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2′,6′-Dihalostyrylanilines, Pyridines, and Pyrimidines for the Inhibition of the Catalytic Subunit of Methionine S-Adenosyltransferase-2

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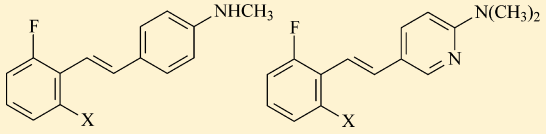
Supporting Information

ABSTRACT: Inhibition of the catalytic subunit of the heterodimeric methionine S-adenosyl transferase-2 (MAT2A) with fluorinated *N,N*-dialkylaminostilbenes (FIDAS agents) offers a potential avenue for the treatment of liver and colorectal cancers where upregulation of this enzyme occurs. A study of structure−activity relationships led to the identification of the most active compounds as those with (1) either a 2,6-difluorostyryl or 2-chloro-6-fluorostyryl subunit, (2) either an *N*-methylamino or *N,N*-dimethylamino group attached in a *para* orientation relative to the 2,6-dihalostyryl subunit, and (3) either an *N*-methylaniline or a 2-(*N,N*-dimethylamino)pyridine ring. These modifications led to FIDAS agents that were active in the low nanomolar range, that formed water-soluble hydrochloride salts, and that possessed the desired property of not inhibiting the human hERG potassium ion channel at concentrations at which the FIDAS agents inhibit MAT2A. The active FIDAS agents may inhibit cancer cells through alterations of methylation reactions essential for cancer cell survival and growth.



INTRODUCTION

The development of antineoplastic agents with novel molecular targets opens the door to new, potentially valuable treatment strategies. We previously reported the effect of difluorinated *N,N′*-dialkylaminostilbenes (FIDAS agents) on the proliferation of colon and liver cancer cells. We identified (*E*)-4-(2′,6′-difluorostyryl)-*N,N*-dimethylaniline (**FIDAS 1a**, Figure 1) as a lead structure with in vitro activity in LS174T cells in the low micromolar range[1−3] and utilized a biotinylated analogue of this FIDAS agent to identify the catalytic subunit of the heterodimeric enzyme, methionine S-adenosyl transferase-2 (MAT2A), as the sole binding partner.[2] We validated MAT2A as the target by suppression using shRNA, which demonstrated in vitro depression in S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) levels in cells exposed to FIDAS agents. Also, we demonstrated in vivo activity of these FIDAS agents on human colon cancer using mouse xenograft studies[1] and in a three-dimensional culture model of primary CRC organoids that mimics the microenvironment of tumors (Supporting Information Figure S2). Other reports describe hydroxy- or methoxy-substituted stilbenes as potential antineoplastic agents,[4−10] but experience directed our study of structure−activity relationships (SAR) away from oxygenated stilbenes because of their potential for redox reactions and off-target biological effects.

Since FIDAS agents offer a potentially new avenue for the treatment of liver and colorectal cancers where MAT2 levels are upregulated,[11−14] we sought analogues of **FIDAS 1a** with improved (nanomolar) potency, water solubility, and the desired property of not interacting with the human ether-à-go-go-related (hERG) potassium channel. In general, drug candidates must avoid hERG activation to progress toward investigational new drug (IND) status, and our commitment to moving FIDAS agents down this pathway led us to incorporate hERG testing in our evaluation of candidates produced in this SAR study. Binding to the hERG channel results in QT interval prolongation in the electrocardiogram and adverse cardiac events.[15] Drug-induced ventricular fibrillation, in these cases, may lead to sudden death and hence the need to identify drug candidates that do not bind hERG at concentrations of the FIDAS agents that inhibit MAT2A.

RESULTS

Synthesis of FIDAS Agents. A Wadsworth−Emmons condensation of 2,6-dihalobenzyl diethyl phosphonates with the appropriate 4-(*N,N*-dimethylamino)benzaldehydes secured the (*E*)-4-(2′,6′-dihalostyryl)-*N,N*-dimethylanilines (**1a−3a**) (Figure 2).[17] Demethylation of **1a−3a** using cyanogen bromide[18] secured the (*E*)-4-(2′,6′-dihalostyryl)-*N*-methylanilines (**1b−3b**). Synthesis of the corresponding pyridine and pyrimidine analogues in the *N,N*-dimethylamino series also employed the Wadsworth−Emmons condensation of the 2,6-dihalobenzyl diethyl phosphonates with the appropriate

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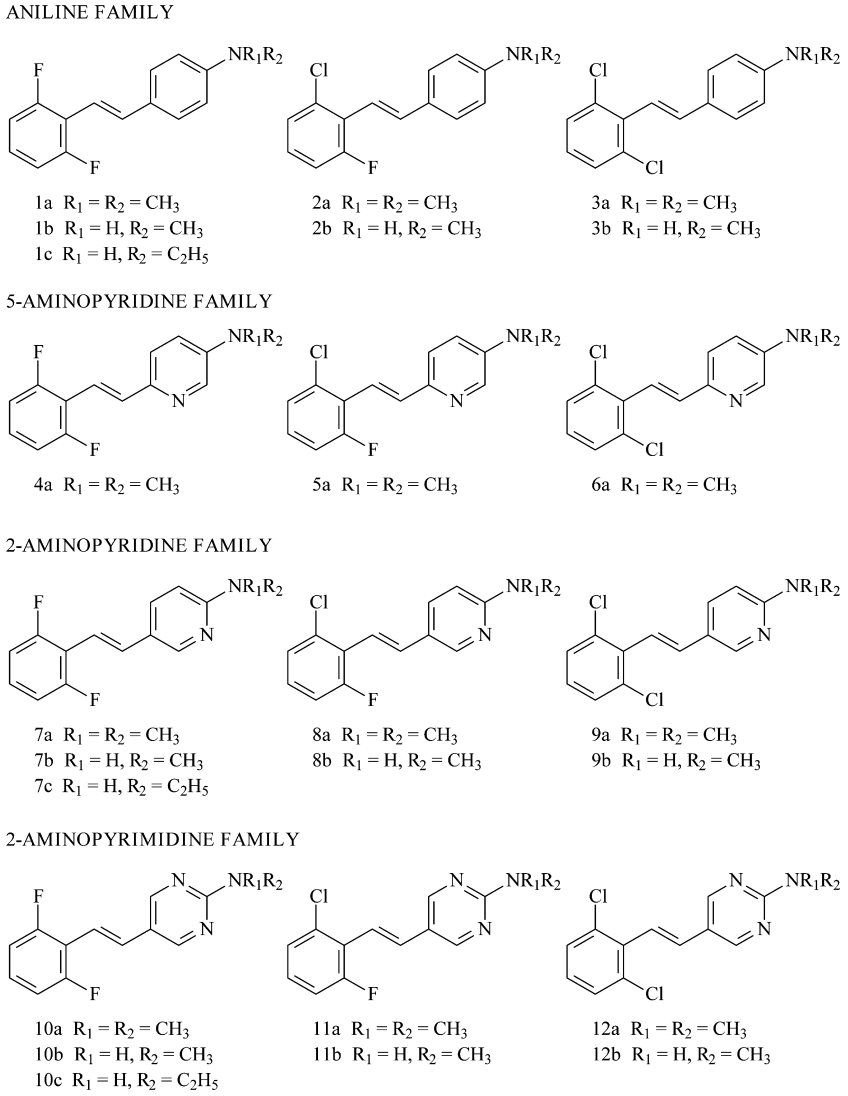

Figure 1. FIDAS agents: Top row contains FIDAS agents in the aniline family; second row contains FIDAS agents in the 5-aminopyridine family; third row contains FIDAS agents in the 2-aminopyridine family; and bottom row contains FIDAS agents in the 2-aminopyrimidine family.

pyridine-2-carboxaldehyde, pyridine-3-carboxaldehyde, and pyrimidine-5-carboxaldehyde and secured (*E*)-2-(2′,6′-dihalostyryl)-5-(*N*,*N*-dimethylamino)pyridines (**4a**−**6a**), (*E*)-5-(2′,6′-dihalostyryl)-2-(*N*,*N*-dimethylamino)pyridines (**7a**−**9a**), and (*E*)-5-(2′,6′-dihalostyryl)-2-(*N*,*N*-dimethylamino)pyrimidines (**10a**−**12a**), respectively (Figure 2). Synthesis of the corresponding pyridine and pyrimidine analogues in the *N*-methylamino series employed the Wadsworth−Emmons condensation of the 2,6-dihalobenzyl diethyl phosphonates with the appropriate pyridine-3-carboxaldehyde and pyrimidine-5-carboxaldehyde and secured (*E*)-5-(2′,6′-dihalostyryl)-2-(*N*-methylamino)pyridines (**7b**−**9b**) and (*E*)-5-(2′,6′-dihalostyryl)-2-(*N*-methylamino)pyrimidines (**10b**−**12b**), respectively (Figure 2). Synthesis of **1c** involved the *N*-monoethylation of (*E*)-4-(2′,6′-difluorostyryl)aniline[3] (Figure 3). Synthesis of **7c** involved the initial Wadsworth−Emmons condensation of 2,6-difluorobenzyl diethyl phosphonate with 2-(*tert*-butyloxycarbonyl)aminopyridine-5-carboxaldehyde, *N*-ethylation, and deprotection of the *tert*-butoxycarbonyl group with trifluoracetic acid (Figure 3). Synthesis of **10c** involved the Wadsworth−Emmons condensation of 2,6-difluorobenzyl diethyl phosphonate with 2-(*N*-ethylamino)pyridine-5-carboxaldehyde (Figure 3). No *E*/*Z* isomerization of these FIDAS agents, as determined by ^{1}H NMR of freshly prepared solutions, occurred when samples were stored as solids in the dark at low temperatures. For biological experiments, freshly prepared DMSO stock solutions were prepared, diluted with buffer, and used immediately as described below.

MAT2A Inhibition Assays. Inherent fluorescence of the 2′,6′-difluorostyryl- and 2′-chloro-6′-fluorostyryl-substituted FIDAS agents at 454 nm facilitated the development of a fluorescence anisotropy assay to evaluate the binding between MAT2A and FIDAS agents.[19] Restriction of this assay to only those FIDAS agents that displayed inherent fluorescence led to the application of another, previously reported, MAT2A inhibition assay to evaluate the activity of FIDAS agents.[2] Recombinant MAT2A holoenzyme was purified and used to synthesize SAM from methionine and ATP.[16] For this assay, each FIDAS analogue was preincubated with MAT2A holoenzyme and then mixed with methionine and ATP. Phosphate P_i concentrations were analyzed to determine the level of MAT2A activity.[20] Final 30 μM concentrations of FIDAS agents were used for these experiments, which were repeated in triplicate. The ratio of MAT2A inhibition for

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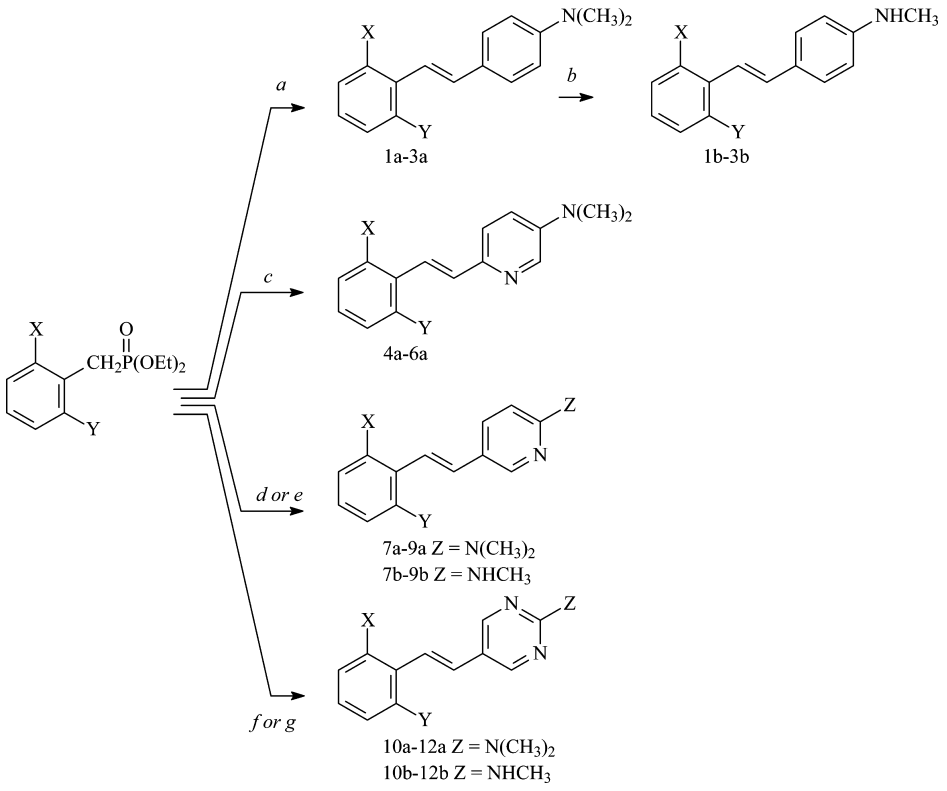

Figure 2. Synthetic route to FIDAS agents with *N*-methylamino or *N*,*N*-dimethylamino groups. Reagents: (a) NaH, DMF followed by 4-$(CH_3)_2N(C_6H_4)CHO$ to give **1a−3a**; (b) CNBr, acetone, 16 h, 56 °C followed by conc. HCl, 3 h, reflux; (c) NaH, DMF followed by 5-$(CH_3)_2N(C_6H_3N)$-2-CHO to give **4a−6a**; (d) NaH, DMF followed by 2-$(CH_3)_2N(C_6H_3N)$-5-CHO to give **7a−9a**; (e) NaH, DMF followed by 2-$CH_3NH(C_6H_3N)$-5-CHO to give **7b−9b**; (f) NaH, DMF followed by 2-$(CH_3)_2N(C_6H_2N_2)$-5-CHO to give **10a−12a**; (g) NaH, DMF followed by 2-$CH_3NH(C_6H_2N_2)$-5-CHO to give **10b−12b**.

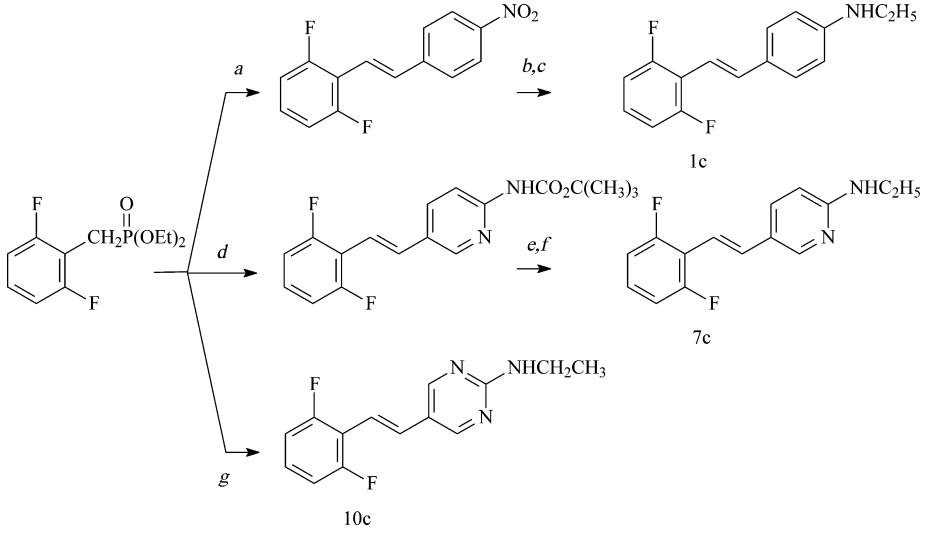

Figure 3. Synthetic route to FIDAS agents with *N*-ethylamino groups. Reagents: (a) NaH, DMF followed by 4-$O_2N(C_6H_4)CHO$; (b) $SnCl_2$, HOAc, conc. HCl; (c) K_2CO_3, CH_3CH_2I, acetone; (d) NaH, DMF followed by 4-*t*BuOCONH$(C_6H_4)CHO$; (e) NaH, C_2H_5I; (f) CF_3CO_2H; (g) NaH, DMF followed by 2-$CH_3CH_2NH(C_6H_2N_2)$-5-CHO.

selected FIDAS agents relative to **FIDAS 1a** is summarized in Figure 4.

In Vitro Testing of FIDAS Agents with LS174T Colorectal Cancer Cells. We treated LS174T colon cancer cells with FIDAS agents **1−3** in the aniline family, **4−6** in the 5-aminopyridine family, **7−9** in the 2-aminopyridine family, and **10−12** in the 2-aminopyrimidine family, and we determined the IC_{50} value of each compound on LS174T cell proliferation (Table 1 and Supporting Information Figure S1). The IC_{50} values were calculated using Prism 5 analysis of the data from the concentration−response curves for each compound. Concentrations of FIDAS agents ranging from 1 to 300 nM were used for these experiments, which were repeated in triplicate at each concentration. Among these four families, only compound **5a** in the 5-aminopyridine family showed an IC_{50} value comparable to that of **FIDAS 1a**. Because this outcome

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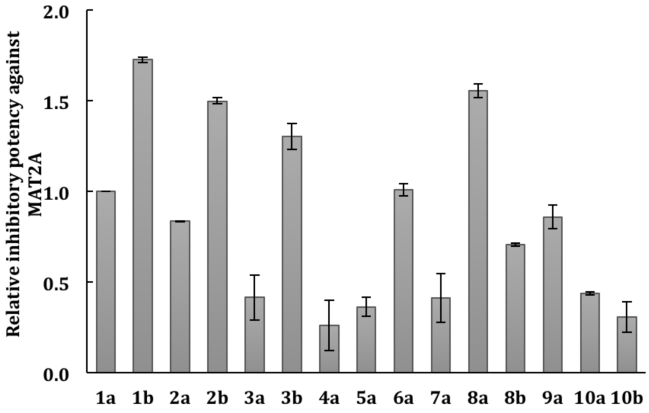

Figure 4. Ratio of MAT2A inhibition for selected FIDAS agents relative to **FIDAS 1a**. Recombinant MAT2A holoenzyme was purified and used to synthesize SAM from methionine and ATP. For inhibition assay, each FIDAS analogue was preincubated with MAT2A holoenzyme and then mixed together with methionine and ATP. The reaction products are SAM and P_i. P_i concentration was analyzed to determine the MAT2A activity. Inhibition activity of each compound was compared with that of **FIDAS 1a**.

Table 1. Comparison of IC_{50} Values for the Inhibition of LS174T Cancer Cells, IC_{50} for hERG Inhibition Values, and the Ratio of IC_{50} for hERG Inhibition to the IC_{50} Values for the Inhibition of LS174T Cancer Cells

FIDAS	inhibition of LS174T cell proliferation IC_{50} (nM)	hERG inhibition IC_{50} (μM)	ratio of IC_{50} for hERG inhibition to IC_{50} for inhibition of LS174T cell proliferation ($\times 10^3$)
1a	26.9 ± 6.5	32.1.1 ± 4.4	1.2
1b	7.6 ± 1.2	49.1 ± 11.1	6.5
1c	199.7 ± 3.3		
2a	19.2 ± 6.4		
2b	7.6 ± 0.5	49.8 ± 5.1	6.6
3a	213.8 ± 7.3		
3b	50.1 ± 6.7		
4a	57.9 ± 11.6	21.1 ± 1.1	0.4
5a	20.4 ± 4.1	20.7 ± 1.8	1.0
6a	59.5 ± 10.2		
7a	13.3 ± 7.8	22.0 ± 3.6	1.7
7b	30.1 ± 5.8		
7c	31.3 ± 5.5		
8a	4.7 ± 0.6	59.5 ± 17.8	12.7
8b	4.0 ± 0.2	19.9 ± 4.1	5.0
9a	30.1 ± 3.1		
9b	19.6 ± 8.5		
10a	>200	40.5 ± 9.7	0.5
10b	>200		
10c	>200		
11a	>200		
11b	>200		
12a	>200		
12b	>200		

did not represent a significant advance, further work with the 5-aminopyridine family was not pursued. Similarly, the 2-aminopyrimidines **10**–**12** displayed IC_{50} values in the 200–900 nM range and also were not pursued in detail.

Replacing the 2′,6′-difluoro-substituents in **FIDAS 1a** with 2′-chloro-6′-fluoro substituents led to compounds with comparable or increased inhibitory activity: IC_{50} value for **1a** ≈ IC_{50} value for **2a**; IC_{50} value for **5a** < IC_{50} value for **4a**; and IC_{50} value for **8a** ≈ IC_{50} value for **7a**. Replacing the 2′,6′-difluoro substituents in **FIDAS 1a** with 2′,6′-dichloro substituents led, in general, to relatively inactive compounds in comparison with that of their difluorinated counterparts: IC_{50} value for **3a** > IC_{50} value for **1a**; IC_{50} value for **6a** ≈ IC_{50} value for **4a**; IC_{50} value for **9a** > IC_{50} value for **7a**; and IC_{50} value for **12a** > IC_{50} value for **10a**.

Replacing the *N*,*N*-(dimethylamino)-substituent in **FIDAS 1a** with an *N*-(methylamino) substituent led, generally, to compounds with increased inhibitory activity: IC_{50} value for **1b** < IC_{50} value for **FIDAS 1a**; IC_{50} value for **2b** ≈ IC_{50} value for **2a**; and IC_{50} value for **3b** < IC_{50} value for **3a**. Replacing the *N*-methylamino substituent in **1b** with the sterically larger *N*-ethylamino-substituent in **1c** led to inactive compounds in some cases (e.g., IC_{50} for **1c** > IC_{50} for **1b**) or compounds with comparable activity (e.g., IC_{50} for **7b** ≈ IC_{50} for **7c**; IC_{50} for **10b** ≈ IC_{50} for **10c**). In summary, the introduction of either the *N*-methylamino substituent in **1b** and **2b** or the introduction of the 2′-chloro-6′-fluorostyryl moiety in **8a** and **8b** led to the most potent compounds with IC_{50} values less than 10 nM (Table 1).

[^{3}H]-Dofetilide Binding to Plasma Membranes Overexpressing the hERG Channel. [^{3}H]-Dofetilide competition binding assays using HEK-293 cell membranes stably expressing the hERG channel (hERG-HEK) correlate well with results from voltage-clamp assays and provide useful predictive screening assays for QT prolongation.[21] We utilized a [^{3}H]-dofetilide binding assay to evaluate FIDAS agent interaction with hERG. Amitriptyline (final concentration, 1 mM) was used as the positive control and exhibited an IC_{50} value (10.7 ± 2.25 μM) that was in agreement with published values.[22] We selected a subset of the FIDAS agents in Figure 1 that possessed potent in vitro inhibition with LS174T colorectal cancer cells and that represented the structural subtypes in this SAR study (i.e., (*E*)-4-(2′,6′-difluorostyryl)-*N*,*N*-(dimethyl)aniline (**FIDAS 1a**); (*E*)-2-(2′,6′-difluorostyryl)-5-*N*,*N*-(dimethylamino)pyridine (**4a**); (*E*)-5-(2′,6′-difluorostyryl)-2-*N*,*N*-(dimethylamino)pyridine (**7a**); and (*E*)-5-(2′,6′-difluorostyryl)-2-*N*,*N*-(dimethylamino)pyrimidine (**10a**) as well as variants with different halogenation and methylation patterns). Concentrations of FIDAS agents ranging from 10^{-9} to 10^{-4} M were assayed in duplicate for these experiments (n = 3 experiments/analogue). IC_{50} values for hERG inhibition of [^{3}H]-dofetilide binding ranged from 20 to 60 μM. Ratios of the IC_{50} values for the hERG inhibition and IC_{50} values for the inhibition of LS174T cell proliferation (Table 1) ranged from 2 to 4 orders of magnitude for the subset of FIDAS agents that were studied.

DISCUSSION

We reported that (*E*)-4-(2′,6′-difluorostyryl)-*N*,*N*-dimethylaniline (**FIDAS 1a**) selectively bound to the catalytic subunit of methionine S-adenosyltranferase-2 (MAT2A), an enzyme upregulated in various liver and colorectal cancers.[1−3] **FIDAS 1a** possessed in vitro and in vivo activity against various liver and colorectal cancer cell lines, possessed good oral bioavailability and a reasonable half-life in vivo, and displayed minimal gross toxicity in terms of body weight loss/death at doses exceeding those that produced in vivo tumor reduction in a xenograft model.[1,2] In seeking analogues with increased potency and improved water solubility relative to that of **FIDAS 1a**, we undertook a SAR study that included pyridine

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and pyrimidine rings into the FIDAS platform (Figure 1). We also focused on the modification of substituents, such as the halogen and the *N*,*N*-dimethylamino substituents in **FIDAS 1a**, as a means of improving potency. Finally, potency alone is no longer sufficient to guide translational drug development. In developing these FIDAS agents as potential drug candidates, we sought FIDAS agents that avoided binding to the hERG potassium channel associated with drug-induced, adverse cardiac events.[15] This study describes our success in meeting these requirements (i.e., nanomolar potency, water solubility, and absence of hERG activation) into the FIDAS platform.

We synthesized and evaluated four families of FIDAS agents, as shown in Figure 1: (*E*)-4-(2′,6′-dihalostyryl)-*N*-alkyl- and (*E*)-4-(2′,6′-dihalostyryl)-*N*,*N*-(dialkyl)anilines (**1**−**3**), which we refer to as the aniline family; (*E*)-2-(2′,6′-dihalostyryl)-5-(*N*-alkylamino)- and (*E*)-2-(2′,6′-dihalostyryl)-5-(*N*,*N*-dialkylamino)pyridines (**4**−**6**), which we refer to as the 5-aminopyridine family; (*E*)-5-(2′,6′-dihalostyryl)-2-(*N*-alkylamino)- and (*E*)-5-(2′,6′-dihalostyryl)-2-(*N*,*N*-dialkylamino)-pyridines (**7**−**9**), which we refer to as the 2-aminopyridine family; and (*E*)-5-(2′,6′-dihalostyryl)-2-(*N*-alkylamino)- and (*E*)-5-(2′,6′-dihalostyryl)-2-(*N*,*N*-dialkylamino)pyrimidines (**10**−**12**), which we refer to as the 2-aminopyrimidine family. Syntheses relied principally on the Wadsworth−Emmons coupling of arylphosphonates with aryl aldehydes (Figures 2 and 3). FIDAS agents were characterized fully, and their purity was established by a combination of ^{13}C nuclear magnetic resonance (NMR) and combustion analyses.

We explored variations in the location, number, and nature of the two halogen substituents within **FIDAS 1a**. As reported previously, alterations in the location of the two halogens, other than the 2,6-dihalostyryl arrangement, invariably led to diminished activity in a cell proliferation in vitro assay using LS174T cells.[1−3] The inclusion of halogen substituents larger than chlorine or the introduction of more than two halogen substituents diminished or produced no appreciable increase in activity (data not shown). We found, however, that the substitution of one, but not both, of the fluorines in **FIDAS 1a** with a chlorine substituent resulted in slightly improved levels of in vitro activity. For example, the 2′-chloro-6′-fluoro analogue, **2a**, was comparable in activity to that of **FIDAS 1a** and was 10-fold better than that of **3a** (Table 1).

Modification of the alkyl groups in the *N*,*N*-(dialkylamino)-phenyl subunit of **FIDAS 1a** produced an even more dramatic improvement in activity than alteration in just the halogen substituents. Prior studies[1−3] had established that only the *para* orientation of the *N*,*N*-dimethylamino group and the 2,6-dihalostyryl group possessed good biological activity; consequently, we did not explore *ortho* or *meta* orientations of these groups in this study. Within the *para* series, the removal of one of the methyl groups, as in the monomethyl analogues, **1b**, **2b**, and **3b**, produced an active series with in vitro potencies in the 7−50 nM range (Table 1). Additional modifications involving substitution of a larger *N*-alkyl group than *N*-methyl, such as *N*-ethyl in **FIDAS 1c**, led to diminished in vitro activity. Inclusion of either *N*-alkylamino- or *N*,*N*-(dialkylamino)-pyridine or pyrimidine rings in place of the *N*,*N*-(dimethylamino)phenyl ring in **FIDAS 1a** offered an attractive option for addressing the water-solubility issue and improving potency. There are some inconsistencies between MAT2A inhibition and cell proliferation inhibition, particularly in the case of **8b**, suggesting that other characteristics, such as stability and solubility, may also contribute to the efficacy of FIDAS agents in cell or in vivo assays. Although the hydrochloride salt of **FIDAS 1a** was readily absorbed in a mouse bioavailability study, the in vitro IC_{50} was only 27 nM in LS174T cells. We found experimentally that the *N*-methyl analogues, **1b** and **2b**, had IC_{50} values for the inhibition of LS174T cells that were 4-fold greater than that of **1a**. We also performed a proof-of-concept experiment using primary CRC cells. We found that **2b** significantly inhibited the growth of CRC organoids (Supporting Information Figure 2), which further suggests that these agents are potential candidates for CRC treatment.

With the objective of developing antineoplastic drugs with limited affinity for human hERG, we utilized a [^3H]-dofetilide binding assay to evaluate the interaction of a subset of these FIDAS agents with hERG. Ratios of the IC_{50} values for the inhibition of LS174T cell proliferation and the IC_{50} values for hERG inhibition (Table 1) were, as desired, larger than 2−4 orders of magnitude, with the exceptions of **4a** and **10a**. Because these latter two FIDAS agents were also in families that were the least active of the four families studied, namely, the 5-aminopyridines (**4**−**6**) and the 2-aminopyrimidines (**10**−**12**), they were set aside for further SAR development. The most active compounds (i.e., **1b**, **2b**, **8a**, and **8b**) did not interact appreciably with hERG and possess ratios of IC_{50} values for hERG inhibition relative to IC_{50} values for inhibition of LS174T cell proliferation (Table 1) that were greater than that of **FIDAS 1a** and that are well within the selectivity range for drugs entering preclinical development.[23]

In summary, four key findings emerged from these studies: (1) FIDAS agents in the aniline family (**1**−**3**) and in the 2-aminopyridine family (**7**−**9**) possess lower IC_{50} values in the inhibition of LS174T cell proliferation than that of the 5-aminopyridines (**4**−**6**) and the 2-aminopyrimidines (**10**−**12**), (2) the most active FIDAS agents possessed either 2,6-difluorostyryl or 2-chloro-6-fluorostyryl subunits, (3) the most active FIDAS agents possessed small *N*-alkyl groups, specifically the *N*-methylamino or the *N*,*N*-dimethylamino groups, in a *para* orientation relative to the 2,6-dihalostyryl subunit, and (4) 2-aminopyridines **8a** and **8b** not only displayed IC_{50} values less than 10 nM but also formed water-soluble hydrochloride salts. In summary, we developed potent analogues of **FIDAS 1a** that exhibited significantly increased ratios of IC_{50} for hERG inhibition to IC_{50} for inhibition of LS174T cell proliferation, thereby opening the door to further development of these compounds as potential antineoplastic drugs.

EXPERIMENTAL PROCEDURES

Chemistry. Chemicals were purchased from Sigma-Aldrich (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA) or were synthesized according to literature procedures. Solvents were used from commercial vendors without further purification unless otherwise noted. Nuclear magnetic resonance spectra were determined on a Varian instrument (^1H, 400 MHz; ^{13}C, 100Mz). High-resolution electrospray ionization (ESI) mass spectra were recorded on a LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Resolution was set at 100 000 (at 400 *m*/*z*). Samples were introduced through direct infusion using a syringe pump with a flow rate of 5 μL/min. Purity of compounds was greater than 95%, as established using combustion analyses determined by Atlantic Microlabs, Inc., Norcross, GA. Compounds were chromatographed on preparative layer Merck silica gel F254 unless otherwise indicated.

General Procedure for the Synthesis of FIDAS Agents. To a solution of 1.65 mmol (1.1 equiv) of diethyl phosphonate in 5 mL of anhydrous DMF at 0 °C was added 2.25 mmol (1.5 equiv) of sodium hydride (washed with anhydrous hexanes to remove oil). The mixture was stirred for 15 min, and 1.5 mmol (1 equiv) of appropriate

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aldehyde dissolved in 1 mL of anhydrous DMF was added dropwise at 0 °C. The mixture was stirred 12 h at 25 °C and quenched by pouring into 30 mL of water with stirring. A precipitate was collected by filtration and purified by recrystallization and/or chromatography as noted for individual compounds described below. Several compounds in this study were reported previously: **FIDAS 1a**,[1−3] **1b**,[2,3] **2a**,[2,3] **2b**,[2,3] **3a**,[3] and **3b**.[3]

(*E*)-4-(2′,6′-Difluorostyryl)-*N*-ethylaniline (1c). To a mixture of 150 mg (0.65 mmol) of (*E*)-4-(2′,6′-difluorostyryl)aniline[3] and 100 mg (0.72 mmol, 1.1 equiv) of K_2CO_3 in 3 mL of acetone was added 101 mg (0.65 mmol, 1 equiv) of iodoethane. The mixture was refluxed for 12 h, poured into water, and extracted with CH_2Cl_2. The combined organic phases were dried over anhydrous $MgSO_4$ and evaporated to give a product that was purified by chromatography using 1:7 ethyl acetate−hexane (R_f = 0.43) to afford 103 mg (61%) of **1c** as a white solid: mp 50−51 °C. ^{1}H NMR ($CDCl_3$): δ 7.38 (d, 2H, J = 8.4 Hz), 7.36 (d, 1H, J = 16.4 Hz), 7.12−7.03 (m, 1H), 6.94−6.84 (m, 3H), 6.59 (d, 2H, J = 8.8 Hz), 3.71 (br s, 1 H), 3.19 (q, 2H, J = 7.2 Hz), 1.27 (t, 3H, J = 7.2 Hz). ^{13}C NMR ($CDCl_3$): δ 160.80 (dd, J_1 = 248.9 Hz, J_2 = 8.4 Hz, two C), 148.55, 135.33 (t, J = 8.4 Hz), 128.02 (two C), 127.71, 126.61 (t, J = 10.0 Hz), 115.58 (t, J = 15.6 Hz), 112.63 (two C), 111.41 (dd, J_1 = 19.8 Hz, J_2 = 6.8 Hz, two C), 110.61, 38.30, 14.80. HRMS (ESI) calcd for $C_{16}H_{16}F_2N$ [MH+], 260.12453; found, 260.12384. Anal. Calcd for $C_{16}H_{15}F_2N$: C, 74.11; H, 5.83. Found: C, 74.20; H, 5.90.

(*E*)-2-(2′,6′-Difluorostyryl)-5-(*N*,*N*-dimethylamino)pyridine (4a). Yield 81%, mp 90−92 °C (from hexane). ^{1}H NMR (DMSO-d_6): δ 8.17 (d, 1H, J = 3.2 Hz), 7.42−7.28 (m, 4H), 7.18−7.11 (m, 2H), 7.06 (dd, 1H, J_1 = 8.4 Hz, J_2 = 3.2 Hz), 2.98 (s, 6H). ^{13}C NMR (DMSO-d_6): δ 160.24 (dd, J_1 = 248.2 Hz, J = 8.4 Hz, two C), 145.47, 142.08, 134.78, 134.42 (t, J = 8.0 Hz), 128.49 (t, J = 11.0 Hz), 123.50, 118.19, 114.20 (t, J = 15.2 Hz), 112.62, 112.00 (dd, J_1 = 19.4 Hz, J_2 = 6.4 Hz, two C), 39.56 (two C). HRMS (ESI) calcd for $C_{15}H_{15}F_2N_2$ [MH+], 261.11978; found, 261.11884. Anal. Calcd for $C_{15}H_{14}F_2N_2$: C, 69.22; H, 5.42. Found: C, 69.33; H, 5.59.

(*E*)-2-(2′-Chloro-6′-fluorostyryl)-5-(*N*,*N*-dimethylamino)-pyridine (5a). Yield 80%, R_f = 0.44 (ethyl acetate−hexane 1:5), mp 78−80 °C. ^{1}H NMR (DMSO-d_6): δ 8.20 (d, 1H, J = 2.4 Hz), 7.53 (d, 1H, J = 16.4 Hz), 7.39−7.25 (m, 5H), 7.06 (dd, 1H, J_1 = 8.4 Hz, J_2 = 3.2 Hz), 2.98 (s, 6H). ^{13}C NMR (DMSO-d_6): δ 160.61 (d, J = 249.7 Hz), 145.52, 141.91, 135.26 (d, J = 12.9 Hz), 134.88, 133.39 (d, J = 6.1 Hz), 128.64 (d, J = 9.9 Hz), 125.96 (d, J = 3.8 Hz), 123.72, 123.55 (d, J = 14.4 Hz), 118.12, 116.88 (d, J = 2.3 Hz), 115.18 (d, J = 23.5 Hz), 39.54 (two C). HRMS (ESI) calcd for $C_{15}H_{15}ClFN_2$ [MH+], 277.09023; found, 277.08939. Anal. Calcd for $C_{15}H_{14}ClFN_2$: C, 65.10; H, 5.10. Found: C, 65.04; H, 5.20.

(*E*)-2-(2′,6′-Dichlorostyryl)-5-(*N*,*N*-dimethylamino)pyridine (6a). Yield 86%, mp 85−86 °C (from hexane). ^{1}H NMR (DMSO-d_6): δ 8.19 (d, 1H, J = 3.2 Hz), 7.52 (d, 2H, J = 7.6 Hz), 7.42−7.36 (m, 2H), 7.30−7.26 (m, 1H), 7.15 (d, 1H, J = 16.0 Hz), 7.07 (dd, 1H, J_1 = 8.8 Hz, J_2 = 3.2 Hz), 2.98 (s, 6H). ^{13}C NMR (DMSO-d_6): δ 145.56, 141.62, 136.25, 134.78, 134.03, 133.53 (two C), 129.00 (two C), 128.78, 123.39, 120.27, 118.20, 39.58 (two C). HRMS (ESI) calcd for $C_{15}H_{15}Cl_2N_2$ [MH+], 293.06068; found, 293.05986. Anal. Calcd for $C_{15}H_{14}Cl_2N_2$: C, 61.45; H, 4.81. Found: C, 61.55; H, 4.75.

(*E*)-5-(2′,6′-Difluorostyryl)-2-(*N*,*N*-dimethylamino)pyridine (7a). Yield 87%, mp 103−104 °C (from hexane). ^{1}H NMR (DMSO-d_6): δ 8.23 (d, 1H, J = 2.4 Hz), 7.89 (dd, 1H, J_1 = 9.0 Hz, J_2 = 2.4 Hz), 7.33−7.24 (m, 2H), 7.16−7.09 (m, 2H), 6.89 (d, 1H, J = 16.8 Hz), 6.68 (d, 1H, J = 8.8 Hz) 3.06 (s, 6H). ^{13}C NMR (DMSO-d_6): δ 159.97 (dd, J_1 = 247.0 Hz, J = 7.2 Hz, two C), 158.70, 148.03, 133.79, 132.60 (t, J = 8.0 Hz), 128.13 (t, J = 10.6 Hz), 120.48, 114.54 (t, J = 15.6 Hz), 111.97 (dd, J_1 = 19.4 Hz, J_2 = 6.4 Hz, two C), 110.14, 106,04, 37.65 (two C). HRMS (ESI) calcd for $C_{15}H_{15}F_2N_2$ [MH+], 261.11978; found, 261.11885. Anal. Calcd for $C_{15}H_{14}F_2N_2$: C, 69.22; H, 5.42. Found: C, 69.32; H, 5.44.

(*E*)-5-(2′,6′-Difluorostyryl)-2-(*N*-methylamino)pyridine (7b). Yield 83%, mp 130−132 °C (from ethanol−water). ^{1}H NMR (DMSO-d_6): δ 8.12 (d, 1H, J = 2.4 Hz), 7.78 (dd, 1H, J_1 = 8.6 Hz, J_2 = 2.6 Hz), 7.32−7.24 (m, 1H), 7.23 (d, 1H, J = 16.8 Hz), 7.16−7.08 (m, 2H), 6.87−6.81 (m, 2H), 6.49 (d, 1H, J = 9.2 Hz), 2.80 (d, 3H, J = 4.8 Hz). ^{13}C NMR (DMSO-d_6): δ 159.95 (dd, J_1 = 247.4 Hz, J = 8.4 Hz, two C), 159.37, 148.44, 133.03, 132.9 (t, J = 8.0 Hz), 127.97 (t, J = 10.6 Hz), 120.60, 114.61 (t, J = 15.9 Hz), 111.96 (dd, J_1 = 19.4 Hz, J_2 = 6.4 Hz, two C), 109.55, 108.31, 27.89. HRMS (ESI) calcd for $C_{14}H_{13}F_2N_2$ [MH+], 247.10413; found, 247.10318. Anal. Calcd for $C_{14}H_{12}F_2N_2$: C, 68.28; H, 4.91. Found: C, 68.12; H, 5.04.

(*E*)-5-(2′,6′-Difluorostyryl)-2-(*N*-ethylamino)pyridine (7c). The general procedure was repeated using 2-(*tert*-butyloxycarbonyl)-aminopyridine-5-carboxaldehyde and 2,6-difluorobenzyl diethyl phosphonate to afford (*E*)-2-(*tert*-butoxycarbonylamino)-5-(2′,6′-difluorostyryl)pyridine: yield 86%, mp 190−191 °C (from dichloromethane). ^{1}H NMR ($CDCl_3$): δ 9.16 (br s, 1H), 8.45 (d, 1H, J = 2.4 Hz), 8.04 (d, 1H, J = 8.8 Hz), 7.89 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.4), 7.36 (d, 1H, J = 16.8 Hz), 7.12−7.20 (m, 1H), 7.08 (d, 1H, J = 16.8 Hz), 6.88−6.95 (m, 2H), 1.58 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 160.95 (dd, J_1 = 250.4 Hz, J = 7.6 Hz, two C), 152.61, 152.15, 146.88, 135.12, 131.10 (t, J = 8.6 Hz), 128.04 (t, J = 10.6 Hz), 115.02, 114.50 (t, J = 15.1 Hz), 112.28, 111.61 (dd, J_1 = 19.4 Hz, J_2 = 6.4 Hz, two C), 81.10, 28.38 (three C). HRMS (ESI) calcd for $C_{18}H_{19}F_2N_2O_2$ [MH+], 333.14091; found, 333.13949. Anal. Calcd for $C_{18}H_{18}F_2N_2O_2$: C, 65.05; H, 5.46. Found: C, 65.12; H, 5.59. To 615 mg of (*E*)-2-(*tert*-butoxycarbonylamino)-5-(2′,6′-difluorostyryl)pyridine (1.85 mmol) in 18 mL of anhydrous *N*,*N*-dimethylformamide at 0 °C was added 163 mg of 60% sodium hydride (4.07 mmol) in portions. The suspension was stirred for 20 min while maintaining the temperature below 5 °C, and 0.16 mL of ethyl iodide (2.04 mmol) was added dropwise. The mixture was stirred at 5 °C for 30 min and allowed to stir at 25 °C for 12 h. The reaction was quenched with water, extracted with dichloromethane, and washed successively with water, 0.1 M hydrochloric acid solution, saturated aqueous $NaHCO_3$ solution, and brine, and dried over anhydrous $MgSO_4$ to afford 633 mg (95%) of (*E*)-2-(*N*-(*tert*-butoxycarbonyl)-*N*-ethylamino)-5-(2′,6′-difluorostyryl)pyridine as a clear, colorless oil that was used in the next step without further purification. To 613 mg (1.7 mmol) of (*E*)-2-(*N*-(*tert*-butoxycarbonyl)-*N*-ethylamino)-5-(2′,6′-difluorostyryl)pyridine in 17 mL of dichloromethane was added 4.4 mL of trifluoroacetic acid (57.1 mmol). The mixture was stirred for 12 h at 25 °C. A precipitate was collected and dissolved in water, cooled to 0 °C, and neutralized with aqueous solution of Na_2CO_3. The product was dissolved in dichloromethane, washed with water and brine, and dried over anhydrous $MgSO_4$. The product was purified by chromatography using 1:3 ethyl acetate−hexane (R_f = 0.23) to afford 398 mg (90%) of **7c** as a white solid. mp 96−97 °C. ^{1}H NMR ($CDCl_3$): δ 8.16 (br s, 1H), 7.70 (d, 1H, J = 7.6 Hz), 7.31 (d, 1H, J = 16.8 Hz), 7.13−7.06 (m, 1H), 6.91−6.86 (m, 3H), 6.39 (d, 1H, J = 8.8 Hz), 4.73 (br s, 1H), 3.33 (m, 2H, J = 7.2 Hz), 1.26 (t, 3H, J = 7.2 Hz). ^{13}C NMR ($CDCl_3$): δ 160.79 (dd, J_1 = 249.7 Hz, J = 7.6 Hz, two C), 158.48, 148.32, 134.06, 132.12 (t, J = 8.3 Hz), 127.10 (t, J = 10.6 Hz), 122.74, 115.11 (t, J = 15.1 Hz), 111.53, 111.47 (dd, J_1 = 19.7 Hz, J_2 = 6.8 Hz, two C), 106.56, 36.92, 14.82. HRMS (ESI) calcd for $C_{15}H_{15}F_2N_2$ [MH+], 261.11978; found, 261.11883. Anal. Calcd for $C_{15}H_{14}F_2N_2$: C, 69.22; H, 5.42. Found: C, 69.27; H, 5.43.

(*E*)-5-(2′-Chloro-6′-fluorostyryl)-2-(*N*,*N*-dimethylamino)-pyridine (8a). Yield 76%, mp 63−65 °C (from hexane). ^{1}H NMR (DMSO-d_6): δ 8.23 (d, 1H, J = 2.4 Hz), 7.87 (dd, 1H, J_1 = 9.0 Hz, J_2 = 2.6 Hz), 7.38−7.35 (m, 1H), 7.29−7.26 (m, 2H), 7.20 (d, 1H, J = 16.8 Hz), 6.97 (d, 1H, J = 16.8 Hz), 6.70 (d, 1H, J = 9.2 Hz), 3.07 (s, 6H). ^{13}C NMR (DMSO-d_6): δ 160.30 (d, J = 248.9 Hz), 158.75, 148.02, 133.81, 133.50 (d, J = 12.1 Hz), 132.97 (d, J = 6.0 Hz), 128.40 (d, J = 10.6 Hz), 125.87 (d, J = 3.1 Hz), 123.95 (d, J = 15.1 Hz), 120.30, 115.14 (d, J = 23.5 Hz), 114.25 (d, J = 2.3 Hz), 106.05, 37.64 (two C). HRMS (ESI) calcd for $C_{15}H_{15}ClFN_2$ [MH+], 277.09023; found, 277.08944. Anal. Calcd for $C_{15}H_{14}ClFN_2$: C, 65.10; H, 5.10. Found: C, 65.01; H, 5.21.

(*E*)-5-(2′-Chloro-6′-fluorostyryl)-2-(*N*-methylamino)pyridine (8b). Yield 86%, mp 106−108 °C (from hexane). ^{1}H NMR (DMSO-d_6): δ 8.12 (d, 1H, J = 2.4 Hz), 7.76 (dd, 1H, J_1 = 9.0 Hz, J_2 = 2.6 Hz), 7.37−7.33 (m, 1H), 7.29−7.24 (m, 2H), 7.17 (d, 1H, J = 16.4 Hz), 6.91 (d, 1H, J = 16.4 Hz), 6.87 (q, 1H, J = 4.6 Hz), 6.51 (d, 1H, J = 8.8

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Hz), 2.80 (d, 3H, J = 4.8 Hz). ^{13}C NMR (DMSO-d_6): δ 160.29 (d, J = 248.1 Hz), 159.44, 148.46, 133.80 (d, J = 11.4 Hz), 133.06, 132.93 (d, J = 5.3 Hz), 128.28 (d, J = 8.3 Hz), 125.86 (d, J = 3.0 Hz), 124.02 (d, J = 15.2 Hz), 120.43, 114.14 (d, J = 22.7 Hz), 113.68, 108.33, 27.90. HRMS (ESI) calcd for $C_{14}H_{13}ClFN_2$ [MH+], 263.07458; found, 263.07375. Anal. Calcd for $C_{14}H_{12}ClFN_2$: C, 64.01; H, 4.60. Found: C, 64.13; H, 4.63.

(*E*)-5-(2′,6′-Dichlorostyryl)-2-(*N*,*N*-dimethylamino)pyridine (9a). Yield 78%, mp 54−56 °C (from hexane). ^{1}H NMR (DMSO-d_6): δ 8.22 (d, 1H, J = 2.8 Hz), 7.87 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.8 Hz), 7.51 (d, 2H, J = 8.0 Hz), 7.29−7.25 (m, 1H), 7.00 (d, 1H, J = 16.8 Hz), 6.92 (d, 1H, J = 16.8 Hz), 6.70 (d, 1H, J = 8.8 Hz), 3.07 (s, 6H). ^{13}C NMR (DMSO-d_6): δ 158.78, 147.82, 134.47, 134.19, 133.98, 133.44 (two C), 128.90 (two C), 128.69, 119.86, 117.80, 106.01, 37.66 (two C). HRMS (ESI) calcd for $C_{15}H_{15}Cl_2N_2$ [MH+], 293.06068; found, 293.05978. Anal. Calcd for $C_{15}H_{14}Cl_2N_2$: C, 61.45; H, 4.81. Found: C, 61.51; H, 4.96.

(*E*)-5-(2′,6′-Dichlorostyryl)-2-(*N*-methylamino)pyridine (9b). Yield 88%, mp 129−131 °C (from hexane). ^{1}H NMR (DMSO-d_6): δ 8.11 (d, 1H, J = 2.0 Hz), 7.77 (dd, 1H, J_1 = 9.0 Hz, J_2 = 2.6 Hz), 7.50 (d, 2H, J = 8.0 Hz), 7.28−7.24 (m, 1H), 6.97 (d, 1H, J = 16.4 Hz), 6.88−6.83 (m, 2H), 6.50 (d, 1H, J = 8.4 Hz), 2.80 (d, 3H, J = 4.8 Hz). ^{13}C NMR (DMSO-d_6): δ 159.44 148.23, 134.52, 134.47, 133.43 (two C), 133.24, 128.89 (two C), 128.60, 119.97, 117.21, 108.22, 27.89. HRMS (ESI) calcd for $C_{14}H_{13}Cl_2N_2$ [MH+], 279.04503; found, 279.04430. Anal. Calcd for $C_{14}H_{12}Cl_2N_2$: C, 60.23; H, 4.33. Found: C, 60.35; H, 4.51.

(*E*)-5-(2′,6′-Difluorostyryl)-2-(*N*,*N*-dimethylamino)pyrimidine (10a). Yield 85%, mp 134−136 °C (from hexane). ^{1}H NMR (DMSO-d_6): δ 8.65 (s, 2H), 7.35−7.28 (m, 1H), 7.20 (d, 1H, J = 17.2 Hz), 7.17−7.10 (m, 2H), 7.00 (d, 1H, J = 17.2 Hz), 3.16 (s, 6H). ^{13}C NMR (DMSO-d_6): δ 161.61, 160.43 (dd, J_1 = 248.2 Hz, J = 7.6 Hz, two C), 156.55 (two C), 130.23 (t, J = 8.0 Hz), 128.99 (t, J = 10.6 Hz), 118.88, 114.65 (t, J = 15.2 Hz), 112.43 (dd, J_1 = 19.4 Hz, J_2 = 6.4 Hz, two C), 111.75, 37.17 (two C). HRMS (ESI) calcd for $C_{14}H_{14}F_2N_3$ [MH+], 262.11503; found, 262.11417. Anal. Calcd for $C_{14}H_{13}F_2N_3$: C, 64.36; H, 5.02. Found: C, 64.45; H, 5.03.

(*E*)-5-(2′,6′-Difluorostyryl)-2-(*N*-methylamino)pyrimidine (10b). Yield 90%, mp 143−144 °C (from ethanol). ^{1}H NMR (DMSO-d_6): δ 8.60 (s, 2H), 7.42 (q, 1H, J = 4.6 Hz), 7.36−7.28 (m, 1H), 7.21−7.11 (m, 3H), 6.99 (d, 1H, J = 16.8 Hz), 2.83 (d, 3H, J = 4.8 Hz). ^{13}C NMR (DMSO-d_6): δ 162.19, 160.00 (dd, J_1 = 248.2 Hz, J = 7.6 Hz, two C), 156.38 (two C), 129.97 (t, J = 7.6 Hz), 128.50 (t, J = 10.6 Hz), 119.08, 114.26 (t, J = 15.2 Hz), 112.02 (dd, J_1 = 19.4 Hz, J_2 = 6.4 Hz, two C), 111.02, 27.93. HRMS (ESI) calcd for $C_{13}H_{12}F_2N_3$ [MH+], 248.09938; found, 248.09838. Anal. Calcd for $C_{13}H_{11}F_2N_3$: C, 63.15; H, 4.48. Found: C, 63.14; H, 4.55.

(*E*)-5-(2′,6′-Difluorostyryl)-2-(*N*-ethylamino)pyrimidine (10c). Yield 89%, mp 147−149 °C (from ethanol). ^{1}H NMR (DMSO-d_6): δ 8.58 (s, 2H), 7.48 (t, 1H, J = 5.6 Hz), 7.35−7.28 (m, 1H), 7.20−7.10 (m, 3H), 6.98 (d, 1H, J = 16.8 Hz), 3.56−3.29 (m, 2H), 1.12 (t, 3H, J = 7.4 Hz). ^{13}C NMR (DMSO-d_6): δ 161.62, 160.00 (dd, J_1 = 248.2 Hz, J = 7.6 Hz, two C), 156.40 (two C), 129.97 (t, J = 7.6 Hz), 128.51 (t, J = 10.6 Hz), 119.08, 114.27 (t, J = 15.6 Hz), 112.00 (dd, J_1 = 19.4 Hz, J_2 = 6.1 Hz, two C), 110.94, 35.50, 14.65. HRMS (ESI) calcd for $C_{14}H_{14}F_2N_3$ [MH+], 262.11503; found, 262.11424. Anal. Calcd for $C_{14}H_{13}F_2N_3$: C, 64.36; H, 5.02. Found: C, 64.54; H, 4.96.

(*E*)-5-(2′-Chloro-6′-fluorostyryl)-2-(*N*,*N*-dimethylamino)-pyrimidine (11a). Yield 74%, mp 105−106 °C (from hexane). ^{1}H NMR (DMSO-d_6): δ 8.64 (s, 2H), 7.38−7.25 (m, 3H), 7.14 (d, 1H, J = 16.8 Hz), 7.42 (d, 1H, J = 16.8 Hz), 3.16 (s, 6H). ^{13}C NMR (DMSO-d_6): δ 161.22, 160.32 (d, J = 248.9 Hz), 156.13 (two C), 133.12 (d, J = 5.3 Hz), 130.73 (d, J = 11.3 Hz), 128.83 (d, J = 8.4 Hz), 125.91 (d, J = 1.5 Hz), 123.67 (d, J = 14.5 Hz), 118.29, 115.36, 115.17 (d, J = 22.7 Hz), 36.73 (two C). HRMS (ESI) calcd for $C_{14}H_{14}ClFN_3$ [MH+], 278.08548; found, 278.08458. Anal. Calcd for $C_{14}H_{13}ClFN_3$: C, 60.55; H, 4.72. Found: C, 60.66; H, 4.79.

(*E*)-5-(2′-Chloro-6′-fluorostyryl)-2-(*N*-methylamino)-pyrimidine (11b). Yield 78%, mp 163−165 °C (from ethanol). ^{1}H NMR (DMSO-d_6): δ 8.58 (s, 2H), 7.43 (q, 1H, J = 4.6 Hz), 7.38−7.36 (m, 1H), 7.33−7.25 (m, 2H), 7.12 (d, 1H, J = 16.8 Hz), 7.04 (d, 1H, J = 16.8 Hz), 2.83 (d, 3H, J = 4.8 Hz). ^{13}C NMR (DMSO-d_6): δ 162.30, 160.38 (d, J = 248.9 Hz), 156.45 (two C), 133.18 (d, J = 5.3 Hz), 130.97 (d, J = 11.4 Hz), 128.86 (d, J = 9.9 Hz), 125.97 (d, J = 3.1 Hz), 123.79 (d, J = 14.4 Hz), 118.98, 115.36, 115.13, 27.99. HRMS (ESI) calcd for $C_{13}H_{12}ClFN_3$ [MH+], 264.06983; found, 264.06918. Anal. Calcd for $C_{13}H_{11}ClFN_3$: C, 59.21; H, 4.20. Found: C, 59.19; H, 4.34.

(*E*)-5-(2′,6′-Dichlorostyryl)-2-(*N*,*N*-dimethylamino)-pyrimidine (12a). Yield 82%, mp 103−104 °C (from hexane). ^{1}H NMR (DMSO-d_6): δ 8.64 (s, 2H), 7.52 (d, 2H, J = 8.0 Hz), 7.31−7.27 (m, 1H), 7.04 (d, 1H, J = 16.8 Hz), 6.94 (d, 1H, J = 16.8 Hz), 3.16 (s, 6H). ^{13}C NMR (DMSO-d_6): δ 161.28, 156.09 (two C), 134.20, 133.49 (two C), 131.42, 129.03, 128.90 (two C), 118.92, 117.84, 36.76 (two C). HRMS (ESI) calcd for $C_{14}H_{14}Cl_2N_3$ [MH+], 294.05593; found, 294.05516. Anal. Calcd for $C_{14}H_{13}Cl_2N_3$: C, 57.16; H, 4.45. Found: C, 57.26; H, 4.59.

(*E*)-5-(2′,6′-Dichlorostyryl)-2-(*N*-methylamino)pyrimidine (12b). Yield 91%, mp 191−193 °C (from ethanol). ^{1}H NMR (DMSO-d_6): δ 8.58 (s, 2H), 7.52 (d, 2H, J = 8.0 Hz), 7.42 (q, 1H, J = 4.8 Hz), 7.31−7.27 (m, 1H), 7.02 (d, 1H, J = 16.8 Hz), 6.91 (d, 1H, J = 16.8 Hz), 2.83 (d, 3H, J = 5.2 Hz). ^{13}C NMR (DMSO-d_6): δ 162.29, 156.34 (two C), 134.26, 133.50 (two C), 131.57, 128.99, 128.88 (two C), 118.62, 118.46, 27.93. HRMS (ESI) calcd for $C_{13}H_{12}Cl_2N_3$ [MH+], 280.04028; found, 280.03979. Anal. Calcd for $C_{13}H_{11}Cl_2N_3$: C, 55.73; H, 3.96. Found: C, 55.77; H, 4.07.

Biological Studies. MAT2A Inhibition Assay. L-Methionine (50 μM) and ATP (50 μM) were incubated with purified His-tagged MAT2A holoenzyme (3 μg) in 0.3 mL of reaction buffer (50 mM Tris pH8.0, 50 mM KCl, 10 mL $MgCl_2$) at room temperature for 25 min. The P_i released from the reaction was measured with SensoLyte Malachite Green (MG) phosphate assay kit (AnaSpec, 71103). The absorbance was measured at 635 nm on a microplate reader (Spectra MR, DYNEX Technologies). For the inhibition assay, MAT2A holoenzyme was incubated with FIDAS agents at room temperature for 10 min and then mixed with L-methionine and ATP in 0.3 mL of reaction buffer.

Cell Proliferation Studies. LS174T colon cancer cells were plated into 12-well plates (3 × 10^4 cells/mL). On the next day, the cells were treated with FIDAS agents. Effects of FIDAS analogues on cell proliferation were analyzed using Cell Viability Analyzer (Beckman Coulter, Vi-Cell XR).

hERG Binding Studies. The HEK-293 cell line stably expressing the hERG potassium channel (accession no. U04270), referred to as hERG-HEK cells, were received at passage 11 (P11) from Millipore (CYL3006, lot 2, Billerica, MA). [^{3}H]-Dofetilide (specific activity of 80 Ci/mmol; labeled on the *N*-methyl group) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Other chemicals and solvents were obtained from Sigma-Aldrich (Milwaukee, WI) with exceptions of polyethylenimine (PEI), which was obtained from Fluka/Sigma-Aldrich (St. Louis, MO), and minimium essential medium (MEM) with GlutaMAX and phenol red, MEM nonessential amino acids solution (NEAA, 100×), G418 disulfate salt solution, fetal bovine serum (FBS), 0.05% Trypsin-EDTA 1× with phenol red, and Hank's balanced salt solution (HBSS), which were obtained from Life Technologies (Carlsbad, CA).

hERG-HEK Cell Culture. hERG-HEK cells were cultured according to the protocol provided by Millipore. Cells were maintained in MEM (with glutamax and phenol red) supplemented with 10% FBS, 1% NEAA. and 400 μg/mL Geneticin, and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Frozen aliquots of cells were transferred into T-75 cm^2 flasks and allowed to adhere for 4−8 h. The medium was replaced every 2 days. Passages were carried out at least three times at 6 day intervals after thawing. Cells were dissociated with trypsin/EDTA and seeded into new 150 × 25 mm dishes at (2−3) × 10^6 cells per dish and placed at 30 °C, 5% CO_2, for 40−48 h prior to membrane preparation. Membrane preparation occurred 6 days after the last passage (passage 20).

Membrane Preparation. Cell membrane preparation was based on previous methods.[19−22] Cells were rinsed twice with HBSS at 37 °C and collected by scraping the dishes in ∼20 mL of ice-cold 0.32 M

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sucrose and homogenized on ice with a Teflon pestle using a Maximal Digital homogenizer (Fisher Scientific, Pittsburgh, PA) at ∼280 rpm for 30 s. Homogenates were centrifuged at 300*g* and 800*g* for 4 min each at 4 °C. Pellets were resuspended in 9 mL of ice-cold Milli-Q water, and osmolarity was restored by addition of 1 mL of 500 mM Tris buffer (pH 7.4) followed by suspension and centrifugation at 20 000*g* for 30 min at 4 °C. Pellets were homogenized in 2 mL assay buffer (50 mM Tris, 10 mM KCl, and 1 mM $MgCl_2$, 4 °C), and aliquots of cell membrane suspensions were stored at −80 °C and thawed the day of the [^{3}H]-dofetilide binding assay. Protein content was determined prior to the assay using a Bradford protein assay with bovine albumin as the standard.

[^{3}H]-Dofetilide Binding Assay. [^{3}H]-Dofetilide binding assays using hERG-HEK293 cell membranes were based on previous methods.[19] Assays determining concentration−response were conducted in duplicate, and three independent assays were performed for each analogue evaluated. Cell membrane suspension (5 μg) was added to duplicate tubes containing assay buffer, 25 μL of a single concentration of FIDAS agent (concentration range of 10 nM to 100 μM for each experiment), and 25 μL of [^{3}H]-dofetilide (5 nM, final concentration) for an assay volume of 250 μL. Binding occurred for 60 min at 25 °C and was terminated by rapid filtration through Whatman GF/B filters, which were presoaked in 0.25% PEI overnight, using a Brandel cell/membrane harvester (M-48; Brandel Inc., Gaithersburg, MD). Filters were washed three times with ∼1 mL of ice-cold assay buffer. Radioactivity was determined by liquid scintillation spectrometry using the Tri-Carb 2100-TR liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences).

Data Analysis. Compound concentrations that produced 50% inhibition (IC_{50}) in the biological studies were determined from the concentration−response curves via the nonlinear regression one-site competition-fitting program (Prism 5.04; GraphPad Software Inc., San Diego, CA).

■ ASSOCIATED CONTENT

Supporting Information

Examples of IC_{50} determination for the cell proliferation assay and effects of FIDAS agent on colon cancer organoids. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): In accord with University policy, Wen Zhang, Chunming Liu, David S. Watt, and Vitaliy M. Sviripa have disclosed this work to the University's Intellectual Property Committee that has sought patent protection and that has given a private firm, Liu-Watt, LLC, in which the four principals have partial ownership, an option to license this technology.

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■ ABBREVIATIONS USED

ATP, adenosine triphosphate; MAT2A, catalytic subunit of methionine S-adenosyltransferase-2; CRC, colorectal cancer; FIDAS, difluorinated *N*,*N*′-dialkylaminostilbenes; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; hERG-HEK, HEK-293 cell membranes stably expressing the hERG channel; hERG, human ether-à-go-go-related protein; IND, investigational new drug; LC, liver cancer; MEM, minimum essential medium; NMR, nuclear magnetic resonance; NEAA, nonessential amino acids; PEI, polyethylenimine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SAR, structure−activity relationship

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