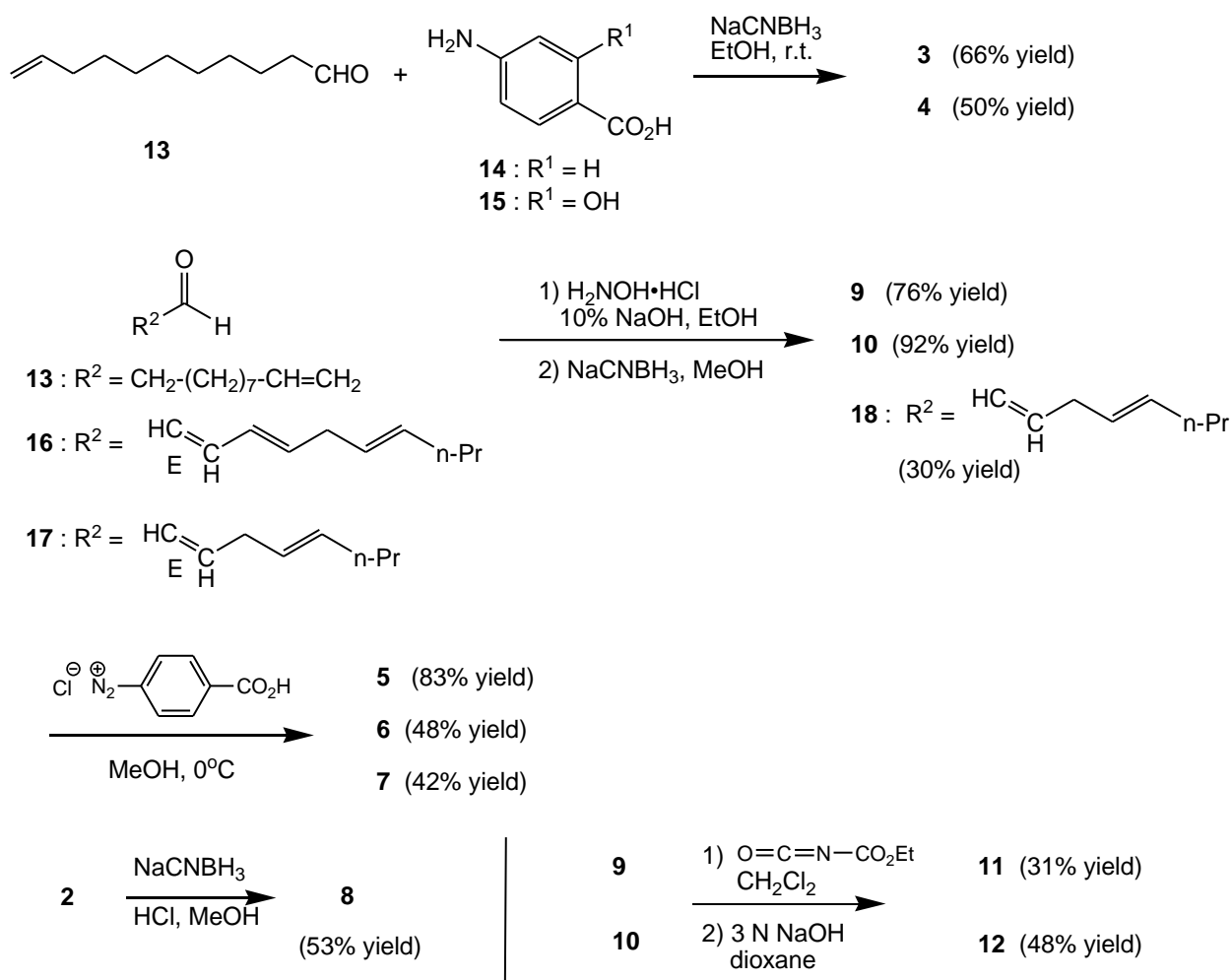
Figure 1. Synthesized and bioevaluated ACSL inhibitors **1** - **12** and triacsin C.



Following the previously reported method,¹⁴ compounds **3** and **4** were readily synthesized in moderate yields from reductive amination reactions of 10-undecenal (**13**) and **14** and **15**, separately, with sodium cyanoborohydride in ethanol at 25°C (Scheme 1). Hydroxyamine **9** was obtained in 76% yield from the coupling of aldehyde **13** and hydroxylamine in ethanol followed by reduction with NaCNBH₃. Similar treatment of aldehydes¹⁴ **16** and **17** afforded hydroxyamines **10** and **18**, respectively. Addition reactions of hydroxyamine **9**, **10**, and **18**, separately, with 4-(hydroxycarbonyl)phenyldiazonium chloride¹⁶ in methanol provided

hydroxytriazene analogs **5**, **6**, and **7** in 83, 48, and 42% yield, respectively. Notably, these three compounds are stable molecules. Because the imino function of **2**¹⁴ hydrolyzes with water slowly, it was reduced with NaCNBH₃ and HCl in methanol to furnish stable sulfonylhydrazine **8** in a 53% yield. Under other reaction conditions including the use of acetic acid as a catalyst in the reduction procedure resulted in a mixture of unidentifiable byproducts. 1,2,4-Oxadiazolidine-3,5-dione is a carboxylic acid bioisostere,¹⁷ hence we synthesized compounds **11** and **12** from **9** and **10**, respectively, by the addition reactions with ethyl isocyanofornate followed by ring closure with NaOH.¹⁷

Scheme 1. Synthesis of compounds **3** - **12**.

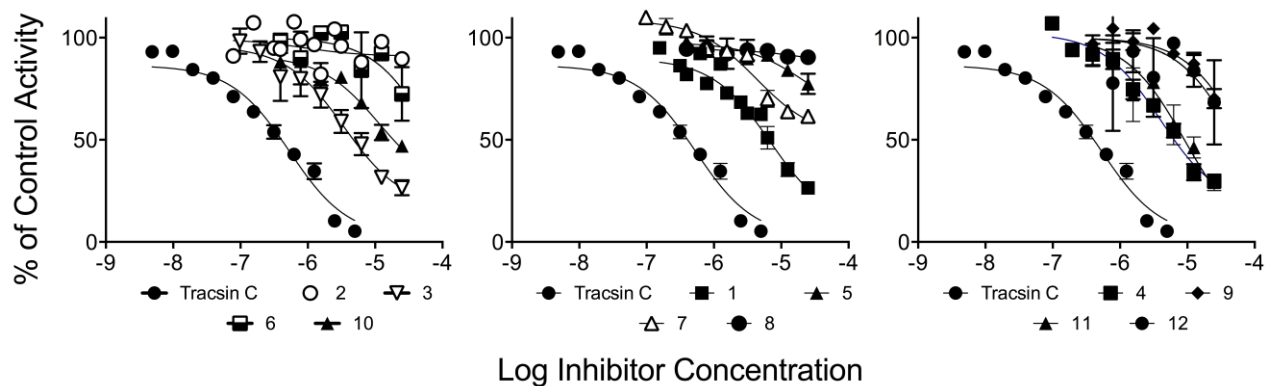


ACSL inhibition *in vitro*: With the exception of Compounds **2** and **8**, all of the triacsin C analogs inhibit ACSL activity in solubilized brain endothelial bEND3 cells, and results and inhibitory curves are shown in Table 1 and Figure 2, respectively. The median effective concentration (EC₅₀) values ranged from ~5 μM to ~170 μM. In contrast, triacsin C is about 10 times more potent than compound **3**, the most active analog of the series. Compound **1** is only one-tenth as potent as triacsin C in the ACSL assay, yet it was at least twice as potent as triacsin C as an inhibitor of rotavirus replication,¹⁴ suggesting that the effect on rotavirus replication may not attribute only by the inhibition of ACSL. Significantly, compound **3**, having EC₅₀ value of 4.95 μM and containing a 10-undecenyl chain, has similar or better inhibitory activity as the trienyl analog, **1** with EC₅₀ value of 5.86 μM. Among the head groups examined, the 4-hydroxycarbonylphenylamino moiety (in compounds **1** and **3**) possesses the greatest activity. The carboxylic acid bioisosteres, 1,2,4-oxadiazolidine-3,5-dione derivatives **11** and **12** do not appear to improve the inhibitory activity. Also, the isothiazolidine dioxides **2** and **8** are >30 folds less active compare with compound **3**.

Table 1. Inhibition of ACSL activity in solubilized brain endothelial bEND3 cells. The EC₅₀ values were calculated by fitting the velocity vs. inhibitor concentration to the constrained three-parameter log (inhibitor) vs. response function of the Prism 6.0 statistical analysis package.

Compound	EC ₅₀ (μM)	Compound	EC ₅₀ (μM)
1	5.86 ± 0.54	7	26.9 ± 3.6
2	171 ± 79.0	8	164 ± 6 0.8
3	4.95 ± 0.74	9	70.4 ± 14.0
4	6.88 ± 0.66	10	14.8 ± 1.6
5	73.1 ± 9.6	11	9.04 ± 1.45
6	74.8 ± 34.8	12	53.5 ± 33.2
Triacsin C	0.358 ± 0.027		

Figure 2. Concentration effect curves of the inhibition of ACSL activity in solubilized brain endothelial bEND3 cells for each of the analogs, as compared to Triacsin C.

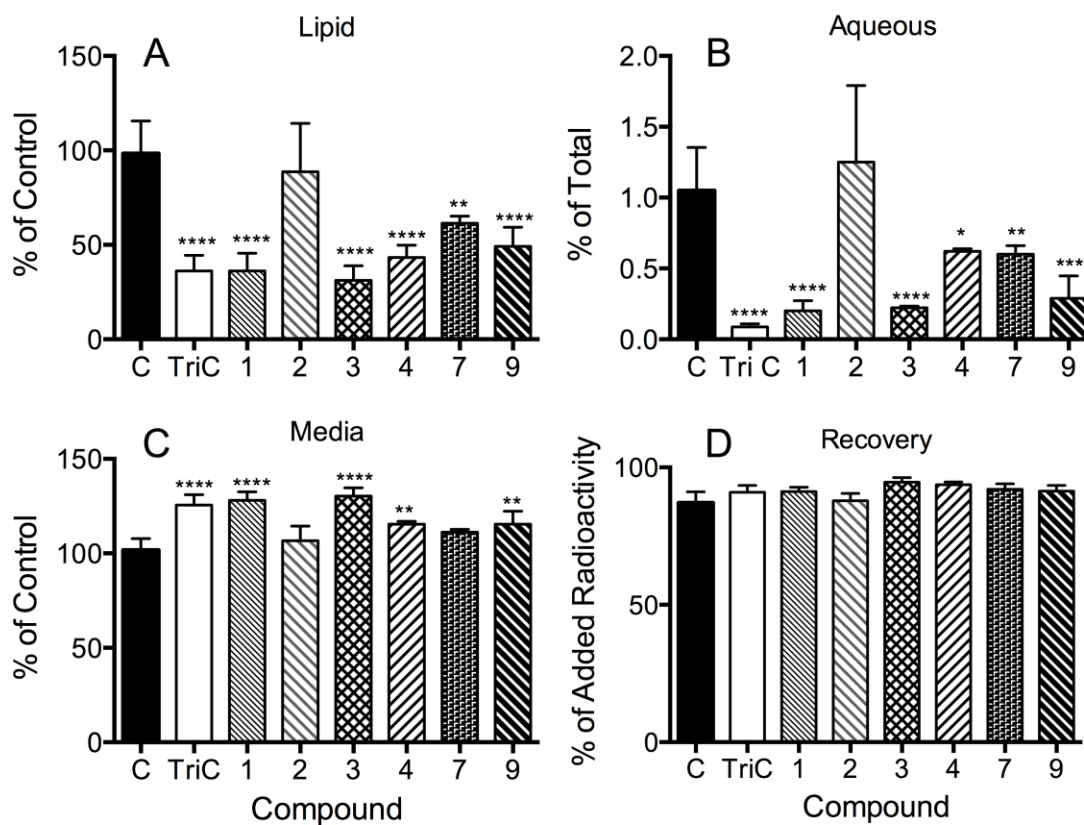


ACSL inhibition *in situ*: Compounds **1**, **2**, **3**, **4**, **7**, and **9** were compared to triacsin C as inhibitors of [¹⁴C]-palmitate incorporation into extractable lipids of bEND 3 cells, and results are shown in Figure 3A. Each of the analogs, with the exception of compound **2**, showed significant inhibition of ACSL, with a potency ratio of triacsin C > **3** > **1** > **4** > **9** = **7** > **2** and mirroring that of *in vitro* ACSL inhibition. [¹⁴C]-Palmitoylated lipids were also measured in aqueous fraction (Figure 3B) and media (Figure 3C), and the total recovered radioactivity (lipid + aqueous + media) was calculated (Figure 3D). The control cultures incorporated 40.2 ± 2.6 fmoles [¹⁴C]-palmitate/ μ g of lipid. Incorporation of radioactive fatty acids into lipid is a two-step process. First, the FAcCoA derivative is formed, a reaction mediated by ACSL and second, the fatty acid moiety is transferred to an available site during *de novo* lipid synthesis or during remodeling of existing lipids. These reactions are mediated by various acyl transferases, so that simply demonstrating that the lesser radioactivity in the lipid fraction is insufficient to distinguish between inhibition of ACSL and the relevant acyl transferase.

Free palmitic acid is insoluble in aqueous systems at neutral pH, while the palmitoyl CoA derivative has appreciable water solubility (about 1.74 g/L). Thus, free palmitic acid is more likely to partition into the CHCl₃ layer of the lipid extraction, while the CoA derivative is more likely to remain in the aqueous layer. As shown in Figure 3B, there was significantly less radioactivity in the aqueous layer for each of the compounds, with the exception of compound **2**. Furthermore, the potency ratio for inhibition of radioactivity in the aqueous layer is similar to that for inhibition of incorporation into extractable lipids. Together, these data are consistent with the inhibition of ACSL and not with inhibition of an acyl transferase. Alternative explanations might include reduced total lipid mass, or loss of radioactivity to β -oxidation. The mass of lipids recovered was slightly greater than control for all compounds tested, with the exception of compound **2**. In cells treated with triacsin C or compounds **1**, **3**, **4**, **7** and **9**, the mean lipid extracted did not differ with treatment, and was $127 \pm 6\%$ of control. The unincorporated radioactivity in the media (with the exception of compound **2**) was greater in treated cells as compared to control cells, as shown in Figure 3C. Finally, the total recovery of radioactive material (media + lipid + aqueous) did not differ among the compounds tested, and was not different from control (Figure 3D). The uniform recovery of radioactivity in both the control and treated cells strongly suggests that treatment did not increase β -oxidation. Thus, the reduced incorporation into lipids is not likely to be the consequence of reduced overall lipid content or increased β oxidation of fatty acid. The possible remaining interpretation of the data is that the reduced incorporation of [¹⁴C]-palmitate into extractable lipids is the consequence of inhibition of ACSL.

Figure 3. *In situ* inhibitions of ACSL by triacsin C and analogs in bEND3 brain endothelial cells, as evaluated by the incorporation of [¹⁴C]-palmitic acid into extractable lipid (A), aqueous

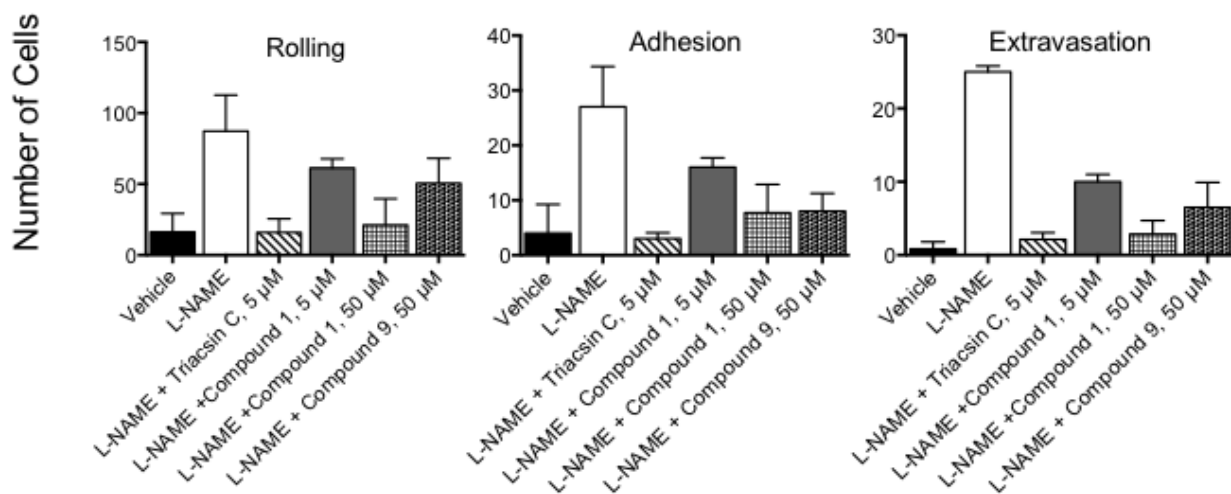
(B), media (C), and recovery (D). Triacsin C was used at 1.5 μM , while all other compounds were evaluated at 15 μM . Differences between the treatment groups and control were evaluated by one-way ANOVA, followed by Dunnett's t test for significance. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$. n = 3 to 9 independent experiments.



Inhibition of leukocyte recruitment: Previously, we demonstrated that 5 μM triacsin C virtually abolished neutrophil recruitment in a model of ischemia.¹⁰ Figure 4 compares the effects of compounds **1** and **9** (as representative analogs) to that of triacsin C. Both compounds **1** and **9** significantly inhibited rolling, adhesion, and extravasation. The magnitudes of inhibition for both compounds were less than that of 5 μM triacsin C, but mirrored the potencies of compounds **1** and **9** to inhibit ACSL activity. Triacsin C inhibits palmitoylation of multiple proteins including eNOS.⁹ This suggests that inhibitors of ACSL exert their effects, at least in part, by inhibiting protein palmitoylation, consequently changing their function and/or sub-cellular location. Protein palmitoylation is a very rapid (half-time of seconds) common pathway

facilitating protein trafficking.^{3,18} Indeed, proteins that are rapidly re-partitioned between cytoplasm and membrane are suspects for palmitoylation.³ Cell adhesion proteins such as PECAM⁵ and P-selectin⁶ require palmitoylation for translocation to the plasma membrane early in the ischemic injury cascade. Our observation that triacsin C and both compounds **1** and **9** inhibit leukocyte recruitment strongly suggest that all three compounds inhibit translocation of one or more cell adhesion molecules from the cytoplasm to the plasma membrane on either the endothelium or leukocyte or both. Furthermore, the potency ratio of these three compounds to inhibit leukocyte recruitment is the same as the potency ratio for inhibition of ACSL activity. Taken together, the data strongly suggest that inhibiting ACSL may attenuate the vascular inflammatory component associated with I/R injury,¹ consequently limiting infarct expansion.

Figure 4. The L-NAME induced leukocyte recruitment at 120 minutes in the presence of compounds **1** and **9**. Rolling, adhesion and extravasation were measured in post-capillary venules of the mesentery, a model of ischemia, and in the presence or absence of 5 or 50 μ M compound **1** or 50 μ M compound **9**. The rolling, adhesion and extravasation rates for 50 μ M compound **1** and 5 μ M triacsin C were not different from each other nor were they different from vehicle alone. When evaluated at 5 μ M, compound **1** inhibited the recruitment to about half the extent as at 50 μ M. Compound **9** at 50 μ M was about as effective as compound **1** at 5 μ M. Each bar represents the mean \pm SEM of 3 to 7 independent experiments.



In conclusion, various triacsin C analogs were synthesized and their ACSL inhibitory activities and amelioration of the vascular inflammatory component associated with ischemia/reperfusion were studied. 4-Akenylaminobenzoic acid **1** and **3** showed the greatest activities. Further optimization of this class of ACSL inhibitors is possible, which may lead to the understanding of the consequence of ACSL inhibition effect in ischemia and treatment of ischemia.

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Supplemental data

Supplemental data (synthetic procedure and biological experiments) associated with this article can be found, in the online version, at

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