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Innovations

Inactivators of Ornithine Aminotransferase for the Treatment of Hepatocellular Carcinoma

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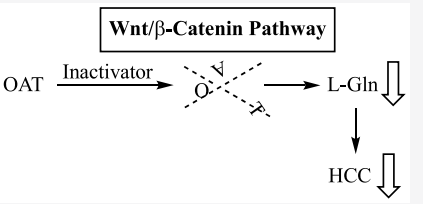
ABSTRACT: Hepatocellular carcinoma (HCC) is the second or third leading cause of cancer mortality worldwide (depending on which statistics are used), yet there is no effective treatment. Currently, there are nine FDA-approved drugs for HCC, five monoclonal antibodies and four tyrosine kinase inhibitors. Ornithine aminotransferase (OAT) has been validated as a target in preclinical studies, which demonstrates that it is a potential target to treat HCC. Currently, there are no OAT inactivators in clinical trials for HCC. This Innovation describes evidence to support inhibition of OAT as a novel approach for HCC tumor growth inhibition. After the mechanism of OAT is discussed, the origins of our involvement in OAT inactivation, based on our previous work on mechanism-based inactivation of GABA-AT, are described. Once it was demonstrated that OAT inactivation does lead to HCC tumor growth inhibition, new selective OAT inactivators were designed and their inactivation mechanisms were elucidated. A summary of these mechanistic studies is presented. Inactivators of OAT provide the potential for treatment of HCC, targeting the Wnt/β-catenin pathway.



KEYWORDS: *Hepatocellular carcinoma, Ornithine aminotransferase, Wnt/β-catenin, Mechanism-based inactivation, X-ray crystal structure*

Hepatocellular carcinoma (HCC) is the sixth or seventh most common cancer and the second or third leading cause of cancer death worldwide (depending on which statistics are used);[1] over 800 000 people worldwide contract HCC each year, and about 700 000 die. Various current treatments include surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy, stem cell transplant, and precision medicine.[2] Despite these numerous approaches available, prognosis for recovery is very poor,[3,4] with an overall 5-year survival rate of <10%[5] (33% for localized HCC, 11% for spread to nearby structures, and 2% if distant spreading[6]), and side effects are extensive, largely because HCC is highly chemotherapy-[7] and radiation-therapy[8]-resistant. Although there are nine FDA-approved drugs for hepatocellular carcinoma,[9] the HCC market still has a high unmet need because of the lack of efficacy of current treatments. Well over 150 clinical trials for HCC have been terminated or withdrawn, mostly because of liver toxicity, flaws in trial design, and marginal potency.

HCC occurs most frequently in Asia and Africa,[10] but because of the spread of hepatitis B and hepatitis C virus infections, which lead to HCC, the incidence of HCC in the United States is rising,[11] where about 90% of primary liver cancers are HCC.[12] HCC also is part of the natural history of nonalcoholic steatohepatitis (NASH).[13] Other causes for the dramatic increase of HCC in the United States include obesity[14] and an increase in diabetes.[15]

SIGNIFICANCE OF THE Wnt/β-CATENIN PATHWAY FOR CANCER GROWTH

Activation of the evolutionarily conserved Wnt/β-catenin signaling pathway[16] in liver[17] correlates with the development of HCC and is essential to normal cellular processes, such as growth, development, survival, and regeneration. The simplified mechanism involves binding of Wnt to co-receptors Frizzled and low-density lipoprotein receptor-related protein5/6 (LRP5/6), which leads to the translocation of β-catenin to the nucleus. Binding of β-catenin to T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors in the nucleus activates transcription of Wnt target genes, such as c-myc, cyclin D1, and AXIN2, important for cell proliferation (Figure 1).[18] However, because of mutations in members of the Wnt/β-catenin pathway, β-catenin also is associated with the initiation and progression of cancer.[19] The development of HCC in liver correlates with the upregulation of Wnt/β-catenin pathway proteins ornithine aminotransferase (OAT), glutamate transporter GLT-1, and glutamine synthetase.[20] OAT converts L-ornithine to L-glutamate, which is transported by GLT-1 to glutamine synthetase to produce L-glutamine. Loss of OAT

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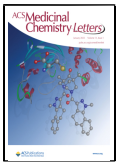

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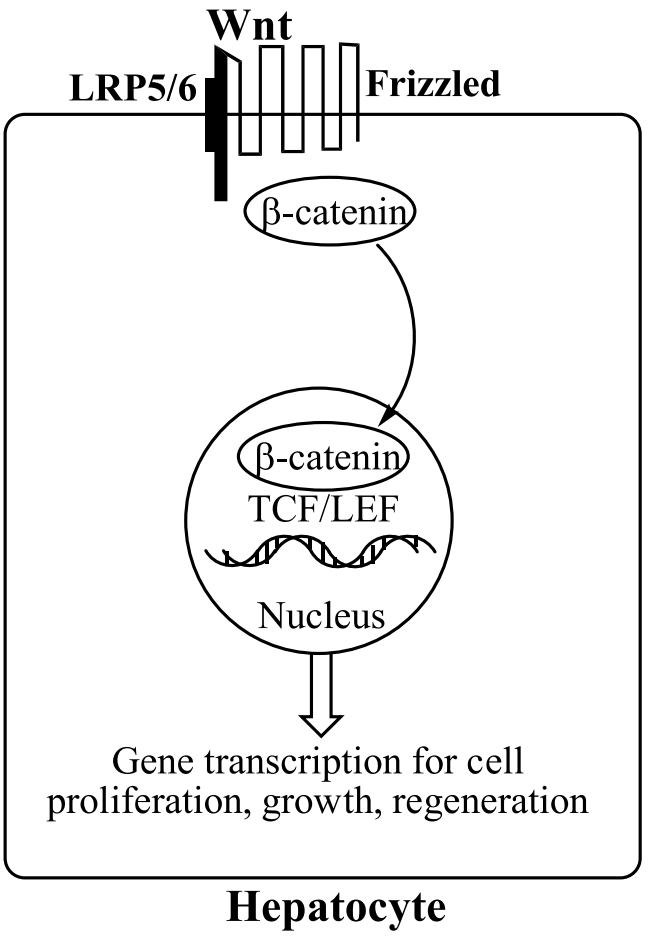


Figure 1. Binding of Wnt to the Frizzled-LRP5/6 co-receptor allows *β*-catenin to be translocated into the nucleus, where it binds to TCF/LEF transcription factors, leading to transcription of Wnt target genes.

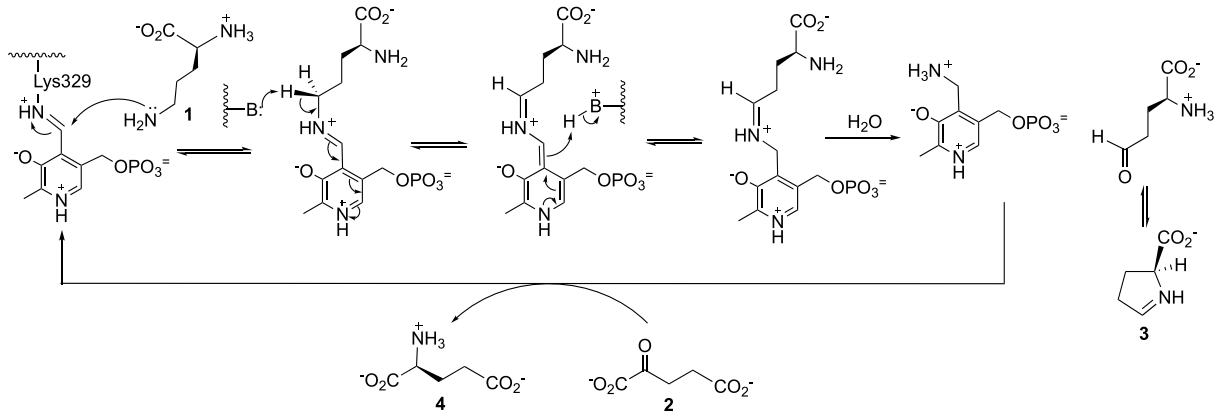
activity shuts down the formation of its product, L-glutamate, and this leads to the blockage of the synthesis of L-glutamine,[21] which is essential for both normal and neoplastic cell growth. Glutamine, however, is taken up more efficiently by tumor cells than normal cells,[22] and tumor growth is more accelerated by glutamine than normal cell growth.[23]

One important function of glutamine is to maintain tricarboxylic acid cycle intermediates (using the carbon atoms of glutamine) and in the biosyntheses of nucleotides, nonessential amino acids, and hexosamine (from the nitrogen atoms in glutamine).[24] Another critical role for glutamine is to suppress oxidative stress by its catabolism to glutathione, an important intracellular antioxidant.[25] Consequently, the survival and proliferation of tumor cells depend on a continuous supply of glutamine. As in the case of glucose metabolism, increased glutamine uptake is controlled by oncogenes, such as c-myc,[26] and the inhibition of glutamine biosynthesis by oncogenes inhibits the growth of cancer.[27] The increased requirement for glutamine by cancer cells is for the purpose of supporting anabolic processes that stimulate proliferation.[28] An increase in glutamine supply by enhancement of OAT activity resulting from Wnt/*β*-catenin activation leads to an increase in tumor cell growth, independent of glutamine supply. Therefore, OAT inhibition to decrease glutamine production has been suggested as a viable mechanism to inhibit tumor growth.[29] Also, it has been found that OAT is required to establish spindle formation in cancer cells, again indicating that inhibition of OAT should be an effective cancer treatment.[30]

■ ORNITHINE AMINOTRANSFERASE

As shown in Scheme 1, OAT, a pyridoxal 5′-phosphate (PLP)-dependent mitochondrial enzyme, catalyzes the reversible interconversion of ornithine (**1**) and *α*-ketoglutarate (*α*-KG, **2**) to L-glutamate semialdehyde, which cyclizes to Δ^1-pyrroline-5-carboxylate (P5C, **3**), and L-glutamate (**4**). OAT also is involved in proline and hydroxyproline metabolism in cancer cell survival, proliferation, and metastasis.[31] Both proline and glutamine are important molecules for tumor growth,[32] and they are interconvertible and linked in their metabolism. Under a hypoxic microenvironment proline metabolism is markedly altered in HCC, with accelerated utilization of proline and accumulation of hydroxyproline, which correlates with secretion of alpha-fetoprotein, a biomarker for HCC, and poor prognosis in disease progression. Accumulation of hydroxyproline further promotes HCC growth and resistance to sorafenib, a primary chemotherapeutic agent for HCC.[33] There is considerable interplay among ornithine, proline, and glutamine, and their metabolites, *α*-KG and P5C, in the regulation of OAT and/or proline dehydrogenase/oxidase-dependent proliferation, apoptosis/autophagy, and metastasis.[34]

Scheme 1. Conversion of L-Ornithine to L-Glutamate Catalyzed by Ornithine Aminotransferase (OAT)



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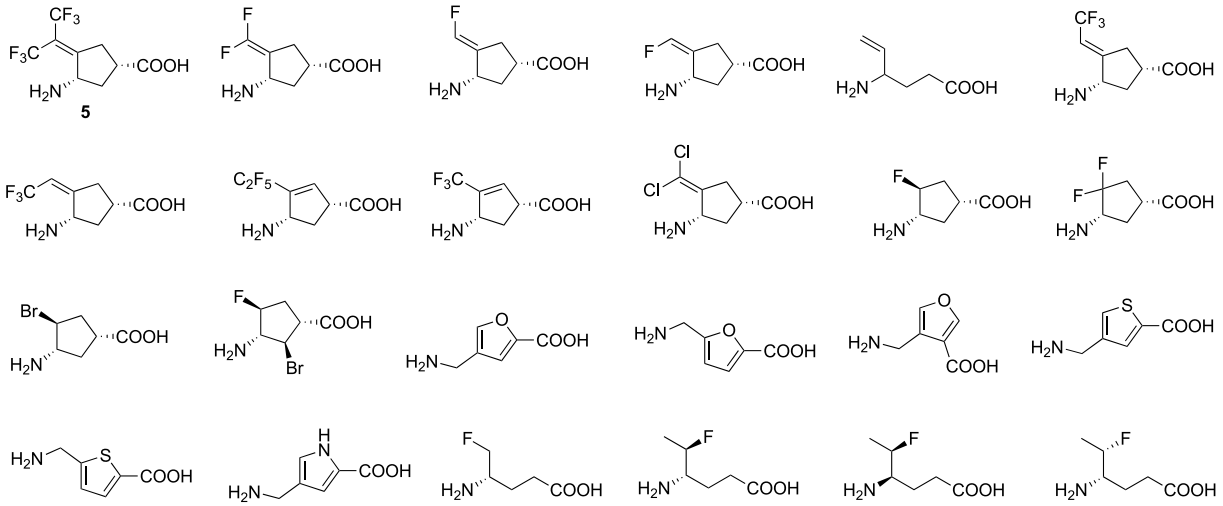

Figure 2. GABA analogues screened for OAT activity. Figure modified from ref 35. Copyright 2015 American Chemical Society.

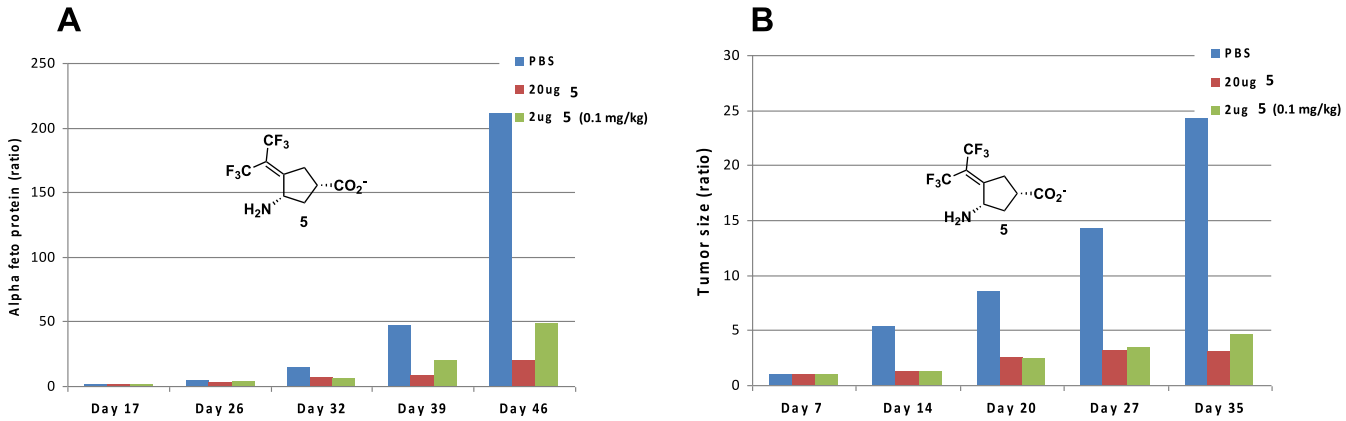

Figure 3. (A) Administration of **5** inhibits serum AFP secretion in vivo. Mice were treated for 27 days, 3 times a week, starting 3 weeks following HCC transplantation with two doses of **1** (0.1 mg/kg [2 μg], green bars or 1 mg/kg [20 μg], red bars), compared with untreated controls (blue bars). Levels are normalized to the starting day of therapy. (B) Compound **5** diminishes tumor volume in both treated groups (0.1 mg/kg [2 μg], green bars and 1.0 mg/kg [20 μg], red bars) compared to untreated controls (blue bars). Figure reproduced from ref 35. Copyright 2015 American Chemical Society.

FROM GABA AMINOTRANSFERASE INACTIVATORS TO OAT INACTIVATORS

About 15 years ago I received an email message from Dr. Yaron Ilan, Professor of Medicine in the Gastroenterology & Liver Units of Hebrew University-Hadassah Medical Center in Jerusalem, asking if I would be willing to send him some of my γ-aminobutyric acid aminotransferase (GABA-AT) inactivators to test in his HCC mouse model. He explained that a DNA microarray analysis was carried out of genes in the liver of a normal rat compared with those in the liver of a rat (*Psammomys obesus*) that spontaneously develops HCC, and seven genes, including the gene encoding for OAT, were overexpressed.[35] He said he wanted to test an OAT inhibitor, but he did not have access to one, and his research assistant had seen my work on GABA-AT inactivators and thought they might work. At first, I thought that it would be futile to test GABA analogue inactivators with OAT because of the low overall sequence identity (17%)[36] between the two enzymes. However, a closer look at the active sites revealed that the residues in the two enzymes are quite similar[37] and share a high structural homology.[38] Also, OAT is a member of the evolutionary aminotransferase subgroup of PLP-dependent enzymes that includes GABA-AT,[39] an enzyme found in glial cells and presynaptic neurons;[40] all aminotransferases have very similar catalytic mechanisms.[41] They both transfer the amino group of their respective substrates (ornithine for OAT and GABA for GABA-AT) to α-ketoglutarate, giving succinic semialdehyde (from GABA)[41] or glutamate γ-semialdehyde (from ornithine)[42] and L-glutamate.[43] But what really got my attention was when I found out that HCC is the most common primary liver malignancy and one of the principal causes of cancer death worldwide.[1] However, I thought I should not just send my most potent GABA-AT inactivators because the active sites are not identical between GABA-AT and OAT. Instead, I chose to send a mixture of mediocre potency, low potency, and inactive inhibitors in addition to potent ones (Figure 2).

OAT INACTIVATORS INHIBIT HCC GROWTH

Using these compounds, we showed, for the first time, that OAT inactivators significantly suppress alpha-fetoprotein (AFP) secretion, a biomarker for HCC, in Hep3B and HepG2 cells; the most potent OAT inactivator at that time (**5**, Figure 2), which was one of the compounds that theoretically could inactivate GABA-AT but did not and, therefore, was OAT selective, significantly suppressed AFP serum levels (Figure 3A) and tumor growth (Figure 3B) in patient-derived HCC-

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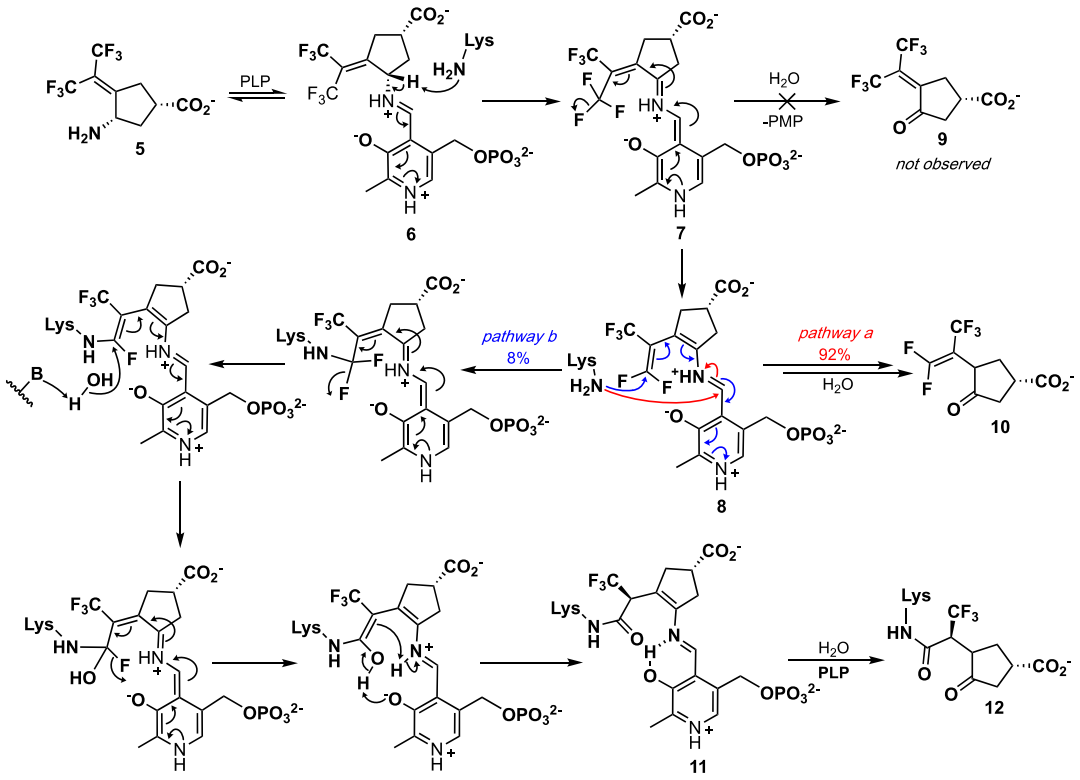
Scheme 2. Inactivation Mechanism for 5 with Human Ornithine Aminotransferase[a]



[a]Scheme reproduced from ref 45. Copyright 2019 American Chemical Society.

harboring mice, even at 0.1 mg/kg.[35] This compound is water-soluble and 42% orally bioavailable, does not inhibit cytochrome P450s or the hERG channel, and is not metabolized by human liver microsomes over 90 min; plasma protein binding is only 62%, maximum tolerated dose is 30 mg/kg (mice), and, in a 7-day repeat study, the no observed adverse effect level (NOAEL) was 10 mg/kg in mice and 30 mg/kg in rats, indicating a therapeutic index of 100−300.

INACTIVATION MECHANISM FOR OUR FIRST OAT INACTIVATOR

Compound **5** is a mechanism-based inactivator[44] of OAT, and the inactivation mechanism, shown in Scheme 2, was elucidated by a combination of intact protein mass spectrometry and crystallography as well as the use of a fluoride ion electrode, partition ratio determination (the number of equivalents of **5** converted to product per inactivation event), and metabolomics.[45] Compound **5** forms a Schiff base with coenzyme PLP to give **6**. Deprotonation to **7** followed by fluoride ion elimination gives **8**. Intermediate **7** does not undergo hydrolysis to **9**, but in 92% of the turnovers **8** is hydrolyzed to **10**. The remainder of turnovers (8%) results in inactivation from Michael addition of Lys-292 with a loss of two fluoride ions followed by hydrolysis to give covalent adduct **11**; hydrolysis of the PLP imine gives **12**. Structure **11** was confirmed by intact protein mass spectrometry and the crystal structure shown in Figure 4.

From the crystal structure it is apparent that only the *syn*-CF_3 group is involved in the inactivation chemistry of **5**. The corresponding *Z*-(**13**) and *E*-monotrifluoromethyl (**14**) analogues, however, had previously been shown to inactivate OAT with comparable inactivation kinetics.[35]

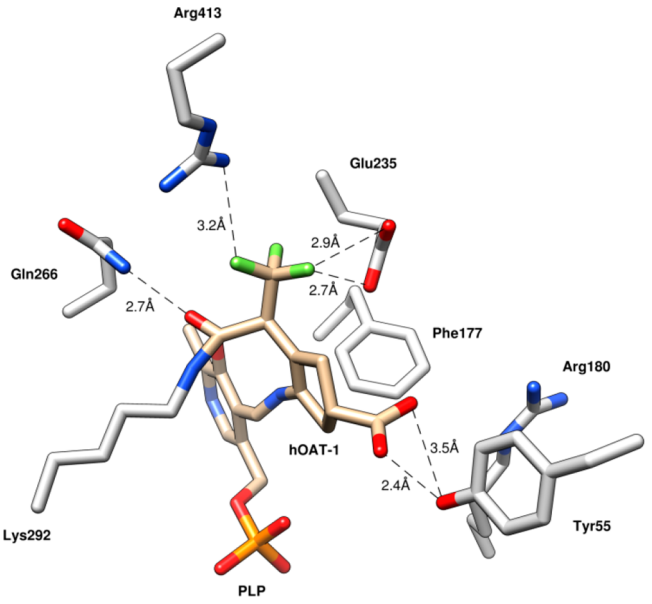


Figure 4. X-ray crystal structure of OAT following inactivation by **5** (PDB code: 6OIA). Figure reproduced from ref 45. Copyright 2019 American Chemical Society.

This was rationalized as the result of interconversion of the intermediates derived from **13** and **14** during inactivation. To prevent rotation and interconversion, the corresponding *syn*-(**15**) and *anti*-monotrifluoromethyl esters (**16**) were synthesized (modeling showed that the ester group could bind with the enzyme to prevent rotation), and, indeed, **15** was comparable in inactivation efficiency to **5** and **16** was inactive.

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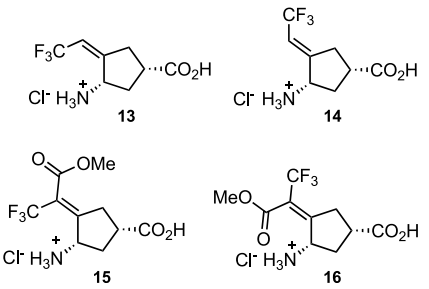
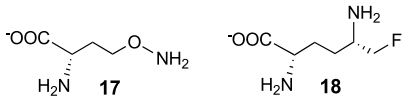


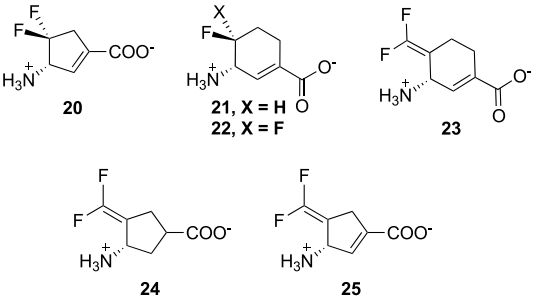
EARLY INACTIVATORS OF OAT

Compound **5** is not the first compound to inactivate OAT; L-canaline ((*S*)-2-amino-4-amino-oxybutyric acid, **17**), a natural product analogue of ornithine,[46] and L-fluoromethylornithine (5-FMO; **18**)[47] were reported in 1965 and 1988, respectively, as inactivators of OAT. However, **17** was shown to strongly inhibit the activity of seven PLP-dependent enzymes,[48] which could account for its toxicity.[49] The alkoxylamine group of **17** forms an oxime with the PLP coenzyme of OAT, causing irreversible inhibition.[50] The crystal structure of **17** with human OAT confirmed the oxime structure.[51] 5-FMO (**18**) also irreversibly inhibits OAT, but selectively, and long-term inhibition of OAT does not produce apparent detrimental effects in mice,[52] possibly because 10−20% of OAT is refractory to inactivation by **18**.[53] Of the four possible stereoisomers of 5-FMO, only the (2*S*,5*S*) isomer inactivates OAT.[54] The crystal structure of human OAT inactivated by 5FMO[55] shows that the PLP is modified to give the expected product of mechanism-based inactivation by an enamine mechanism (**19**, Scheme 3).[54] The enamine inactivation mechanism was first introduced by Metzler and co-workers for two other PLP-dependent enzymes, glutamate decarboxylase[56] and aspartate aminotransferase,[57] both inactivated by L-serine *O*-sulfate.

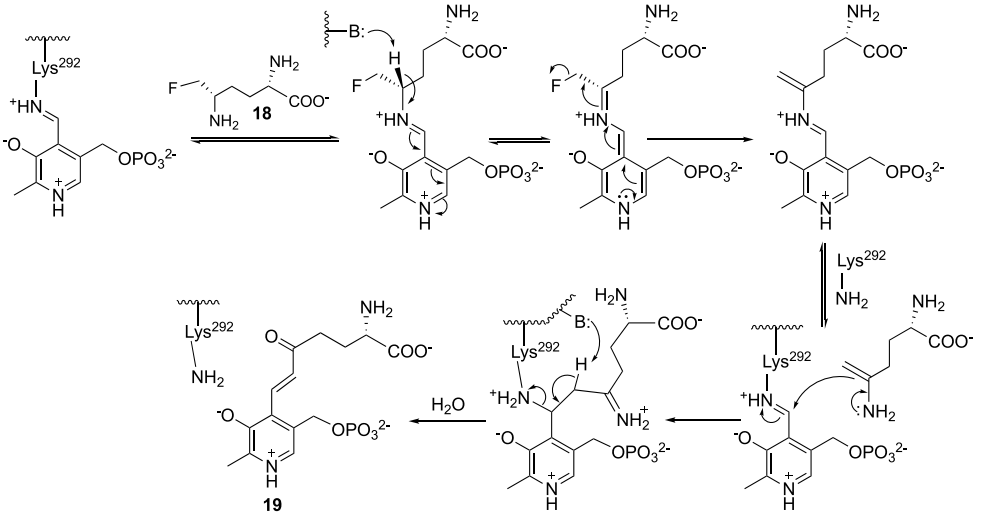


NEWER OAT INACTIVATORS AND INACTIVATION MECHANISMS

We have designed several new OAT mechanism-based inactivators on the basis of organic chemical intuition and computer modeling, including **20**−**23**. (*S*)-3-Amino-4,4-difluorocyclopent-1-enecarboxylic acid (**20**)[58] is an irreversible inhibitor of OAT, comparable in inactivation efficiency to **5**. The corresponding cyclopentane analogue is a very weak inactivator, but that was expected on the basis of our earlier studies showing that incorporation of a double bond into CPP-115 (**24**)[59] to give OV329 (**25**) enhances inactivation efficiency 10-fold.[60] With the aid of intact protein mass spectrometry, metabolomics, and protein crystallography, the turnover and inactivation mechanisms for **20** were elucidated (Scheme 4).[61] Transient-state kinetic measurements, using rapid-mixing spectrophotometry, were used that supported an initial direct E2 elimination of HF from the PLP complex to give **27** (pathway a) over the typical E1cB mechanism (**26**, pathway b). The major pathway (97%) from **27** involves an attack by Lys292 to release, after hydrolysis of the enamine, **28** (pathway c); only 3% of turnovers proceed by water attack to give **29** (pathway d). The loss of a fluoride ion from **29** gives **30**, which, based on transient-state spectrophotometry and intact protein mass spectrometry, undergoes tautomerization to give noncovalent adducts **31**/**32** 84% of the turnovers (pathway e) and attack by Lys292 to give covalent adducts **33**/**34** 16% of the turnovers (pathway f). In the conversion of **27** to **28**, evidence for Lys292 attack (pathway c) was obtained by soaking **20** into OAT crystals and determining the X-ray crystal structure (Figure 5A); the co-crystallization of **20** with OAT gave the structures of **31**/**32** (Figure 5B).



Scheme 3. Mechanism of Inactivation of OAT by 5-Fluoromethylornithine (18)



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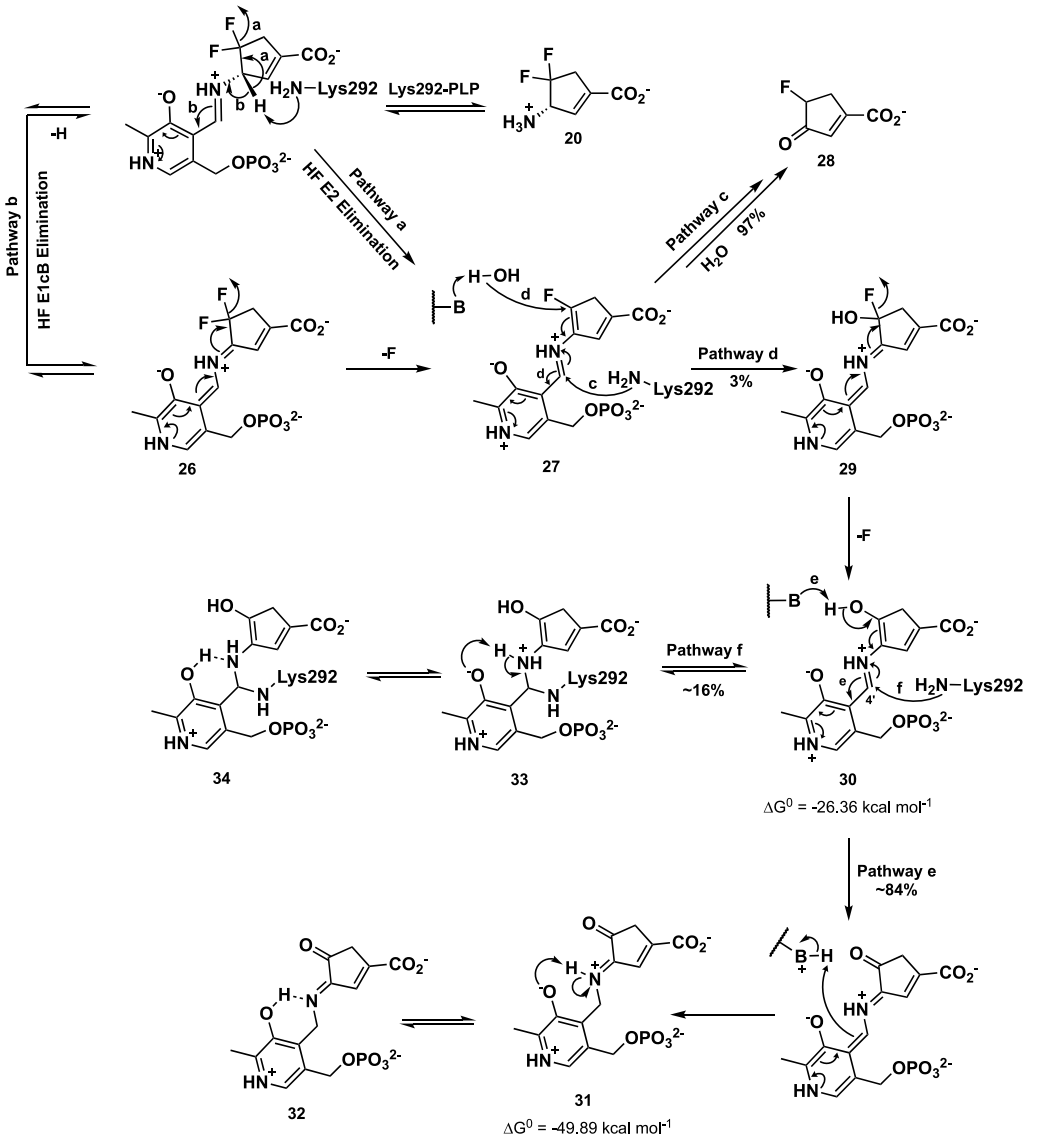
Scheme 4. Mechanism Proposed for the Inactivation of OAT by 20[a]



[a]Scheme modified from ref 61. Copyright 2021 American Chemical Society.

Because of the larger active site pocket of OAT relative to that of GABA aminotransferase (GABA-AT), CPP-115[35] and OV329[60a] are nonselective inactivators of both enzymes. By increasing the size of the fluorine substituents on these inactivators to trifluoromethyl groups (to give **5**), selective OAT inactivation was realized. Another approach to gain selectivity over GABA-AT based on size was taken by increasing the ring size from cyclopentene to cyclohexene (**21**/**22**).[62] Kinetic analyses revealed that **21** was 2.9 times more efficient as an inactivator of OAT than **5** and **22** is 23.4 times more efficient than **5**. Although **21** and **22** also inactivate GABA-AT, they do so much less efficiently: 65.6 times and 13.3 times less efficiently, respectively. With the aid of fluoride ion release studies, intact protein mass spectrometry, and protein crystallography, inactivation mechanisms were proposed for **21** and **22**. Three potential mechanisms are shown in Scheme 5. They all are initiated by Schiff base formation with the active site PLP followed by deprotonation and elimination of a fluoride ion to give **35**. Pathway a is an enamine mechanism; when X = H, enamine adduct **36** results, and when X = F enamine addition is followed by elimination of HF and aromatization (**37**). Pathway b results in aromatization without loss of X (**38**). Pathway c is a Michael addition mechanism, leading to either covalent adduct **39** when X = H or aromatized adduct **40** when X = F. On the basis of intact protein mass spectrometry, **21** formed a covalent adduct with OAT with an additional mass (369.29) that corresponds to either **36a** in pathway a or **39** (pathway c, Scheme 5); **22** added an additional 366.34 mass units to OAT, which corresponds to either **37** (pathway a) or **40** (pathway c). Protein crystallography clarified the structures, clearly showing adduct **36a** (pathway a) from **21** and adduct **40** (pathway c) from **22** (Figure 6). A difference of a single fluorine atom made the difference between an enamine mechanism (**21**) and a Michael addition/aromatization mechanism (**22**). Furthermore, shrinkage of the ring from cyclohexene (**22**) to cyclopentene (**20**) was sufficient to again change the inactivation mechanism (Scheme 4), this time to Michael addition by water followed by tautomerization (**31**/**32**) or attack by Lys292 (**33**/**34**). The major pathway (97%) for **20**, however, was the release of metabolite **28** (pathway c).

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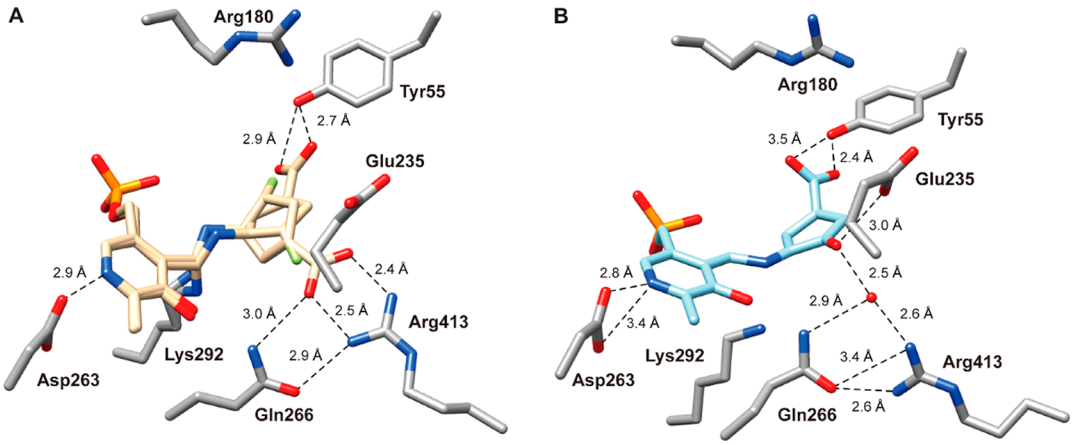

Figure 5. (A) Crystal structure of OAT resulting from soaking of **20** over 1 h, shown in two alternate conformations (beige): one in which the carboxylate group interacts with Tyr55 (conformation A) and the other in which the carboxylate forms a salt bridge with Arg413 (conformation B); PDB code: 7LK1. (B) Co-crystal structure of OAT with **20**; PDB code: 7LK0. Carbon atoms in the residues are colored gray, nitrogen in dark blue, and oxygen in red; the water molecule is shown as a red sphere. Hydrogen bonding distances between atoms are in Ångstroms (Å) and are shown as black dashed lines. Figure reproduced ref 61. Copyright 2021 American Chemical Society.

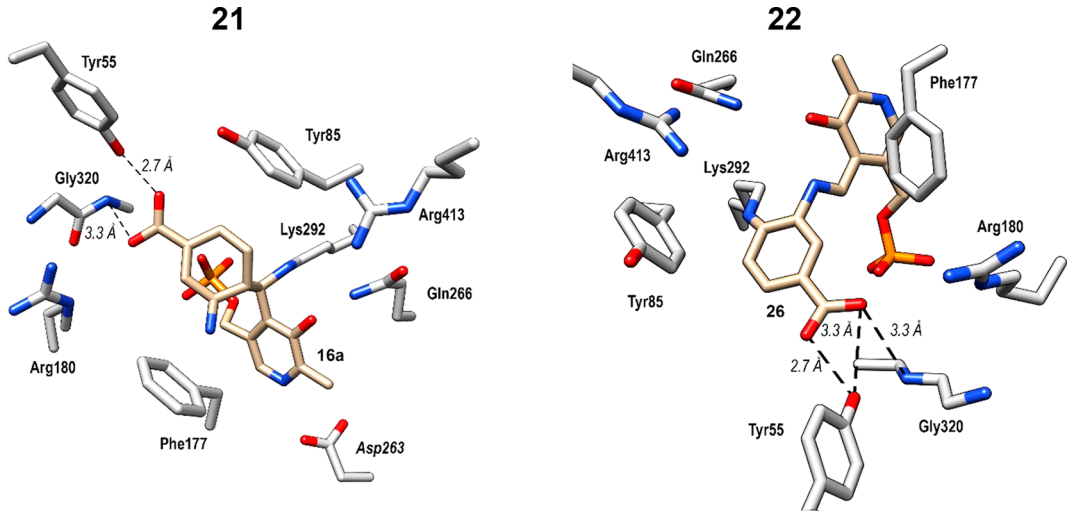

Figure 6. Crystal structures of **21** (PDB code: 6 V8D) and **22** (PDB code: 6 V8C) cocrystallized with OAT. Amino acid residues are in silver; compounds bound to PLP are in gold; oxygen is red, and nitrogen is blue. Figure reproduced from ref 62. Copyright 2020 American Chemical Society.

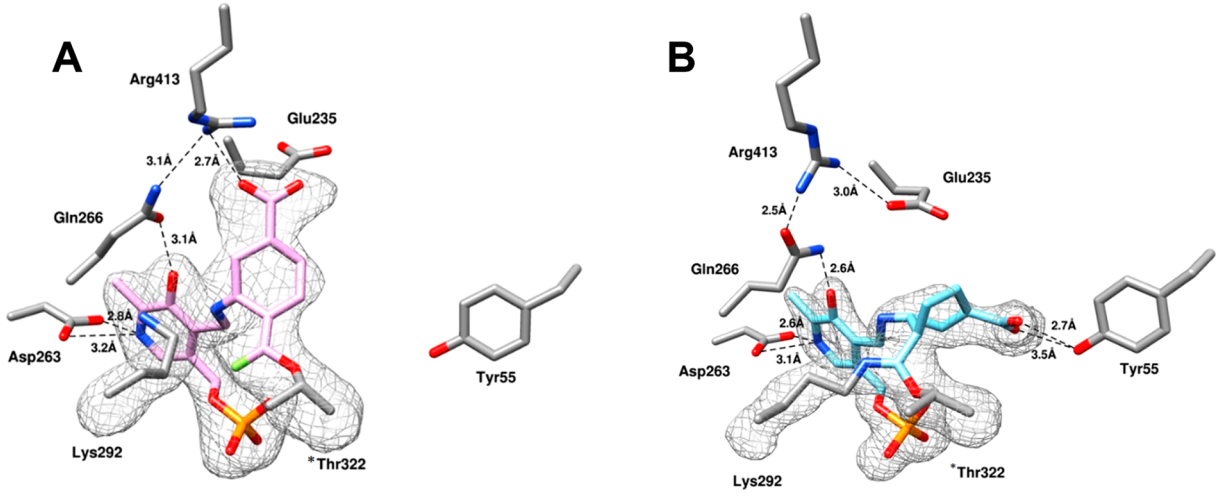

Figure 7. (A) X-ray crystal structure of first intermediate (**50**) from soaking **23** into OAT; PDB code: 7LOM. (B) X-ray cocrystal structure of double covalently bonded **23** (**52**) with Thr322 and Lys292 in OAT; PDB code: 7LON. Figure reproduced from ref 63. Copyright 2021 American Chemical Society.

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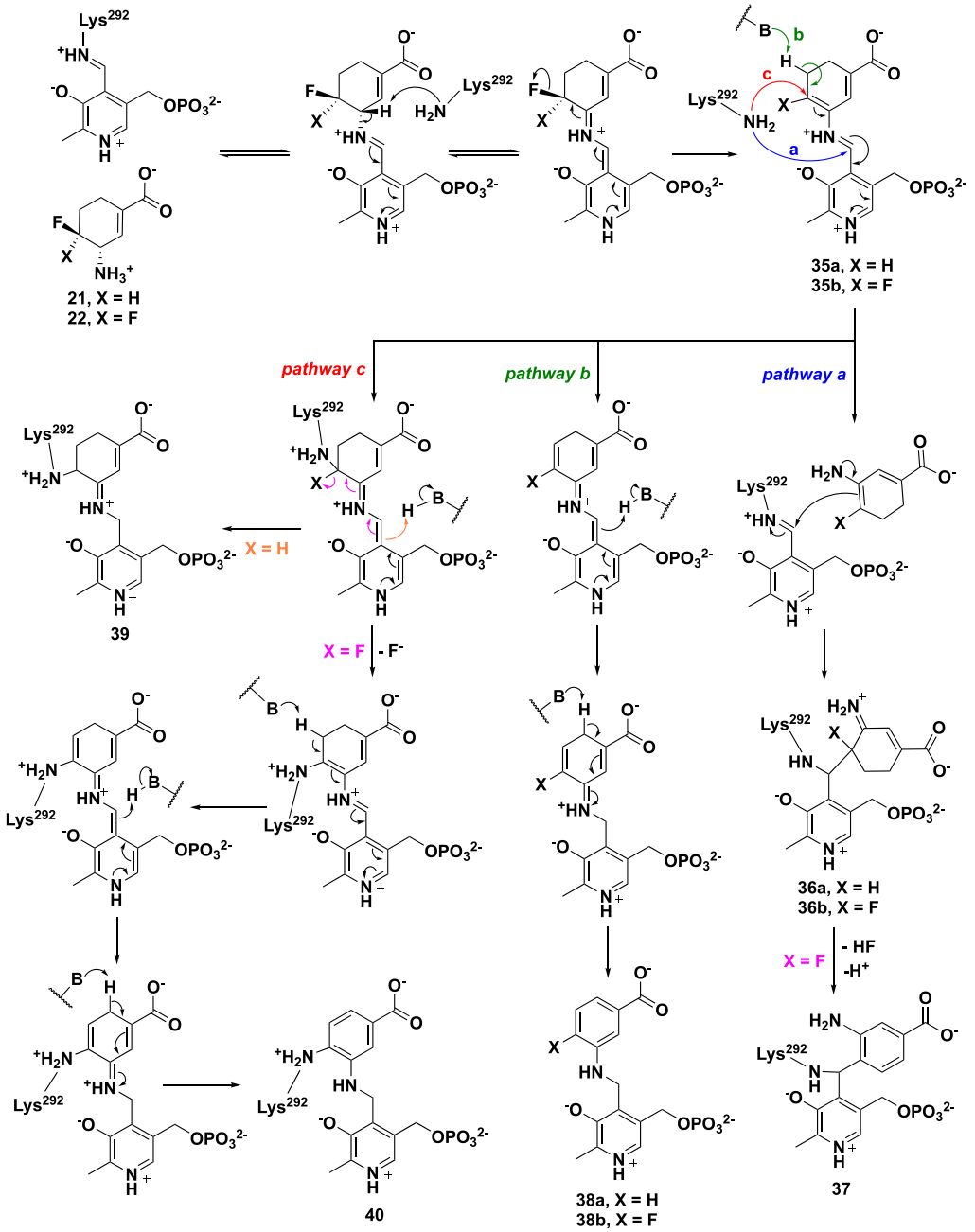
Scheme 5. Possible Inactivation Mechanisms by 21 and 22[a]



[a]Scheme reproduced from ref 62. Copyright 2020 American Chemical Society.

A chimera of **22** and OV329 (**25**) resulted in **23**, which was expected, if it were an inactivator, to have an inactivation mechanism similar to that for **22** or **25**. Compound **23** is 22 times more efficient as an inactivator of OAT than **5** and comparable to **22**; however, another completely different inactivation mechanism was revealed.[63] On the basis of native mass spectrometry (to preserve noncovalent substrate binding and protein quaternary structure), intact protein mass spectrometry (to identify covalent adducts), top-down tandem mass spectrometry (to fragment the intact protein), protein crystallography, and metabolomics, the most plausible mechanisms for turnover and inactivation are shown in Scheme 6. The methods used to identify each of the intermediates and final adducts are given next to the structures. Schiff base formation of **23** with the active site PLP gives **41**. Deprotonation to **42** leads to partitioning of the pathway, with 63% (pathway a) leading to inactivation intermediate **43** and 37% (pathway b) leading to turnover intermediate **44**. Attack by Lys292 on **44** gives **45**, which eliminates F^- to give **46**; deprotonation leads to aromatic metabolite **47**. Inactivation intermediate **43** undergoes bond rotation because of steric hindrance with the difluoromethylenyl group to give **48**. Tautomerization results in reactive intermediate **49**, which first is attacked by Thr322, displacing a F^- to give **50**, whose crystal structure was obtained by soaking **23** into crystalline OAT (Figure 7A). Attack by Lys292 gives intermediate **51**; protonation leads to the final double covalent adduct (**52**, Figure 7B), the first example of a mechanism-based inactivator leading to a single adduct with two covalent bonds

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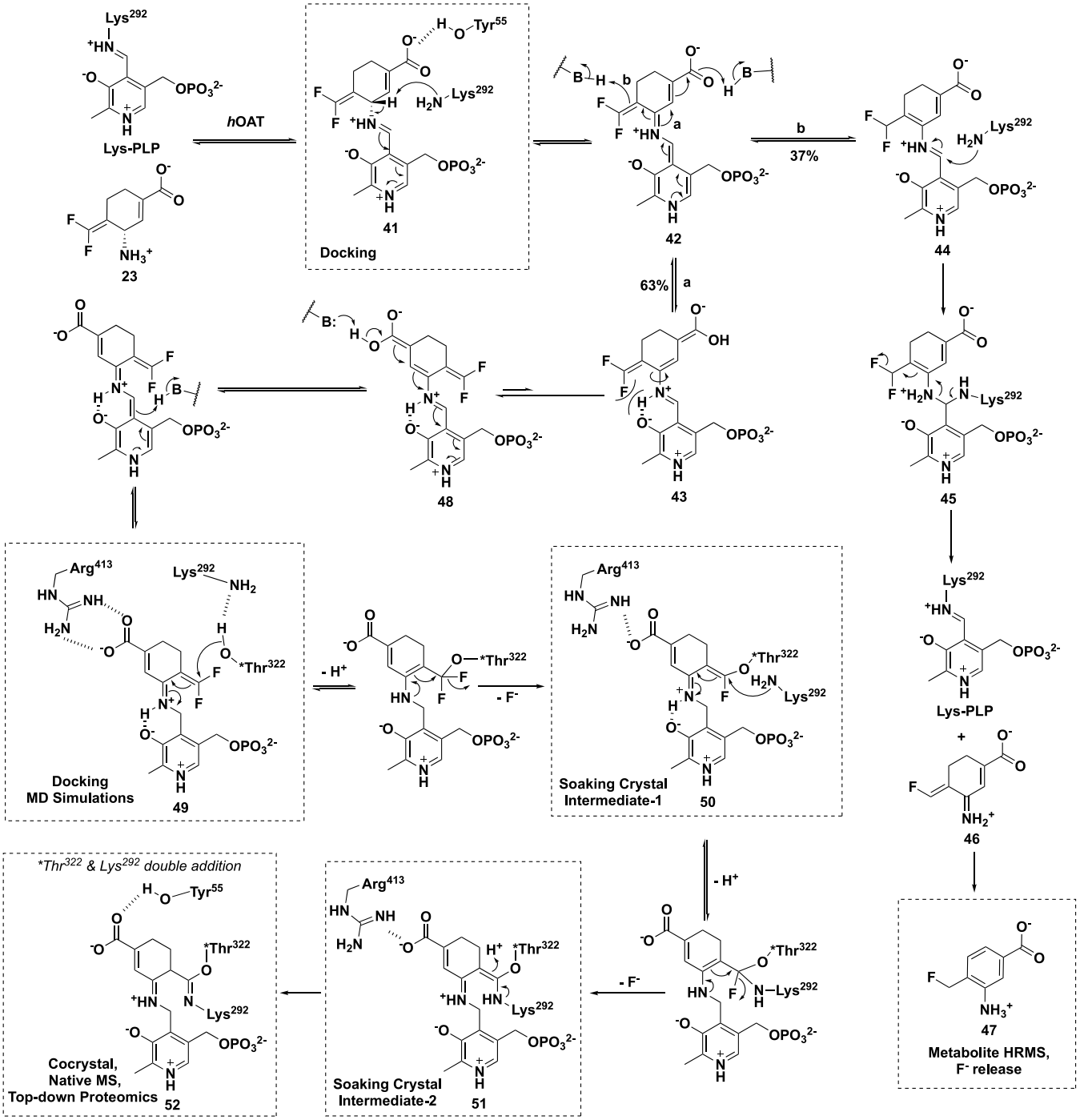
Scheme 6. Plausible turnover and Inactivation Mechanisms for the Inactivation of OAT by 23[a]



[a]Scheme modified from ref 63. Copyright 2021 American Chemical Society.

from two different active site residues, one of which is not catalytic.

The compounds described above have been designed to allow for a variety of potential inactivation mechanisms to occur. Inactivators with related structures demonstrate how minor structural differences can have a major impact on inactivation mechanisms. Enzymes evolve to catalyze highly specific chemistry on specific molecules, but when enzymes are presented with compounds having unnatural structures, unexpected catalytic chemistry can result. This exemplifies the ongoing struggle between Man and Nature, each trying to outwit the other. With the assistance of mechanism-based inactivators, it is sometimes possible to get the better of Mother Nature.

Currently, there are nine FDA-approved drugs for HCC. Five are monoclonal antibodies that either act as checkpoint inhibitors to trigger the immune system to fight the cancer or act by binding to VEGF, leading to a reduction in microvascular growth of tumor blood vessels and limiting the blood supply to the tumor. The other four drugs are tyrosine kinase inhibitors. OAT is a novel target for drugs to treat HCC. Inactivators of OAT provide the promise of treatment for this dreaded disease by targeting a yet untapped point of a new pathway, the Wnt/β-

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catenin pathway. Possibly a combination of drugs acting on different pathways will be the way forward to successfully inhibit progression of this disease.

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Notes

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ABBREVIATIONS

AFP, alpha-fetoprotein; α-KG, α-ketoglutarate; 5-FMO, L-fluoromethylornithine; GABA-AT, gamma-aminobutyric acid aminotransferase; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; hERG, human ether-à-go-go-related gene; NASH, nonalcoholic steatohepatitis; NOAEL, no observed adverse effect level; OAT, ornithine aminotransferase; PLP, pyridoxal 5′-phosphate; P5C, Δ^1-pyrroline-5-carboxylate; VEGF, vascular endothelial growth factor

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