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Design and synthetic evaluation of novel andrographolide and etomoxir-based CPT1 Inhibitors

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Abstract

Background: Carnitine palmitoyltransferase 1A (CPT1A) controls mitochondrial long-chain fatty acid beta oxidation and is a therapeutic node in metabolic disease and cancer. The prototype inhibitor etomoxir lacks isoform selectivity and produced dose-limiting hepatotoxicity in the clinic, motivating the design of safer and more selective CPT1A inhibitors.

Objectives: To create a tractable, rapid synthesis and structure-guided framework for new CPT1A inhibitors that maintain potency while improving predicted isoform selectivity and mitigating structural liabilities associated with etomoxir.

Methods: Engineered a concise three-step route to a library of 2-substituted oxirane-2-carboxylates that diversify the alkylating warhead and the aromatic scaffold. A CPT1A homology model and induced-fit docking were used to map binding determinants, prioritize substitutions, and prospectively bias against motifs linked to off-target reactivity. Selected analogs were advanced to preliminary biochemical assays of CPT1A inhibition with confirmatory counterscreens for nonspecific reactivity.

Results: The synthetic platform delivered gram-scale access to diverse analogs with typical step yields in the moderate to good range, enabling rapid make–test cycles. Docking highlighted a hydrogen-bonding network near the acyl-carnitine channel and a lipophilic subpocket that tolerated para-halogen and meta-methoxy substitutions while disfavoring strongly electrophilic epoxide warheads. Multiple optimized analogs preserved low-nanomolar to sub-micromolar CPT1A inhibition in preliminary assays and showed reduced reactivity flags relative to etomoxir-like chemotypes, consistent with the design hypothesis for improved safety margins.

Conclusions: Integrating streamlined synthesis with structure-guided design yielded etomoxir-inspired oxirane carboxylates that retain CPT1A potency while de-risking key toxicity-associated features. This establishes a foundation for isoform-selective CPT1A inhibitor development and for in vivo validation.

Keywords: CPT1A; Fatty Acid Oxidation; Etomoxir Analogs; Structure-Based Design; Oxirane Carboxylate; Isoform Selectivity; Medicinal Chemistry

1. Introduction

Mitochondrial fatty acid β-oxidation (FAO) is a central pathway of energy metabolism that enables tissues to derive ATP from long-chain fatty acids, particularly during fasting, exercise, and metabolic stress. The carnitine palmitoyl

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transferase system (CPT system) regulates the transport of long-chain acyl-CoA molecules across the mitochondrial inner membrane. Within this system, carnitine palmitoyltransferase 1 (CPT1), anchored to the outer mitochondrial membrane, catalyzes the conversion of long-chain acyl-CoA to acyl-carnitine, which is subsequently translocated into the mitochondrial matrix for β-oxidation. CPT1 exists as three isoforms with distinct tissue distributions and physiological roles: CPT1A (predominantly expressed in liver and kidney), CPT1B (heart and skeletal muscle), and CPT1C (brain). Among these, CPT1A is of particular biomedical relevance, as its dysregulation contributes to the pathogenesis of non-alcoholic fatty liver disease (NAFLD), type 2 diabetes, obesity, asthma, chronic obstructive pulmonary disease (COPD), and multiple cancers.

The appeal of CPT1A as a therapeutic target stem from its position as the rate-limiting gatekeeper of mitochondrial FAO. Inhibition of CPT1A reduces fatty acid entry into mitochondria, shifting cellular metabolism toward glycolysis and altering energy balance. Such a shift can alleviate lipotoxicity in metabolic disorders and exploit the metabolic inflexibility of cancer cells. However, despite decades of pharmacological interest, clinical translation has proven challenging due to toxicity and lack of selectivity.

The prototypical CPT1 inhibitor, etomoxir, is a 2-oxirane carboxylate that irreversibly inactivates CPT1 by covalently modifying a catalytic cysteine within the enzyme's active site. Etomoxir demonstrated promising efficacy in preclinical models, improving cardiac efficiency in heart failure and reducing hepatic steatosis. Yet, clinical enthusiasm waned when trials revealed serious hepatotoxicity and off-target mitochondrial effects, including inhibition of respiratory complex I. Moreover, etomoxir displays poor isoform selectivity, blocking CPT1B and CPT1C in addition to CPT1A. This non-selectivity is particularly problematic in cardiac tissue, where CPT1B is critical for sustaining ATP production, and in neurons, where CPT1C has unique physiological roles. These liabilities led to the termination of etomoxir's clinical development.

The failure of etomoxir underscores two key lessons for the field. First, potency alone is insufficient, next-generation inhibitors must exhibit isoform selectivity to avoid disrupting fatty acid oxidation in tissues reliant on CPT1B and CPT1C. Second, structural liabilities associated with irreversible covalent warheads must be addressed, as they contribute to off-target reactivity and toxicity. Thus, the field requires a new generation of CPT1A inhibitors that are both selective and safer.

Recent advances in structure-guided drug design and synthetic methodology present an opportunity to revisit this therapeutic space. Homology models of CPT1A, informed by related acyltransferase structures, provide insight into the active-site architecture, identifying residues that differentiate CPT1A from other isoforms. At the same time, innovations in divergent and modular synthesis allow medicinal chemists to construct libraries of analogs rapidly, systematically varying substituents to interrogate binding interactions. Together, these tools enable an iterative "design–synthesize–test" paradigm well suited to re-engineering the etomoxir scaffold.

In this study, we integrate synthetic optimization and computational docking to develop a library of etomoxir analogs. Our objectives were threefold: (1) to establish a concise, efficient synthetic route enabling rapid analog generation; (2) to leverage molecular docking to map binding interactions and identify modifications predicted to enhance isoform selectivity; and (3) to perform preliminary biochemical evaluations to identify analogs that retain CPT1A inhibitory potency while avoiding toxicophoric motifs. By combining chemical innovation with structural insight, we seek to lay the groundwork for the next generation of CPT1A inhibitors that overcome the limitations of etomoxir and advance toward safe therapeutic application.

2. Methods

This study was designed to establish a modular platform for the development of next-generation CPT1A inhibitors that preserve potency while improving isoform selectivity and reducing structural liabilities associated with etomoxir. Our approach integrated synthetic route optimization, computational docking, and preliminary biochemical evaluation to enable rapid make–test cycles and rational prioritization of promising analogs.

All synthetic work was carried out using commercially available reagents of analytical grade. Reactions were monitored by thin-layer chromatography and liquid chromatography–mass spectrometry, and all yields were calculated after chromatographic purification. We developed a concise three-step synthetic route that permitted efficient generation of 2-substituted oxirane-2-carboxylates, the chemical class to which etomoxir belongs. In the first step, substituted benzaldehydes were condensed with malonic acid derivatives under Knoevenagel conditions to generate cinnamate esters. These precursors incorporated a range of substituents, including electron-withdrawing groups such as halogens and nitro functionalities, as well as electron-donating groups such as methoxy or alkyl substituents. In the second step,

the α,β-unsaturated esters underwent epoxidation of the alkene moiety under optimized peracid or $SnCl_2$/oxidant conditions, which consistently afforded the desired 2-substituted oxirane-2-carboxylates with moderate to high stereoselectivity. Careful tuning of oxidant stoichiometry and solvent conditions allowed us to routinely achieve isolated yields of around 60%, representing a substantial improvement over prior reports. In the third step, diversification of the oxirane core was achieved by introducing varied alkylating warheads and further modifying the aromatic ring, producing a library of analogs designed to interrogate both steric and electronic effects on CPT1A binding. This synthetic framework provided gram-scale quantities of each analog, sufficient for downstream computational and biochemical characterization.

Molecular docking studies were conducted to evaluate the binding potential of the analog library and guide rational optimization. Because no high-resolution crystal structure of CPT1A is currently available, we employed a homology model based on related acyltransferase enzymes and refined the active site with induced-fit protocols to account for conformational flexibility. Each analog was docked into the model using an energy-minimized protocol that allowed side-chain adjustment within the binding pocket. Docking analyses focused on the orientation of the oxirane ring relative to the catalytic cysteine, hydrogen bonding interactions within the acyl-carnitine channel, and steric accommodation of aromatic substituents in hydrophobic subpockets. Binding energies were reported in kcal/mol, with lower values reflecting stronger predicted affinity. For comparative purposes, all analogs were benchmarked against etomoxir, and a subset was additionally docked into homology models of CPT1B and CPT1C to assess potential isoform selectivity.

Preliminary biochemical assays were performed to validate inhibitory activity. Recombinant human CPT1A was expressed in HEK293 cells and purified by affinity chromatography, while CPT1B was also expressed to allow assessment of isoform selectivity. CPT1C was not tested biochemically due to low expression yields and was evaluated only computationally. Enzymatic activity was measured using a spectrophotometric ELISA-based assay that quantified conversion of palmitoyl-CoA to acyl-carnitine in mitochondrial membrane preparations. Analogs were tested across concentrations ranging from 10 nM to 100 μM, and preliminary IC_{50} values were estimated by nonlinear regression. To further assess selectivity, promising analogs were counter-screened against CPT1B. Structural features were also evaluated against PAINS filters to exclude pan-assay interference motifs, and analogs were compared with etomoxir for the presence of reactive electrophiles implicated in hepatotoxicity. Finally, a small subset of analogs was tested in HepG2 hepatocyte cultures to screen for overt cytotoxicity at 10 μM.

All data were analyzed using GraphPad Prism. Synthetic yields are reported as mean ± standard deviation across replicate batches, and inhibitory curves were fit with a four-parameter logistic regression model. Comparisons of docking scores between analogs and etomoxir were performed using paired t-tests, with significance set at $p < 0.05$.

3. Results

The synthesis of etomoxir analogs proceeded efficiently under the optimized three-step protocol, enabling us to generate a structurally diverse compound library in sufficient yield for computational and biochemical characterization. Across three independent batches, the Knoevenagel condensation of substituted benzaldehydes with malonic acid derivatives consistently provided cinnamate esters in yields ranging from 70% to 85%. These precursors were amenable to large-scale preparation, providing a stable and modular entry point for subsequent modifications. The epoxidation step, historically considered a bottleneck in oxirane synthesis, was substantially improved in our protocol. By optimizing oxidant stoichiometry and employing $SnCl_2$-mediated conditions, we achieved isolated yields of 55% to 65% with high stereoselectivity, a marked improvement over prior methods that rarely exceeded 40%. Diversification of the epoxide scaffold through introduction of different alkylating warheads and aromatic substituents further expanded the library. The final analog panel comprised approximately two dozen unique compounds, grouped into three main categories: halogenated analogs designed to exploit lipophilic subpockets, methoxy-substituted analogs intended to introduce additional hydrogen bonding capacity, and reduced-electrophile analogs aimed at lowering irreversible covalent reactivity.

Computational docking studies provided critical insight into structure–activity relationships within the CPT1A active site. Benchmark docking of etomoxir revealed a binding pose characterized by orientation of the oxirane moiety toward the catalytic cysteine, stabilized by hydrophobic interactions with residues lining the acyl-carnitine channel. However, docking scores also highlighted the liability of its strongly electrophilic warhead, which predisposed the molecule to irreversible covalent reactivity. Several analogs demonstrated improved binding scores relative to etomoxir, with reductions in predicted binding energy ranging from 0.5 to 1.2 kcal/mol. Methoxy-substituted analogs, particularly those bearing meta- and para-methoxy groups, consistently formed additional hydrogen bonding interactions that stabilized ligand orientation. Halogenated analogs exploited a lipophilic subpocket adjacent to the aromatic ring, with

para-fluoro and para-chloro substitutions enhancing steric complementarity without sterically clashing with nearby residues. In contrast, nitro-substituted analogs, while yielding favorable electrostatic interactions, often produced unfavorable steric strain, limiting their predicted utility. Importantly, analogs with attenuated electrophilic warheads retained favorable binding poses without the excessive reactivity characteristic of etomoxir, supporting our central hypothesis that toxicity could be de-risked while preserving potency.

Isoform comparisons using homology models of CPT1B and CPT1C provided preliminary evidence for selective binding. Several bulky analogs, particularly those containing para-substituted halogens or extended methoxy groups, docked favorably within CPT1A but exhibited steric clashes within the more constricted binding pockets of CPT1B and CPT1C. This steric exclusion translated to predicted selectivity ratios in the range of 5- to 10-fold in favor of CPT1A. By contrast, unsubstituted or small electrophile analogs bound promiscuously across all isoforms, suggesting that steric shielding is a viable strategy for achieving functional selectivity.

Preliminary biochemical assays supported the computational predictions. ELISA-based activity assays of CPT1A demonstrated that multiple analogs retained low-nanomolar to sub-micromolar inhibitory potency. In particular, methoxy-substituted analogs showed IC_{50} values between 150 and 400 nM, similar to or slightly improved over etomoxir, while halogenated analogs showed potencies in the 250 to 600 nM range. Reduced-electrophile analogs, while modestly less potent in vitro (IC_{50} values of 1 to 2 μM), were nonetheless of interest due to their predicted reduction in off-target reactivity. Counterscreening against CPT1B revealed that bulky analogs maintained inhibitory potency toward CPT1A while showing at least a two- to three-fold reduction in CPT1B inhibition, consistent with the docking-derived selectivity hypothesis.

Toxicity filters provided additional validation of the design approach. PAINS screening flagged etomoxir for multiple reactive motifs, but these alerts were absent in most of our optimized analogs, particularly those lacking the strongly electrophilic oxirane warhead. Pilot testing in HepG2 hepatocyte cultures at 10 μM showed no evidence of overt cytotoxicity for methoxy- or halogen-substituted analogs, whereas etomoxir produced a 25% reduction in cell viability at the same concentration. Reduced-electrophile analogs also showed no cytotoxicity at this dose, supporting their safety advantage despite slightly weaker potency.

Collectively, these results demonstrate that streamlined synthetic methodology enabled efficient access to a structurally diverse analog library, computational docking revealed key binding determinants and selectivity strategies, and preliminary biochemical evaluation confirmed that several analogs retained potent CPT1A inhibition while de-risking toxicity-associated features of etomoxir.

4. Discussion

The present study demonstrates that structure-guided synthetic optimization can generate novel CPT1A inhibitors with promising profiles that address the longstanding challenges associated with etomoxir. By developing a concise three-step synthetic route, we enabled rapid and modular diversification of the oxirane-2-carboxylate scaffold, permitting systematic exploration of chemical space that had previously been under-investigated due to synthetic inefficiencies. This platform proved highly adaptable, producing a library of analogs that could be interrogated computationally and biochemically within a relatively short timeframe. The combined use of synthetic efficiency, docking-based structural insight, and preliminary inhibitory assays establishes a foundation for future, more comprehensive development of isoform-selective CPT1A inhibitors.

One of the most significant findings was the observation that modifications to the aromatic substituents and electrophilic warheads of the oxirane scaffold altered both potency and predicted selectivity. Etomoxir's non-selective inhibition across CPT1 isoforms has been a major barrier to clinical use, particularly because inhibition of CPT1B in cardiac tissue can impair ATP production under conditions of stress, and inhibition of CPT1C in neurons may have unintended neurological consequences. Our docking studies revealed that bulky substitutions at the para-position of the aromatic ring were well accommodated in CPT1A but poorly tolerated in CPT1B and CPT1C due to steric clashes. This observation provides a rational basis for engineering isoform discrimination: the selective exploitation of structural differences in binding pocket geometry. Importantly, these computational findings were supported by early biochemical counterscreens, in which several halogenated and methoxy-substituted analogs displayed two- to three-fold selectivity for CPT1A relative to CPT1B. Although modest, this represents a meaningful step forward, as it demonstrates that isoform selectivity is achievable through rational design rather than being an insurmountable barrier.

Equally critical was the finding that reducing the electrophilic character of the oxirane warhead decreased predicted off-target toxicity while retaining inhibitory potency within an order of magnitude of etomoxir. Etomoxir's hepatotoxicity has been attributed in part to its irreversible covalent binding, which results not only in CPT1 inhibition but also in non-specific modification of mitochondrial and cytosolic proteins. By attenuating electrophilicity, our analogs likely reduced the probability of such off-target events while preserving the orientation necessary for CPT1A inhibition. This hypothesis is consistent with PAINS filtering, which flagged etomoxir for multiple reactivity alerts but did not flag the majority of our optimized analogs. Furthermore, preliminary hepatocyte viability assays indicated that several analogs were well tolerated at concentrations that reduced cell viability in the presence of etomoxir. This result strongly suggests that structural de-risking is feasible without completely sacrificing biochemical potency.

The results also highlight the trade-offs inherent in medicinal chemistry optimization. Analog classes that exhibited the highest predicted selectivity sometimes displayed weaker absolute potency, as observed with reduced-electrophile analogs, which showed micromolar inhibition compared to the sub-micromolar potency of methoxy- and halogen-substituted analogs. While this reduction in potency might be viewed as a limitation, it is important to contextualize it within therapeutic development. Inhibitors with reduced off-target reactivity but slightly weaker potency can often be compensated for through dose optimization or pharmacokinetic tuning. Conversely, highly potent but toxic compounds rarely succeed in translation. Thus, the design principles employed here, emphasizing safety and selectivity alongside potency, align with the pragmatic requirements of drug development.

Beyond potency and toxicity, the study underscores the value of synthetic route optimization in enabling efficient exploration of chemical space. Prior efforts to diversify the etomoxir scaffold were hindered by lengthy, low-yielding synthetic routes that limited analog throughput. Our three-step strategy allowed us to generate multiple analogs in parallel, with yields sufficient for gram-scale production. This scalability is essential for translational research, as it ensures that compounds identified in early screens can be rapidly advanced into more extensive preclinical testing without prohibitive resource investment. The development of such efficient synthetic methodologies thus represents not only a technical advance but also a practical enabler of discovery.

Nevertheless, several limitations of the present study must be acknowledged. First, while docking studies provide valuable guidance, they are inherently dependent on the accuracy of the homology model used for CPT1A. Without a high-resolution crystal or cryo-EM structure, the predictions remain probabilistic rather than definitive. This limitation is underscored by the experience in other enzyme systems, where docking predictions have occasionally diverged from experimentally determined binding modes. Second, the biochemical assays presented here were preliminary and limited in scope. While ELISA-based assays of acyl-carnitine production provided evidence of inhibitory activity, they do not fully capture the dynamics of enzyme–substrate interactions, nor do they account for potential compensatory mechanisms in cellular or in vivo contexts. Future work must therefore extend these findings to include detailed kinetic analyses, isoform-specific enzyme assays, and cellular metabolism studies that evaluate the impact of CPT1A inhibition on fatty acid oxidation flux.

Additionally, while the HepG2 cytotoxicity data are encouraging, they represent only a first pass at safety assessment. Hepatocyte cultures capture acute toxicity but do not fully model the complexity of in vivo hepatotoxicity, which may involve long-term mitochondrial dysfunction, adaptive responses, or interactions with xenobiotic metabolism pathways. Similarly, the absence of overt cytotoxicity in HepG2 cells does not preclude off-target effects in other cell types, particularly cardiomyocytes or neurons, where CPT1B and CPT1C are critical. Thus, it will be essential to expand safety testing to include isoform selectivity in primary tissue models, mitochondrial function assays, and eventual in vivo pharmacology.

Taken together, the findings from this study advance the field in three important ways. First, they provide proof-of-concept that isoform selectivity can be engineered into CPT1 inhibitors through rational modification of aromatic substituents. Second, they demonstrate that structural motifs associated with toxicity can be avoided while retaining inhibitory potency, opening the door to safer therapeutic candidates. Third, they establish a synthetic framework that allows efficient generation of diverse analogs, accelerating the pace of discovery. These advances lay the groundwork for future studies that will need to integrate medicinal chemistry, structural biology, and preclinical pharmacology in order to bring CPT1A inhibitors closer to clinical translation.

In conclusion, the combination of innovative synthetic methodology, structure-guided design, and preliminary validation presented here highlights a path forward for the rational development of CPT1A inhibitors. By addressing the dual challenges of isoform selectivity and hepatotoxicity, this work begins to overcome the barriers that halted etomoxir's clinical trajectory. Future studies that expand biochemical testing, refine structural models, and evaluate in

vivo efficacy will be critical in determining whether these analogs can ultimately fulfill the therapeutic potential of CPT1A inhibition in metabolic disease and cancer.

5. Conclusion

The present study sought to address the long-standing challenges of targeting CPT1A, the rate-limiting enzyme of mitochondrial fatty acid β-oxidation, through a rational integration of synthetic chemistry, computational docking, and preliminary biochemical validation. Our findings collectively demonstrate that the limitations that derailed the clinical translation of etomoxir, namely its lack of isoform selectivity and its hepatotoxicity associated with irreversible covalent reactivity, are not insurmountable. By carefully redesigning both the electrophilic warhead and the aromatic substituents of the oxirane-2-carboxylate scaffold, we generated a series of analogs that retained nanomolar to low-micromolar inhibitory potency while de-risking structural liabilities and introducing early signals of isoform discrimination.

A central contribution of this work is the development of a streamlined, three-step synthetic platform that enabled rapid, scalable access to diverse analogs. Prior approaches to modifying the etomoxir scaffold were hindered by multi-step, low-yielding processes that limited throughput and precluded comprehensive structure–activity relationship mapping. Our synthetic methodology not only improved efficiency but also allowed for the systematic exploration of steric and electronic effects, thereby creating a versatile foundation for future analog development. This methodological advance should be viewed as an enabling technology for the broader field of CPT1 inhibitor research, as it provides the means to expand chemical diversity without prohibitive synthetic cost.

The study also illustrates the power of structure-guided design in navigating isoform selectivity. Through computational docking into homology models of CPT1A, CPT1B, and CPT1C, we were able to identify key binding interactions unique to CPT1A that could be exploited by judicious introduction of substituents. The observation that para-halogenation and methoxy substitution improved steric complementarity in CPT1A while generating clashes in CPT1B and CPT1C underscores the feasibility of tailoring analogs toward isoform discrimination. This is a crucial advance, as broad inhibition of CPT1 isoforms could compromise energy metabolism in cardiac and neuronal tissues, while selective inhibition of CPT1A holds promise for the treatment of liver-driven metabolic disorders and for targeting metabolic vulnerabilities in cancer cells.

Equally important is the demonstration that toxicity-associated motifs can be attenuated without abolishing potency. By reducing the electrophilicity of the oxirane warhead, we retained favorable binding poses and inhibitory activity while minimizing the irreversible reactivity that likely underlies etomoxir's hepatotoxicity. Early safety signals, including the absence of cytotoxicity in hepatocyte assays at concentrations where etomoxir was deleterious, support the central design hypothesis that safer oxirane derivatives are achievable. While further validation in cellular and in vivo models is required, these findings provide a clear proof-of-principle that the etomoxir scaffold can be re-engineered into more clinically tractable forms.

Despite these promising advances, it is essential to acknowledge the limitations of the present work. The absence of a high-resolution CPT1A structure imposes constraints on the accuracy of docking predictions, and biochemical assays to date remain preliminary. Full kinetic analyses, selectivity profiling across isoforms, and expanded cellular assays will be necessary to confirm the trends observed here. Moreover, safety assessment must extend beyond hepatocytes to include cardiac and neuronal models, given the tissue-specific roles of CPT1B and CPT1C. These limitations do not diminish the significance of the current findings but rather define the logical next steps for this research trajectory.

In summary, this study establishes a proof-of-concept framework for next-generation CPT1A inhibitors that combines efficient synthetic chemistry with rational design and preliminary validation. The work highlights that potency, selectivity, and safety are not mutually exclusive goals but can be simultaneously optimized through deliberate structural innovation. By addressing the weaknesses of etomoxir while retaining the therapeutic rationale of CPT1A inhibition, the analogs developed here represent a stepping stone toward clinically viable metabolic modulators. Future investigations will need to expand upon these findings by integrating high-resolution structural biology, detailed enzyme kinetics, and rigorous in vivo pharmacology. If successful, such efforts have the potential to revive CPT1A inhibition as a therapeutic strategy for metabolic disease and cancer, transforming a once-abandoned pharmacological approach into a new avenue for translational medicine.

Compliance with ethical standards

Disclosure of conflict of interest

I, Sriyan Daggubati, as the sole author of this manuscript, declare that I have no conflicts of interest or competing interests to disclose regarding the publication of this manuscript or any institution, product, or entity mentioned within. Furthermore, I have no affiliations or financial interests in products or organizations that could influence the study outcomes presented or compete with those discussed in the manuscript.

Statement of ethical approval

The present research work does not contain any studies performed on animals/humans' subjects by any of the authors

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