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Original article

UNC1062, a new and potent Mer inhibitor

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ABSTRACT

Abnormal activation of Mer kinase has been implicated in the oncogenesis of many human cancers including acute lymphoblastic and myeloid leukemia, non-small cell lung cancer, and glioblastoma. We have discovered a new family of small molecule Mer inhibitors, pyrazolopyrimidine sulfonamides, that potently inhibit the kinase activity of Mer. Importantly, these compounds do not demonstrate significant hERG activity in the PatchXpress assay. Through structure–activity relationship studies, **35** (**UNC1062**) was identified as a potent (IC_{50} = 1.1 nM) and selective Mer inhibitor. When applied to live tumor cells, **UNC1062** inhibited Mer phosphorylation and colony formation in soft agar. Given the potential of Mer as a therapeutic target, **UNC1062** is a promising candidate for further drug development.

1. Introduction

Mer belongs to the TAM (***T***yro3, ***A***xl, and ***M***er) family of receptor tyrosine kinases (RTKs) [1]. Under normal physiological conditions, Mer kinase promotes cell proliferation and survival, platelet aggregation, macrophage clearance of apoptotic cells, and cytokine release [2]. Abnormal activation of Mer has been implicated in the oncogenesis of many human cancers and is often associated with poor prognostic indicators [2,3]. For example, Mer is ectopically expressed in both B- and T-cell acute lymphoblastic leukemias (ALL) [4] but not in normal mouse and human T- and B-lymphocytes at any stage of development. Similarly, Mer is expressed in the majority of acute myeloid leukemia (AML) cell lines and patient samples but not in normal bone marrow myeloid cells [5]. Finally, Mer is frequently overexpressed in solid tumors, including non-small cell lung cancer (NSCLC) cell lines [6] and patient samples, even though normal human bronchial epithelial (NHBE) cells and lung tissue adjacent to tumors do not express Mer protein [6]. Similar findings have been reported in other solid tumors, including glioblastoma (GBM) [3b,7] and metastatic melanoma [8]. This tumor specific expression pattern may confer a large therapeutic window – at least in these tumor types. Expression of a Mer transgene in hematopoietic cells leads to development of leukemia and/or lymphoma in mice [9]. In addition, when Mer is inhibited by sh-RNA knockdown in leukemia [4c], NSCLC [6], or GBM cells [7a], they are more susceptible to apoptotic death and exhibit reduced colony formation in soft agar. Moreover, in orthotropic ALL and AML xenograft mouse models, onset and progression of disease were delayed and survival was significantly increased in mice transplanted with Mer knockdown leukemia cell lines relative to mice transplanted with wild-type cell lines [4c,5]. Similarly, in an

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NSCLC xenograft mouse model growth of tumors is markedly slowed by Mer knockdown. In addition, the combination of standard cytotoxic chemotherapies with shRNA-mediated Mer knockdown results in synergistic tumor cell killing. Taken together, these data demonstrate important roles of Mer for oncogenesis and anti-apoptotic activity in multiple tumor types. Therefore, Mer provides a novel therapeutic target for the treatment of ALL, AML, NSCLC, and other Mer-related diseases. Consequently, Mer inhibitors are expected to both mediate direct anti-tumor, proapoptotic effects and augment chemosensitivity in combination with standard therapies.

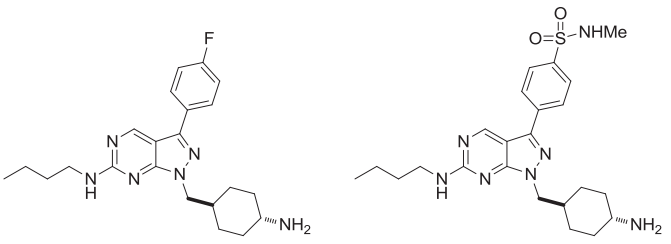
Few small molecule Mer inhibitors have been reported [10], among them compound **1** (**UNC569**) (Fig. 1) is the most promising one demonstrating inhibition of Mer at sub-nanomolar concentrations [11]. However, **UNC569** has undesirable activity toward the human *ether-a-go-go*-related gene potassium channel (hERG) in the conventional PatchXpress assay ($EC_{50} = 1.7$ μM) [12]. Inhibition of hERG could lead to long QT syndrome and sudden death and is the reason for the withdrawal of several FDA-approved drugs [13]. In an effort to decrease the hERG activity of **UNC569** through analog synthesis, we discovered that sulfonamide **2** (Fig. 1) had similar activity against Mer kinase with better physical properties. Consequently, compound **2** is a better starting point than **UNC569** to develop potent Mer inhibitors as potential therapeutics. Herein we report the structure–activity relationship (SAR) of a series of compounds related to **2** on inhibiting the TAM family kinases and the discovery of potent Mer inhibitors with minimal hERG activity and excellent cellular activity.

2. Synthesis

The synthetic routes to sulfonamide analogs (Scheme 1) are similar to what we have previously developed for the synthesis of analogs of **UNC569** [11]. Briefly, 3-chlorobenzoperoxoic acid (*m*-CPBA) oxidation of the known compound **3** [14] followed by the displacement of the methyl sulfinyl/sulfonyl group by amines provided the intermediate **4**, which was subsequently alkylated at the N1 position of the pyrazole ring to yield **5**. Suzuki coupling between **5** and a boronic acid provided the final product **6.**

3. Results and discussion

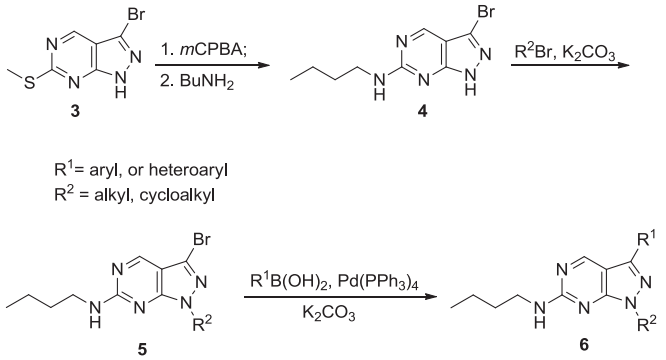
Most hERG inhibitors contain a basic amine group and we thus speculate that the hERG activity of **1** may be due to the presence of a primary amine (NH_2) at the R^2 position (Table 1). Consequently, we first synthesized a focused library of compounds with different R^2 groups while keeping R^1 fixed as *N*-methylbenzenesulfonamide. Inhibition of Mer kinase activity by analogs was tested at the K_m for ATP using a microfluidic capillary electrophoresis (MCE) assay [15]



1 (UNC569)
IC_{50} Mer 2.9 nM
Axl 37 nM
Tyro3 48 nM

2
IC_{50} Mer 3.5 nM
Axl 59 nM
Tyro3 27 nM

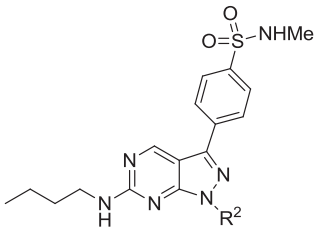
Fig. 1. The structures and enzymatic activities of **1** and **2**.

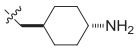
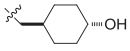
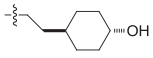
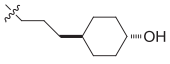
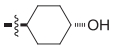
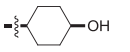
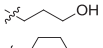
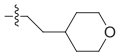


Scheme 1. The synthetic routes for sulfonamide analogs.

in which phosphorylated and unphosphorylated substrate peptides were separated and analyzed using a LabChip EZ Reader. For comparison, inhibition of other members in the TAM RTK family, Tyro3 and Axl, was also measured. As shown in Table 1, when the

Table 1
Preliminary SAR at the R^2 position.



Compound	R^2	IC_{50} (nM)[a]		
		Mer	Axl	Tyro3
2		3.5	59	27
7		18	140	130
8		35	120	200
9		50	190	82
10		2.4	74	47
11		4.4	70	44
12		250	2400	860
13		130	5000	730
14		23	450	280
15		47	620	810
16		54	360	430
17		100	26000	720

[a] Values are the mean of two or more independent assays performed at the ATP Km.

NH_2 group at the R^2 position (**2**) was replaced by a hydroxyl (OH) group (**7**), the activity of the analog **7** was decreased 5-fold. Increasing the length of the linker between the pyrazole ring and the cyclohexyl group also decreased the activity of compounds (**8**, **9** vs **2**). In contrast, when the length of the linker was shortened and R^2 was replaced with a *trans*-4-hydroxycyclohexyl group, the corresponding analog **10** had enhanced activity compared to the parent **7**. More specifically, the IC_{50} value was changed from 18 nM for **7** to 2.4 nM for **10**, suggesting that the conformational rigidity and the size of R^2 was important for Mer inhibition. Consistent with this notion, analogs **12** and **13**, which featured open chain versions of cyclohexanol at the R^2 position, had decreased Mer inhibition. Using a tetrahydropyran to replace the 4-hydroxycyclohexyl group provided analogs that were less active (**14**, **15** and **16**). Furthermore, a cyclohexyl group alone at the R^2 position resulted in a 28-fold weaker inhibitor **17**, which further confirmed our previous observation: a hydrogen bond between the polar substituent at the R^2 position with the carbonyl of Arg727 of Mer protein was important [11]. The configuration of the hydroxyl group was not critical, as the *cis*-analog **11** had similar activity as *trans*-**10**.

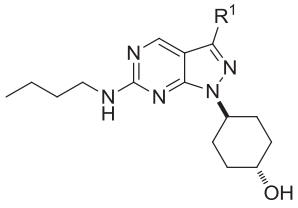
We also tested the activity of the synthetic analogs against Axl and Tyro3 to monitor their selectivity for TAM family members and develop a pan-TAM SAR. In general, these analogs showed modest selectivity within the TAM family and are generally less potent against Axl and Tyro3. In addition, to test our hypothesis regarding on the cause of the hERG activity, compounds **2** and **10** were characterized in the hERG binding assay. Indeed, **10** had no hERG activity in the PatchXpress assay while **2** was active with an EC_{50} of 3.2 μM. Removal of the primary amine at R^2 position eliminated the binding of our compounds to the hERG channel.

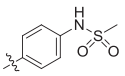
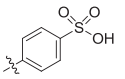
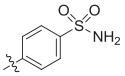
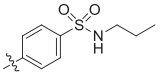
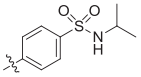
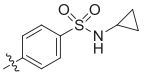
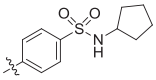
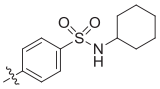
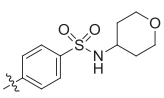
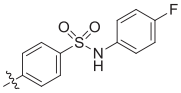
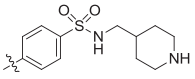
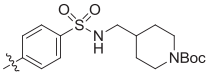
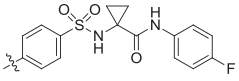
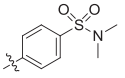
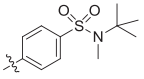
Next, the SAR at the R^1 site was explored (Table 2). In general, these analogs are very potent against Mer (at sub-nanomolar concentrations) and have good selectivity for Mer over Axl (>16 fold) and Tyro3 (>9 fold). A reversed sulfonamide functional group (**18**), sulfonic acid (**19**) and unsubstituted sulfonamide (**20**) at the R^1 site were tolerated. When one of the acidic hydrogens in sulfonamide was substituted with a small alkyl group such as propyl (**21**) or iso-propyl (**22**), the activity of the analogs remained the same. In contrast, a cycloalkyl group such as cyclopropyl (**23**), cyclopentyl (**24**) and cyclohexyl (**25**) at this position yielded a more active analog. A tetrahydropyran (**26**) or 4-piperidinylmethyl (**28**) group was also well tolerated at this position while a *para*-fluorophenyl ring (**27**) was less suitable. Interestingly, **29**, the precursor of **28** with a Boc protection group at piperidinyl nitrogen, was equally potent, which could be explained by the fact that the R^1 position was exposed to solvents as observed in the X-ray structure of **UNC569** complexed with Mer [11]. To further confirm this explanation, we introduced a large side chain at this position and found that the corresponding derivative **30** was still active. Finally, when the acidic hydrogen on the sulfonamide functional group was replaced by a variety of alkyl groups, the activity and TAM RTK-selectivity of the resulting analogs **31**–**36** were maintained [16].

Compared to **UNC569**, **35** (**UNC1062**) had improved selectivity and activity against Mer relative to other TAM family RTKs (78-fold selectivity over Axl and 54-fold over Tyro3). Morrison tight-binding inhibition studies revealed that **UNC1062** had a K_i of 0.33 nM against Mer [17]. These outstanding inhibitory profiles of **UNC1062** prompted us to further investigate its activity in intact cell systems.

The ability of **UNC1062** to inhibit Mer auto-phosphorylation in different cell lines was first tested. Briefly, human pre-B leukemia 697 cells were grown in suspension and treated with various concentrations of **UNC1062** for 1 h prior to addition of a phosphatase inhibitor to stabilize phospho-Mer. Mer protein was immunoprecipitated from lysates and phospho-Mer and total Mer proteins were detected by western blot. In this cell-based assay, the

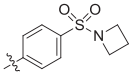
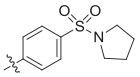
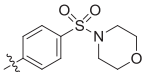
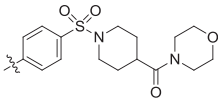
Table 2
SAR study of R^1.



Compound	R^1	IC_{50} (nM)[a]		
		Mer	Axl	Tyro3
18		4.0	110	35
19		1.8	130	55
20		1.4	29	54
21		1.8	62	35
22		3.2	120	68
23		1.1	75	38
24		0.80	67	31
25		1.1	350	45
26		1.2	69	28
27		9.7	6500	290
28		1.1	55	47
29		1.4	73	150
30		18	780	320
31		1.8	71	47
32		1.2	110	160

(*continued on next page*)

Table 2 (*continued*)

Compound	R^1	IC_{50} (nM)[a]		
		Mer	Axl	Tyro3
33		1.0	42	27
34		0.65	46	22
35		1.1	85	60
36		1.4	23	76

[a] Values are the mean of two or more independent assays performed at the ATP Km.

IC_{50} value for inhibition of phospho-Mer by **UNC1062** was 6.4 nM (Fig. 2). In comparison, the IC_{50} of **UNC569** in the same assay was 141 nM [11], suggesting that **UNC1062** was significantly more potent than **UNC569** both with purified Mer kinase and in intact cells.

To determine if **UNC1062** inhibits Mer phosphorylation in adherent cells, similar studies were performed using a human, adherent, pediatric rhabdoid brain tumor cell line (BT-12) and two NSCLC cell lines (A549 and Colo699). Inhibition of Mer phosphorylation was evident in cells when the concentration of **UNC1062** was above 300 nM (Fig. 3A). Similarly, phosphorylation of Mer was stably inhibited over 72 h of culture in A549 and Colo699 NSCLC cells treated with 250 nM or 500 nM **UNC1062** (Fig. 3B). Thus, while solid tumor cells require significantly higher concentrations of **UNC1062** compared to non-adherent leukemia cells, possibly as a result of decreased exposure across adherent cell membranes, treatment with **UNC1062** at sub-micromolar concentrations was sufficient to inhibit Mer phosphorylation in adherent cell lines.

To determine if **UNC1062** can mediate functional anti-tumor effects, BT-12, A549, and Colo699 cells were cultured in soft agar overlaid with medium containing **UNC1062** or vehicle only. Treatment with 1.0 μM **UNC1062** was sufficient to completely abrogate growth of BT-12 rhabdoid tumor colonies in soft agar (Fig. 4A). Similarly, treatment with **UNC1062** inhibited colony formation in both A549 and Colo699 NSCLC cultures (Fig. 4B). Moreover, inhibition of colony formation in long-term soft agar assays and inhibition of Mer phosphorylation in short-term assays occurred at similar concentrations of **UNC1062** in these cell lines, consistent with a causal relationship.

4. Conclusions

In summary, we have identified and synthesized a new family of small molecule Mer inhibitors, pyrazolopyrimidine sulfonamides, which potently inhibit the kinase activity of Mer. Importantly, this new generation of inhibitors do not show significant hERG activity in the PatchXpress assay. Through structure–activity relationship studies, **35** (**UNC1062**) was identified as both a potent (K_i = 0.33 nM) and selective Mer inhibitor. When applied to live cells, **UNC1062** inhibited Mer phosphorylation and colony formation in soft agar. These results provide the first evidence of anti-tumor activity mediated by a member of this novel class of inhibitors, thus further validate Mer as a drug target for cancer.

5. Experimental

5.1. General

Microwave reaction was carried out using a Discover-S reactor with a vertically-focused IR external temperature sensor and an Explorer 72 autosampler. The dynamic mode was used to set up the desired temperature and hold time with the following fixed parameters: Prestirring, 1 min; Pressure, 200 psi; Power, 200 W; PowerMax, off; stirring, high. Flash chromatography was carried out on pre-packed silica gel disposable columns. Analytical thin-layer chromatography (TLC) was performed with silica gel 60 F_{254}, 0.25 mm pre-coated TLC plates. TLC plates were visualized using UV_{254} or phosphomolybdic acid with charring. All ^1H NMR spectra were obtained with a 400 MHz spectrometer and ^{13}C NMR spectra were obtained with a 100 MHz spectrometer. Preparative HPLC was performed with the UV detection at 220 or 254 nm. LC–MS was performed with the UV detection at 220 nm, 254 nm, and 280 nm, and a single quadrupole mass spectrometer using electrospray ionization (ESI) source. High-resolution (positive ion) mass spectra (HRMS) were acquired using a LCMS-TOF mass spectrometer.

5.2. Synthesis

5.2.1. 3-Bromo-N-butyl-1H-pyrazolo[3,4-d]pyrimidin-6-amine (*4*)

To a mixture of 3-bromo-6-(methylthio)-1*H*-pyrazolo[3,4-*d*] pyrimidine (24.5 g, 100 mmol) in THF (100 mL) was added *meta*-chloroperoxybenzoic acid (33.7 g, 77%, 150 mmol) in portions at room temperature. The white mixture was stirred for 2 h and transferred into a THF (50 mL) solution of *n*-butylamine (49.4 mL, 500 mmol) at 0 °C. The resulting solution was heated at 60 °C for 2.0 h. After removal of the solvent under reduced pressure, MeOH was added and the mixture was filtered. The white solid was

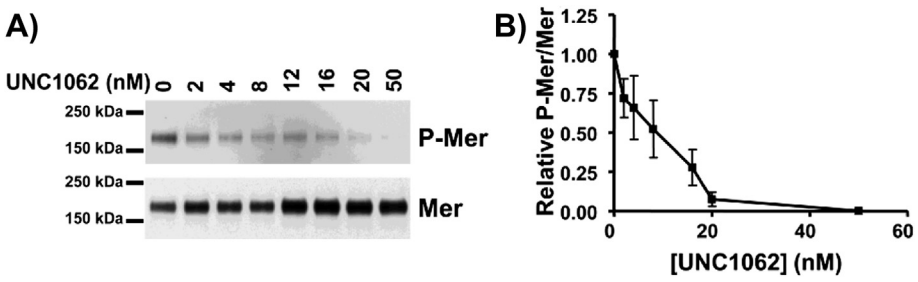


Fig. 2. UNC1062 inhibits accumulation of activated Mer protein in acute leukemia cells. 697 Cell cultures were treated with the indicated concentrations of **UNC1062** for 1 h. Pervanadate was added to cultures for 3 min to stabilize the phosphorylated form of Mer. Mer was immunoprecipitated from cell lysates and total Mer protein and Mer phosphoprotein were detected by immunoblot. A) Representative western blots. B) Relative levels of phospho-Mer and Mer proteins were determined. Mean values +/− standard error derived from 4 independent experiments are shown. IC_{50} = 6.4 nM with a 95% confidence interval of 3.8–10.7 nM.

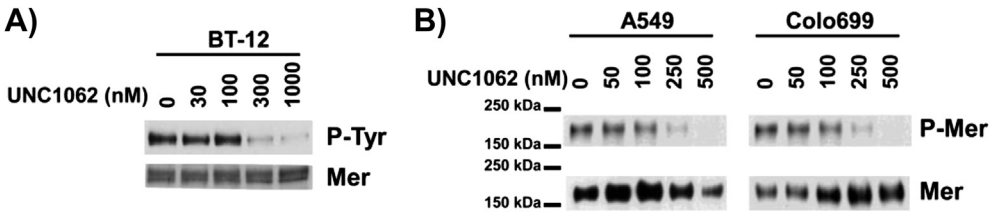


Fig. 3. UNC1062 inhibits Mer activation in adherent tumor cell lines. A) BT-12 pediatric rhabdoid tumor cells were treated with **UNC1062** or vehicle only for 1 h prior to the addition of pervanadate for 15 min. Cell lysates were prepared, Mer protein was immunoprecipitated, and phospho-tyrosine and Mer protein were detected by western blot. B) The indicated NSCLC cell lines were treated with **UNC1062** or an equivalent volume of vehicle only for 72 h prior to treatment with pervanadate for 1.0 (Colo699) or 5.0 (A549) min. Whole cell lysates were prepared, Mer protein was immunoprecipitated, and phosphorylated and total Mer proteins were detected by western blot. The results shown are representative of 3 independent experiments.

washed with MeOH (3×) and dried to afford the title compound **4** (23.6 g, 87%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.59 (s, 1H), 7.63 (s, 1H), 3.37–3.11 (m, 3H), 1.54–1.41 (m, 2H), 1.29 (dq, J = 14.4, 7.3 Hz, 2H), 0.84 (t, J = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.0, 157.4, 153.1, 120.5, 107.1, 41.0, 31.0, 20.1, 14.2; MS m/z 270.1 $[M + H]^+$.

*5.2.2. 4-(1-((trans-4-Aminocyclohexyl)methyl)-6-(butylamino)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-methylbenzenesulfonamide (**2**)*

A mixture of **4** (0.054 g, 0.20 mmol), K_2CO_3 (0.11 g, 0.8 mmol), DMF (2.0 mL), and *tert*-butyl (*trans*-4-(chloromethyl)cyclohexyl) carbamate (0.062 g, 0.25 mmol) in a 10 mL microwave tube was heated under microwave irradiation at 150 °C for 10 min. After cooling to room temperature, (4-(*N*-methylsulfamoyl) phenyl)boronic acid (0.065 g, 0.30 mmol), $Pd(PPh_3)_4$ (0.023 g, 0.020 mmol), and H_2O (1.0 mL) were added sequentially. The resulting mixture was stirred at room temperature for 1.0 min and then heated under microwave irradiation at 150 °C for 15 min. After cooling to room temperature, the mixture was quenched with H_2O and extracted with EtOAc (3×). The combined organic layer was dried (Na_2SO_4) and concentrated. The residue was purified by an ISCO silica gel column to provide *tert*-butyl (*trans*-4-((3-(4-(*N*-methylsulfamoyl)phenyl)-6-(butylamino)-1*H*-pyrazolo[3,4-*d*]pyrimidin-1-yl)methyl)cyclohexyl) carbamate as a white solid. This white solid was dissolved in CH_2Cl_2 (2.0 mL), then was added TFA (0.5 mL) at room temperature. The resulting solution was stirred for 1.0 h and was concentrated. The residue was purified by prep-HPLC to provide the title compound **2** as a white solid (0.053 g, 48% over 3 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.17 (s, 1H), 8.20–8.14 (m, 2H), 8.01–7.95 (m, 2H), 4.26 (d, J = 6.8 Hz, 2H), 3.52 (t, J = 7.1 Hz, 2H), 3.13–3.00 (m, 1H), 2.81 (s, 3H), 2.15–2.01 (m, 3H), 1.86 (d, J = 12.0 Hz, 2H), 1.68 (dt, J = 12.7, 7.4 Hz, 2H), 1.48 (dt, J = 14.8, 7.3 Hz, 2H), 1.41–1.21 (m, 4H), 1.00 (t, J = 7.4 Hz, 3H); LC–MS: >97% purity, t_R = 4.379 min; MS m/z 472.3 $[M + 1]^+$.

*5.2.3. 4-(1-((trans-4-Hydroxycyclohexyl)methyl)-6-(butylamino)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-methylbenzenesulfonamide (**7**)*

General procedure A: A mixture of **4** (0.054 g, 0.20 mmol), K_2CO_3 (0.11 g, 0.8 mmol), DMF (2.0 mL), and *tert*-butyl((*trans*-4-(chloromethyl)cyclohexyl)oxy)dimethylsilane (0.066 g, 0.25 mmol) in a 10 mL microwave tube was heated under microwave irradiation at 150 °C for 10 min. After the reaction mixture was cooled to room temperature, (4-(*N*-methylsulfamoyl)phenyl)boronic acid (0.065 g, 0.30 mmol), $Pd(PPh_3)_4$ (0.023 g, 0.020 mmol), and H_2O (1.0 mL) were added sequentially. The mixture was stirred at room temperature for 1.0 min and then heated under microwave irradiation at 150 °C for 15 min. After cooling to room temperature, the mixture was quenched with H_2O and extracted with EtOAc (3×). The combined organic layers were dried (Na_2SO_4) and concentrated. The residue was purified by an ISCO silica gel column to provide 4-(1-((*trans*-4-((*tert*-butyldimethylsilyl)oxy)cyclohexyl) methyl)-6-(butylamino)-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-yl)-*N*-methylbenzenesulfonamide as a white solid. This white solid was dissolved in THF (2.0 mL) and was added TBAF (1.0 M in THF, 1 mL) at room temperature. The resulting solution was refluxed for 2.0 h and was concentrated. The residue was purified by prep-HPLC to provide the title compound **7** (0.040 g, 44% over 3 steps) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.30 (s, 1H), 8.20 (d, J = 8.0 Hz, 2H), 8.01 (d, J = 8.0 Hz, 2H), 4.26 (d, J = 7.0 Hz, 2H), 3.58 (t, J = 7.2 Hz, 2H), 3.55–3.45 (m, 1H), 2.58 (s, 3H), 2.11–1.93 (m, 3H), 1.73 (td, J = 14.9, 7.9 Hz, 4H), 1.49 (dq, J = 14.8, 7.5 Hz, 2H), 1.32–1.14 (m, 4H), 1.02 (t, J = 7.7Hz, 3H); LC–MS: >97% purity, t_R = 6.629 min; MS m/z 473.3 $[M + 1]^+$.

*5.2.4. 4-(1-(2-(trans-4-Hydroxycyclohexyl)ethyl)-6-(butylamino)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-methylbenzenesulfonamide (**8**)*

The title compound **8** (0.040 g, 42%) was prepared according to general procedure A from *tert*-butyl((*trans*-4-(2-chloroethyl) cyclohexyl)oxy)dimethylsilane (0.069 g, 0.25 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.79 (s, 1H), 7.92 (d, J = 8.6 Hz,

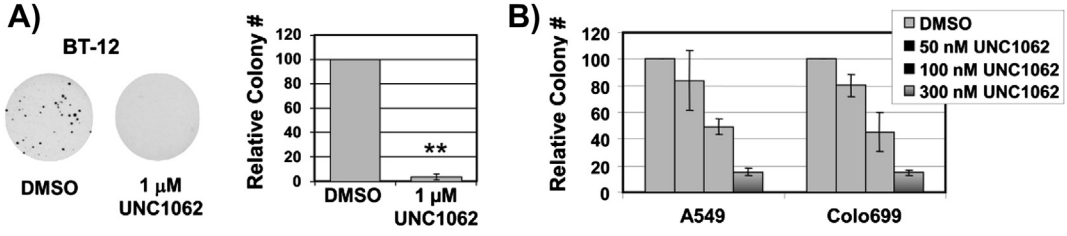


Fig. 4. UNC1062 inhibits colony formation in solid tumor cell lines. A) BT-12 rhabdoid tumor cells were cultured in soft agar containing 1.0 μM **UNC1062** or vehicle only and overlaid with medium containing **UNC1062** or vehicle only. Medium and **UNC1062** were refreshed twice weekly. Colonies were stained and counted. Representative plates are shown. B) A549 and Colo699 NSCLC cells were cultured in 0.35% soft agar overlaid with medium containing **UNC1062** or vehicle only. Medium and **UNC1062** were refreshed 3 times per week. Colonies were stained and counted. Mean values ± SEM derived from 3 independent experiments are shown. *Statistically significant results were determined using the student's paired t test (*$p < 0.02$, **$p < 0.001$ relative to vehicle only).

2H), 7.83 (d, J = 8.6 Hz, 2H), 4.24 (t, J = 7.0 Hz, 2H), 3.45–3.30 (m, 3H), 2.50 (s, 3H), 1.81 (t, J = 11.5 Hz, 3H), 1.71 (dd, J = 13.7, 6.8 Hz, 2H), 1.53 (dt, J = 14.9, 7.3 Hz, 2H), 1.40–1.27 (m, 2H), 1.08 (dd, J = 23.1, 12.5 Hz, 4H), 1.00–0.89 (m, 2H), 0.86 (t, J = 7.9 Hz, 3H); LC–MS: >97% purity, t_R = 5.759 min; MS m/z 487.3 $[M + 1]^+$.

5.2.5. 4-(1-(3-(trans-4-Hydroxycyclohexyl)propyl)-6-(butylamino)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-methylbenzenesulfonamide (**9**)

The title compound **9** (0.055 g, 56%) was prepared according to general procedure B from *tert*-butyl((*trans*-4-(3-chloropropyl) cyclohexyl)oxy)dimethylsilane (0.069 g, 0.25 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.03 (s, 1H), 8.17–8.09 (m, 2H), 7.99–7.92 (m, 2H), 4.32 (t, J = 6.9 Hz, 2H), 3.48 (t, J = 7.0 Hz, 2H), 3.42 (dt, J = 10.8, 4.2 Hz, 1H), 2.58 (s, 3H), 2.02–1.84 (m, 4H), 1.76 (d, J = 11.6 Hz, 2H), 1.66 (dt, J = 14.8, 7.3 Hz, 2H), 1.52–1.39 (m, 2H), 1.29–1.13 (m, 5H), 0.99 (t, J = 7.4 Hz, 3H), 0.96–0.86 (m, 2H); LC–MS: >97% purity, t_R = 5.856 min; MS m/z 501.3 $[M + 1]^+$.

5.2.6. 4-(1-(trans-4-Hydroxycyclohexyl)-6-(butylamino)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-methylbenzenesulfonamide (**10**) and 4-(1-(cis-4-hydroxycyclohexyl)-6-(butylamino)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-methylbenzenesulfonamide (**11**)

General procedure B: A mixture of **4** (0.054 g, 0.20 mmol), K_2CO_3 (0.11 g, 0.8 mmol), DMF (2.0 mL), and 4-chlorocyclohexanol (0.081 g, 0.6 mmol) in a 10 mL microwave tube was heated under microwave irradiation at 200 °C for 30 min. After cooling to room temperature, (4-(*N*-methylsulfamoyl)phenyl)boronic acid (0.065 g, 0.30 mmol), $Pd(PPh_3)_4$ (0.023 g, 0.020 mmol), and H_2O (1.0 mL) were added sequentially. The resulting mixture was stirred at room temperature for 1.0 min and then heated under microwave irradiation at 150 °C for 15 min. After cooling to room temperature, the mixture was quenched with H_2O and extracted with EtOAc (3×). The combined organic layer was dried (Na_2SO_4) and concentrated. The residue was purified by prep-HPLC to provide the title compounds **10** (0.011 g, 13% over 3 steps) and **11** (0.020 g, 23% over 3 steps) as white solids. Compound **10**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.78 (s, 1H), 7.92 (d, J = 8.4 Hz, 2H), 7.82 (d, J = 8.3 Hz, 2H), 4.51 (s, 1H), 3.64 (s, 1H), 3.37 (t, J = 7.0 Hz, 2H), 2.50 (s, 3H), 2.17–1.86 (m, 6H), 1.61–1.29 (m, 6H), 0.87 (t, J = 7.3 Hz, 3H); LC–MS: >97% purity, t_R = 5.402 min; HRMS (TOF, ESI$^+$) m/z: $[M + H]^+$ calculated for $C_{22}H_{31}N_6O_3S$, 459.2178; found 459.2155. Compound **11**: ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3OD) δ 8.82 (s, 1H), 7.95 (dd, J = 8.2, 6.2 Hz, 2H), 7.84 (dd, J = 8.5, 1.8 Hz, 2H), 4.66–4.48 (m, 1H), 3.44–3.33 (m, 3H), 2.53 (s, 3H), 2.46–2.32 (m, 1H), 1.99–1.81 (m, 3H), 1.79–1.61 (m, 3H), 1.55 (dt, J = 14.8, 7.4 Hz, 2H), 1.36 (td, J = 14.8, 7.4 Hz, 3H), 0.88 (t, J = 7.3 Hz, 3H); LC–MS: >97% purity, t_R = 5.590 min; MS m/z 459.2 $[M + 1]^+$.

5.2.7. 4-(1-(2-Hydroxyethyl)-6-(butylamino)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-methylbenzenesulfonamide (**12**)

General procedure C: A mixture of **4** (0.054 g, 0.20 mmol), K_2CO_3 (0.11 g, 0.8 mmol), DMF (2.0 mL), and 2-bromoethanol (0.038 g, 0.30 mmol) in a 10 mL microwave tube was heated under microwave irradiation at 150 °C for 10 min. After cooling to room temperature, (4-(*N*-methylsulfamoyl)phenyl)boronic acid (0.065 g, 0.30 mmol), $Pd(PPh_3)_4$ (0.023 g, 0.020 mmol), and H_2O (1.0 mL) were added sequentially. The resulting mixture was stirred at room temperature for 1.0 min and then heated under microwave irradiation at 150 °C for 15 min. After cooling to room temperature, the mixture was quenched with H_2O and extracted with EtOAc (3×). The combined organic layers were dried (Na_2SO_4) and concentrated. The residue was purified by an ISCO silica gel column to provide the title compound **12** (0.044 g, 57%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.86 (s, 1H), 8.00–7.92 (m, 2H), 7.91–7.83 (m, 2H), 4.42 (t, J = 5.0 Hz, 2H), 4.01 (t, J = 5.0 Hz, 2H), 3.38 (t, J = 7.1 Hz, 3H), 2.56 (s, 3H), 1.63–1.50 (m, 2H), 1.44–1.30 (m, 2H), 0.90 (t, J = 7.3 Hz, 3H); LC–MS: >97% purity, t_R = 5.244 min; MS m/z 405.2 $[M + 1]^+$.

5.2.8. 4-(1-(3-Hydroxypropyl)-6-(butylamino)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-methylbenzenesulfonamide (**13**)

The title compound **13** (0.045 g, 56%) was prepared according to general procedure C from 3-chloropropan-1-ol (0.029 g, 0.30 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.88 (s, 1H), 7.98 (d, J = 8.6 Hz, 2H), 7.90 (d, J = 8.6 Hz, 2H), 4.43 (t, J = 6.2 Hz, 2H), 3.50 (t, J = 5.8 Hz, 2H), 3.40 (t, J = 7.1 Hz, 2H), 2.59 (s, 3H), 2.10–2.00 (m, 2H), 1.60 (dt, J = 14.9, 7.4 Hz, 2H), 1.40 (dq, J = 14.5, 7.3 Hz, 2H), 0.92 (t, J = 7.3 Hz, 3H); LC–MS: >97% purity, t_R = 5.336 min; MS m/z 419.2 $[M + 1]^+$.

5.2.9. N-Methyl-4-(6-(butylamino)-1-(tetrahydro-2H-pyran-4-yl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)benzenesulfonamide (**14**)

General procedure D: A mixture of **4** (0.054 g, 0.20 mmol), K_2CO_3 (0.11 g, 0.8 mmol), DMF (2.0 mL), and 4-chlorotetrahydro-2*H*-pyran (0.072 g, 0.60 mmol) in a 10 mL microwave tube was heated under microwave irradiation at 200 °C for 30 min. After cooling to room temperature, (4-(*N*-methylsulfamoyl)phenyl) boronic acid (0.065 g, 0.30 mmol), $Pd(PPh_3)_4$ (0.023 g, 0.020 mmol), and H_2O (1.0 mL) were added sequentially. The resulting mixture was stirred at room temperature for 1.0 min and then heated under microwave irradiation at 150 °C for 15 min. After cooling to room temperature, the mixture was quenched with H_2O and extracted with EtOAc (3×). The combined organic layers were dried (Na_2SO_4) and concentrated. The combined organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by an ISCO silica gel column to provide the title compound **14** as a white solid (0.052 g, 61% over 2 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.96 (s, 1H), 8.07 (d, J = 8.5 Hz, 2H), 7.97 (d, J = 8.5 Hz, 2H), 5.33 (s, 1H), 4.92–4.79 (m, 1H), 4.32 (dd, J = 10.8, 5.4 Hz, 1H), 4.17 (dd, J = 11.7, 3.3 Hz, 2H), 3.64 (dd, J = 11.9, 10.2 Hz, 2H), 3.52 (dd, J = 12.9, 6.9 Hz, 2H), 2.71 (d, J = 5.4 Hz, 3H), 2.48 (ddd, J = 25.0, 12.5, 4.7 Hz, 2H), 1.97 (d, J = 10.8 Hz, 2H), 1.66 (dt, J = 14.8, 7.2 Hz, 2H), 1.47 (dq, J = 14.5, 7.3 Hz, 2H), 0.99 (t, J = 7.3 Hz, 3H); LC–MS: 96% purity, t_R = 5.689 min; MS m/z 445.2 $[M + 1]^+$.

5.2.10. N-Methyl-4-(6-(butylamino)-1-((tetrahydro-2H-pyran-4-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)benzenesulfonamide (**15**)

The title compound **15** (0.062 g, 70% over 2 steps) was prepared according to general procedure C from 4-(bromomethyl)tetrahydro-2*H*-pyran (0.045 g, 0.25 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.95 (s, 1H), 8.10–8.03 (m, 2H), 8.00–7.93 (m, 2H), 5.37 (br s, 1H), 4.41–4.30 (m, 1H), 4.24 (d, J = 7.1 Hz, 2H), 4.03–3.91 (m, 2H), 3.51 (dd, J = 13.0, 6.9 Hz, 2H), 3.38 (td, J = 11.5, 2.6 Hz, 2H), 2.71 (d, J = 5.4 Hz, 3H), 2.40–2.24 (m, 1H), 1.71–1.61 (m, 2H), 1.60–1.56 (m, 1H), 1.54–1.38 (m, 5H), 0.99 (t, J = 7.3 Hz, 3H); LC–MS: >97% purity, t_R = 5.714 min; MS m/z 459.3 $[M + 1]^+$.

5.2.11. N-Methyl-4-(6-(butylamino)-1-(2-(tetrahydro-2H-pyran-4-yl)ethyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)benzenesulfonamide (**16**)

The title compound **16** (0.058 g, 63% over 2 steps) was prepared according to general procedure C from 4-(2-bromoethyl) tetrahydro-2*H*-pyran (0.048 g, 0.25 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.95 (s, 1H), 8.10–8.03 (m, 2H), 7.99–7.93 (m, 2H), 5.38 (br s, 1H), 4.47–4.33 (m, 3H), 3.95 (dd, J = 11.5, 3.5 Hz, 2H), 3.49 (dd, J = 13.1, 6.8 Hz, 2H), 3.33 (td, J = 11.7, 1.8 Hz, 2H), 2.71 (d, J = 5.4 Hz, 3H), 1.97–1.86 (m, 2H), 1.76 (d, J = 12.7 Hz, 2H), 1.65 (dt, J = 12.7, 7.4 Hz, 2H), 1.55–1.30 (m, 5H), 0.98

(t, J = 7.3 Hz, 3H); LC–MS: 97% purity, t_R = 5.835 min; MS m/z 473.3 $[M + 1]^+$.

*5.2.12. 4-(1-Cyclohexyl-6-(butylamino)-1H-pyrazolo[3,4-d] pyrimidin-3-yl)-N-methylbenzene sulfonamide (**17**)*

The title compound **17** (0.035 g, 40% over 2 steps) was prepared according to general procedure D from chlorocyclohexane (0.071 g, 0.60 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.94 (s, 1H), 8.10–8.03 (m, 2H), 7.99–7.92 (m, 2H), 5.30 (br s, 1H), 4.68–4.56 (m, 1H), 4.33 (q, J = 5.4 Hz, 1H), 3.52 (dd, J = 12.9, 7.0 Hz, 2H), 2.70 (d, J = 5.4 Hz, 3H), 2.16–1.89 (m, 6H), 1.77 (d, J = 12.7 Hz, 1H), 1.66 (dt, J = 14.8, 7.3 Hz, 2H), 1.54–1.28 (m, 5H), 0.99 (t, J = 7.3 Hz, 3H); LC–MS: >97% purity, t_R = 6.358 min; MS m/z 443.2 $[M + 1]^+$.

5.2.13. trans-4-(3-Bromo-6-(butylamino)-1H-pyrazolo[3,4-d] pyrimidin-1-yl)cyclohexanol

To a solution of **4** (2.70 g, 10 mmol) and *cis*-4-chlorocyclohexanol (4.04 g, 30 mmol) in DMF (50 mL) was added K_2CO_3 (5.52 g, 40 mmol). The reaction mixture was heated at 150 °C for overnight, quenched with water at room temperature and extracted with EtOAc (3×). The combined organic layer was dried (Na_2SO_4) and concentrated. The residue was purified by an ISCO silica gel column to afford the title compound (1.83 g, 52%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.49 (s, 1H), 4.59–4.41 (m, 1H), 3.69–3.56 (m, 1H), 3.42 (t, J = 7.1 Hz, 2H), 2.13–2.00 (m, 4H), 1.98–1.89 (m, 2H), 1.66–1.56 (m, 2H), 1.52–1.36 (m, 4H), 0.96 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.6, 155.0, 153.4, 119.1, 110.3, 107.7, 68.3, 55.5, 34.6, 30.9, 29.8, 20.0, 14.1; MS m/z 369.1 $[M + H]^+$.

*5.2.14. trans-N-(4-(6-(Butylamino)-1-(4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenyl)methanesulfonamide (**18**)*

The title compound **18** (0.008 g, 18%) was prepared according to general procedure B from (4-(methylsulfonamido)phenyl)boronic acid (0.033 g, 0.15 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.76 (s, 1H), 7.76–7.72 (m, 2H), 7.28–7.24 (m, 2H), 4.56–4.48 (m, 1H), 3.71–3.61 (m, 1H), 3.42–3.39 (m, 1H), 3.29–3.27 (m, 2H), 2.92 (s, 3H), 2.14–2.02 (m, 4H), 1.97–1.90 (m, 2H), 1.61–1.53 (m, 2H), 1.47–1.32 (m, 6H), 0.90 (t, J = 7.36 Hz, 3H); LC–MS: >97% purity, t_R = 5.538 min; MS m/z 459.3 $[M + 1]^+$.

*5.2.15. 4-(1-(trans-4-Hydroxycyclohexyl)-6-(butylamino)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)benzenesulfonic acid (**19**)*

General procedure E: A mixture of *trans*-4-(3-bromo-6-(butylamino)-1*H*-pyrazolo[3,4-*d*]pyrimidin-1-yl)cyclohexanol (0.055 g, 0.15 mmol), K_3PO_4 (0.063 g, 0.30 mmol), 4-boronobenzenesulfonic acid (0.045 g, 0.225 mmol), $Pd(PPh_3)_4$ (0.017 g, 0.015 mmol), dioxane (2.0 mL) and H_2O (0.5 mL) in a 10 mL microwave tube was heated under microwave irradiation at 150 °C for 10 min. After cooling to room temperature, the mixture was quenched with H_2O and extracted with EtOAc (3×). The combined organic layer was dried (Na_2SO_4) and concentrated. The residue was filtered through a plug of Celite and then purified by prep-HPLC to afford the title compound **19** (0.027 g, 30%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 9.19 (s, 1H), 8.05–7.93 (m, 4H), 4.77–4.61 (m, 1H), 3.73 (tt, J = 10.7, 4.1 Hz, 1H), 3.57 (t, J = 7.1 Hz, 2H), 2.31–2.02 (m, 6H), 1.77–1.67 (m, 2H), 1.61–1.44 (m, 4H), 1.03 (t, J = 7.4 Hz, 3H); LC–MS: >97% purity, t_R = 4.890 min; MS m/z 444.2 $[M - 1]^-$.

*5.2.16. trans-4-(6-(Butylamino)-1-(4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)benzenesulfonamide (**20**)*

The title compound **20** (0.064 g, 71%) was prepared according to general procedure E from 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzenesulfonamide (0.086 g, 0.306 mmol) as a white solid. ^{1}H NMR (400 MHz, CD_3OD + $CDCl_3$) δ 8.74 (s, 1H), 7.87–7.78 (m, 4H), 4.46–4.43 (m, 1H), 3.59–3.55 (m, 1H), 3.33–3.28 (m, 2H), 2.03–1.93 (m, 4H), 1.90–1.85 (m, 2H), 1.50–1.45 (m, 2H), 1.39–1.33 (m, 2H), 1.30–1.25 (m, 2H), 1.08–1.06 (m, 3H), 0.80 (t, J = 7.4 Hz, 3H); LC–MS: 97% purity, t_R = 5.147 min; MS m/z 445.2 $[M + 1]^+$.

*5.2.17. trans-4-(6-(Butylamino)-1-(4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-propylbenzenesulfonamide (**21**)*

The title compound **21** (0.019 g, 13%) was prepared according to general procedure B from (4-(*N*-propylsulfamoyl)phenyl)boronic acid (0.109 g, 0.45 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 (s, 1H), 7.98 (s, 4H), 4.68–4.62 (m, 1H), 4.42 (t,, J = 6.16 Hz, 1H), 3.87–3.81 (m, 1H), 3.57–3.51 (m, 2H), 2.98 (dd, J = 6.95, 13.46 Hz, 2H), 2.25–2.16 (m, 4H), 2.10–2.07 (m, 2H), 1.71 (dt, J = 7.43, 14.83 Hz, 2H), 1.60–1.42 (m, 6H), 1.00 (t, J = 7.36 Hz, 3H), 0.89 (t, J = 7.40 Hz, 3H); LC–MS: >97% purity, t_R = 6.217 min; MS m/z 487.3 $[M + 1]^+$.

*5.2.18. trans-4-(6-(Butylamino)-1-(4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-isopropylbenzenesulfonamide (**22**)*

The title compound **22** (0.016 g, 13%) was prepared according to general procedure B from (4-(*N*-isopropylsulfamoyl)phenyl) boronic acid (0.095 g, 0.39 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.91 (s, 1H), 8.00 (dd, J = 8.62, 20.77 Hz, 4H), 4.69–4.61 (m, 1H), 4.26 (d, J = 7.61 Hz, 1H), 3.88–3.80 (m, 1H), 3.57–3.48 (m, 2H), 2.27–2.18 (m, 4H), 2.07 (d, J = 10.85 Hz, 2H), 1.71–1.53 (m, 6H), 1.47 (dd, J = 7.36, 14.90 Hz, 2H), 1.11 (d, J = 6.52 Hz, 6H), 0.99 (t, J = 7.34 Hz, 3H); LC–MS: >97% purity, t_R = 5.826 min; MS m/z 487.3 $[M + 1]^+$.

*5.2.19. 4-(6-(Butylamino)-1-(trans-4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-cyclopropylbenzenesulfonamide (**23**)*

The title compound **23** (0.003 g, 4%) was prepared according to general procedure E from (4-(*N*-cyclopropylsulfamoyl)phenyl) boronic acid (0.090 g, 0.375 mmol) as a white solid. ^{1}H NMR (400 MHz, CD_3OD). δ 9.11 (s, 1H), 8.17 (d, J = 8.6 Hz, 2H), 8.00 (d, J = 8.6 Hz, 2H), 4.73–4.63 (m, 1H), 3.79–3.67 (m, 1H), 3.51 (t, J = 7.1 Hz, 2H), 2.30–2.01 (m, 7H), 1.69 (td, J = 14.7, 7.4 Hz, 2H), 1.61–1.42 (m, 4H), 1.01 (t, J = 7.4 Hz, 3H), 0.62–0.47 (m, 4H); LC–MS: 95% purity, t_R = 5.957 min; MS m/z 485.0 $[M + 1]^+$.

*5.2.20. trans-4-(6-(Butylamino)-1-(4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-cyclopentylbenzenesulfonamide (**24**)*

The title compound **24** (0.015 g, 10%) was prepared according to general procedure B from (4-(*N*-cyclopentylsulfamoyl)phenyl) boronic acid (0.121 g, 0.45 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 7.99 (q, J = 8.67 Hz, 4H), 4.68–4.64 (m, 1H), 4.44 (d, J = 7.34 Hz, 1H), 3.86–3.82 (m, 1H), 3.67 (dd, J = 6.82, 13.53 Hz, 1H), 3.55–3.50 (m, 2H), 2.22–2.16 (m, 4H), 2.10–2.07 (m, 2H), 1.84 (dd, J = 5.70, 12.36 Hz, 2H), 1.71 (dd, J = 7.63, 14.79 Hz, 2H), 1.63–1.57 (m, 4H), 1.54–1.48 (m, 4H), 1.40–1.35 (m, 2H), 1.00 (t, J = 7.35 Hz, 3H); LC–MS: 95% purity, t_R = 6.186 min; MS m/z 513.3 $[M + 1]^+$.

*5.2.21. trans-4-(6-(Butylamino)-1-(4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-cyclohexylbenzenesulfonamide (**25**)*

The title compound **25** (0.015 g, 11%) was prepared according to general procedure B from (4-(*N*-cyclohexylsulfamoyl)phenyl) boronic acid (0.110 g, 0.39 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.95 (s, 1H), 8.04 (d, J = 8.61 Hz, 2H), 7.97 (d, J = 8.59 Hz, 2H), 5.36 (br s, 1H), 4.67–4.64 (m, 1H), 4.34 (d, J = 7.55 Hz, 1H), 3.87–3.80 (m, 1H), 3.52 (dd, J = 7.03, 12.98 Hz, 2H), 3.24–3.15 (m, 1H), 2.28–2.18 (m, 4H), 2.09–2.06 (m, 2H), 1.82–1.79 (m, 2H), 1.67–1.56 (m, 6H), 1.52–1.46 (m, 4H), 1.27–1.14 (m, 6H),

1.00 (t, $J = 7.35$ Hz, 3H); LC–MS: >97% purity, $t_R = 6.171$ min; MS m/z 527.0 $[M + 1]^+$.

*5.2.22. trans-4-(6-(Butylamino)-1-(4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-(tetrahydro-2H-pyran-4-yl)benzenesulfonamide (**26**)*

The title compound **26** (0.016 g, 10%) was prepared according to general procedure B from (4-(*N*-(tetrahydro-2*H*-pyran-4-yl)sulfamoyl)phenyl)boronic acid (0.128 g, 0.45 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.44 (br s, 1H), 8.92 (s, 1H), 8.00 (q, $J = 8.67$ Hz, 4H), 4.69–4.61 (m, 2H), 3.90–3.84 (m, 4H), 3.55 (t, $J = 7.04$ Hz, 2H), 3.43–3.33 (m, 2H), 2.21–2.07 (m, 6H), 1.79–1.68 (m, 4H), 1.60–1.44 (m, 6H), 1.00 (t, $J = 7.36$ Hz, 3H); LC–MS: 95% purity, $t_R = 5.747$ min; MS m/z 529.3 $[M + 1]^+$.

*5.2.23. trans-4-(6-(Butylamino)-1-(4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-(4-fluorophenyl)benzenesulfonamide (**27**)*

The title compound **27** (0.009 g, 6%) was prepared according to general procedure B from 4-(*N*-(4-fluorophenyl)sulfamoyl)phenyl) boronic acid (0.133 g, 0.45 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.38 (s, 1H), 8.82 (s, 1H), 7.94–7.89 (m, 2H), 7.85–7.81 (m, 2H), 7.09–7.05 (m, 2H), 6.98–6.94 (m, 2H), 6.57 (s, 1H), 4.67–4.61 (m, 1H), 3.98–3.80 (m, 1H), 3.57–3.51 (m, 2H), 2.22–2.08 (m, 6H), 1.71 (dt, $J = 7.29$, 14.74 Hz, 2H), 1.62–1.57 (m, 2H), 1.45 (dt, $J = 7.37$, 14.45 Hz, 2H), 0.99 (t, $J = 7.38$ Hz, 3H); LC–MS: >97% purity, $t_R = 6.198$ min; MS m/z 539.3 $[M + 1]^+$.

*5.2.24. trans-tert-Butyl 4-((4-(6-(butylamino)-1-(4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenylsulfonamido)methyl) piperidine-1-carboxylate (**29**)*

The title compound **29** (0.011 g, 9%) was prepared according to general procedure B from (4-(*N*-((1-(*tert*-butoxycarbonyl)piperidin-4-yl)methyl)sulfamoyl)phenyl)boronic acid (0.119 g, 0.30 mmol, 1.5 equiv) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3OD) δ 8.78 (s, 1H), 7.90 (d, $J = 8.44$ Hz, 2H), 7.80 (d, $J = 7.34$ Hz, 2H), 4.46–4.43 (m, 1H), 3.91 (d, $J = 12.95$ Hz, 2H), 3.63–3.61 (m, 1H), 2.36 (t, $J = 7.08$ Hz, 2H), 3.22 (dt, $J = 1.61$, 3.24 Hz, 3H), 2.65 (d, $J = 6.62$ Hz, 2H), 2.54–2.51 (m, 2H), 2.08–2.00 (m, 4H), 1.93–1.90 (m, 2H), 1.58–1.46 (m, 4H), 1.31 (s, 12H), 0.97–0.91 (m, 2H), 0.89 (t, $J = 7.35$ Hz, 3H); LC–MS: 96% purity, $t_R = 6.305$ min; MS m/z 642.4 $[M + 1]^+$.

*5.2.25. trans-4-(6-(Butylamino)-1-(4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-(piperidin-4-ylmethyl)benzenesulfonamide (**28**)*

A mixture of **29** (20 mg, 0.03 mmol) and TFA (2.3 mL) in dichloromethane (4.7 mL) was stirred at room temperature for 2.0 h, then diluted with dichloromethane (20 mL) and washed with a saturated aqueous $NaHCO_3$ solution, water and brine. The organic layer was dried (Na_2SO_4) and concentrated to afford the title compound **28** (12.7 mg, 75%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$+CD_3OD) δ 8.25 (s, 1H), 7.23 (d, $J = 8.45$ Hz, 2H), 7.14 (d, $J = 8.20$ Hz, 2H), 3.85–3.80 (m, 1H), 2.96–2.91 (m, 1H), 2.72 (t, $J = 7.15$ Hz, 2H), 2.54–2.52 (m, 3H), 2.07–1.99 (m, 4H), 1.41–1.32 (m, 4H), 1.26–1.23 (m, 2H), 1.12 (d, $J = 13.63$ Hz, 2H), 0.98–0.94 (m, 1H), 0.91–0.84 (m, 2H), 0.79–0.75 (m, 2H), 0.67–0.56 (m, 4H), 0.17 (t, $J = 7.35$ Hz, 3H); LC–MS: >97% purity, $t_R = 4.975$ min; MS m/z 542.3 $[M + 1]^+$.

*5.2.26. trans-1-(4-(6-(Butylamino)-1-(4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenylsulfonamido)-N-(4-fluorophenyl)cyclopropanecarboxamide (**30**)*

The title compound **30** (0.009 g, 7%) was prepared according to general procedure B from (4-(*N*-(1-((4-fluorophenyl)carbamoyl) cyclopropyl)sulfamoyl)phenyl)boronic acid (0.160 g, 0.42 mmol, 2.0 equiv) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 9.17 (s, 1H), 8.95 (s, 1H), 8.06 (d, $J = 8.54$ Hz, 2H), 7.96 (d, $J = 8.33$ Hz, 2H), 7.34–7.31 (m, 2H), 6.90–6.86 (m, 2H), 4.68–4.62 (m, 1H), 3.75–3.70 (m, 1H), 3.50 (t, $J = 7.90$ Hz, 2H), 2.24–2.13 (m, 4H), 2.06–2.03 (m, 2H), 1.71–1.64 (m, 2H), 1.58–1.38 (m, 6H), 1.10 (dd, $J = 4.67$, 7.89 Hz, 2H), 1.00 (t, $J = 7.36$ Hz, 3H); LC–MS: 96% purity, $t_R = 6.051$ min; MS m/z 622.3 $[M + 1]^+$.

*5.2.27. 4-(6-(Butylamino)-1-(trans-4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N,N-dimethylbenzenesulfonamide (**31**)*

The title compound **31** (0.052 g, 74%) was prepared according to general procedure E from (4-(*N*,*N*-dimethylsulfamoyl)phenyl) boronic acid (0.052 g, 0.225 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.94 (s, 1H), 8.07 (d, $J = 8.4$ Hz, 2H), 7.88 (d, $J = 8.3$ Hz, 2H), 5.34 (br s, 1H), 4.73–4.59 (m, 1H), 3.90–3.76 (m, 1H), 3.52 (dd, $J = 13.0$, 6.8 Hz, 2H), 2.75 (s, 6H), 2.29–2.15 (m, 4H), 2.13–2.02 (m, 2H), 1.66 (dt, $J = 14.9$, 7.3 Hz, 2H), 1.59–1.52 (t, $J = 10.2$ Hz, 3H), 1.47 (dd, $J = 15.0$, 7.4 Hz, 2H), 1.00 (t, $J = 7.3$ Hz, 3H); LC–MS: 97% purity, $t_R = 6.011$ min; MS m/z 473.3 $[M + 1]^+$.

*5.2.28. N-(tert-Butyl)-4-(6-(butylamino)-1-(trans-4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-N-methylbenzenesulfonamide (**32**)*

The title compound **32** (0.058 g, 76%) was prepared according to general procedure E from ((4-(*N*-(*tert*-butyl)-*N*-methylsulfamoyl) phenyl)boronic acid (0.061 g, 0.225 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.93 (s, 1H), 8.07–7.97 (m, 2H), 7.96–7.89 (m, 2H), 5.40 (br s, 1H), 4.73–4.58 (m, 1H), 3.91–3.76 (m, 1H), 3.52 (dd, $J = 12.9$, 6.9 Hz, 2H), 3.01 (s, 3H), 2.30–2.14 (m, 4H), 2.07 (d, $J = 10.8$ Hz, 2H), 1.68–1.44 (m, 7H), 1.38 (d, $J = 4.8$ Hz, 9H), 0.99 (t, $J = 7.3$ Hz, 3H); LC–MS: 96% purity, $t_R = 6.328$ min; MS m/z 515.3 $[M + 1]^+$.

*5.2.29. trans-4-(3-(4-(Azetidin-1-ylsulfonyl)phenyl)-6-(butylamino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)cyclohexanol (**33**)*

The title compound **33** (0.054 g, 74%) was prepared according to general procedure E from (4-(azetidin-1-ylsulfonyl)phenyl)boronic acid (0.054 g, 0.225 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.96 (s, 1H), 8.11 (d, $J = 8.3$ Hz, 2H), 7.95 (d, $J = 8.2$ Hz, 2H), 5.33 (s, 1H), 4.75–4.57 (m, 1H), 3.92–3.74 (m, 5H), 3.53 (dd, $J = 13.1$, 6.7 Hz, 2H), 2.32–2.16 (m, 4H), 2.15–2.03 (m, 4H), 1.66 (dt, $J = 14.8$, 7.2 Hz, 2H), 1.55–1.50 (m, 3H), 1.45 (dd, $J = 14.6$, 7.3 Hz, 2H), 1.00 (t, $J = 7.3$ Hz, 3H); LC–MS: >97% purity, $t_R = 5.992$ min; MS m/z 485.3 $[M + 1]^+$.

*5.2.30. trans-4-(6-(Butylamino)-3-(4-(pyrrolidin-1-ylsulfonyl) phenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)cyclohexanol (**34**)*

The title compound **34** (0.020 g, 27%) was prepared according to general procedure E from (4-(pyrrolidin-1-ylsulfonyl)phenyl) boronic acid (0.096 g, 0.375 mmol) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 9.20 (s, 1H), 8.20 (d, $J = 8.5$ Hz, 2H), 7.98 (d, $J = 8.5$ Hz, 2H), 4.75–4.64 (m, 1H), 3.78–3.67 (m, 1H), 3.55 (t, $J = 7.1$ Hz, 2H), 3.32–3.23 (m, 4H), 2.30–2.02 (m, 6H), 1.85–1.64 (m, 6H), 1.62–1.42 (m, 4H), 1.02 (t, $J = 7.4$ Hz, 3H); LC–MS: >97% purity, $t_R = 6.100$ min; MS m/z 499.0 $[M + 1]^+$.

*5.2.31. trans-4-(6-(Butylamino)-3-(4-(morpholinosulfonyl)phenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)cyclohexanol (**35**, **UNC1062**)*

The title compound **35** (0.063 g, 82%) was prepared according to general procedure E from 4-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)sulfonyl)morpholine (0.079 g, 0.225 mmol) as a white solid. ^{1}H NMR (400 MHz, CD_3OD). δ 9.20 (s, 1H), 8.26–8.20 (m, 2H), 7.95–7.89 (m, 2H), 4.75–4.64 (m, 1H), 3.79–3.68 (m, 1H), 3.73 (dd, $J = 10.2$, 5.7 Hz, 4H), 3.55 (t, $J = 7.1$ Hz, 2H), 3.08–2.97 (m, 4H), 2.36–2.01 (m, 6H), 1.76–1.65 (m, 2H), 1.62–1.42

(m, 4H), 1.02 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.6, 155.2, 154.0, 140.6, 136.9, 133.7, 128.3, 127.0, 105.1, 68.0, 65.3, 55.0, 45.9, 40.3, 34.2, 30.6, 29.4, 19.6, 13.7; LC–MS: >97% purity, t_R = 5.657 min; HRMS (TOF, ESI$^+$) m/z: $[M + H]^+$ calculated for $C_{25}H_{35}N_6O_4S$, 515.2440; found 515.2421.

5.2.32. *trans-(1-((4-(6-(Butylamino)-1-(4-hydroxycyclohexyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenyl)sulfonyl)piperidin-4-yl)(morpholino)methanone (**36**)*

The title compound **36** (0.065 g, 66%) was prepared according to general procedure E from (4-((4-(morpholine-4-carbonyl)piperidin-1-yl)sulfonyl)phenyl)boronic acid (0.091 g, 0.24 mmol) as a white solid. ^{1}H NMR (400 MHz, $CD_3OD + CDCl_3$) δ 9.21 (s, 1H), 8.37–8.28 (m, 2H), 8.24–8.16 (m, 2H), 5.04–4.94 (m, 1H), 4.60–4.38 (m, 2H), 4.23–4.07 (m, 3H), 3.99–3.94 (m, 3H), 3.93–3.82 (m, 4H), 3.78–3.68 (m, 2H), 2.91–2.82 (m, 2H), 2.76 (ddd, J = 10.4, 7.2, 3.9 Hz, 1H), 2.61–2.46 (m, 4H), 2.46–2.36 (m, 2H), 2.30–2.17 (m, 2H), 2.15–2.06 (m, 2H), 2.06–1.98 (m, 2H), 1.98–1.86 (m, 2H), 1.85–1.74 (m, 2H), 1.33 (t, J = 7.4 Hz, 3H); LC–MS: 96% purity, t_R = 5.468 min; MS m/z 626.3 $[M + 1]^+$.

5.3. Microfluidic capillary electrophoresis (MCE) assay

Activity assays were performed in a 384 well, polypropylene microplate with a final volume of 50 μL in 50 mM Hepes, pH 7.4 containing 10 mM $MgCl_2$, 1.0 mM DTT, 0.01% Triton X-100, 0.1% bovine serum albumin (BSA), using 1.0 μM fluorescent substrate (Table 3) and ATP at K_m for each enzyme (Table 3). All reactions were terminated by addition of 20 μL of 70 mM EDTA. Phosphorylated and unphosphorylated substrate peptides (Table 3) were separated following a 180 min incubation on a LabChip EZ Reader equipped with a 12-sipper chip in separation buffer supplemented with 1 × CR-8 and analyzed using EZ Reader software.

5.4. Morrison K_i measurement

Inhibition of Mer kinase by **UNC1062** was measured in the MCE assay using the Morrison tight-binding method [17b]. Reactions were performed in a 384 well, polypropylene microplate in a final volume of 80 μL in 50 mM Hepes, pH 7.4 containing 10 mM $MgCl_2$, 1.0 mM DTT, 0.01% Triton X-100, 0.1% bovine serum albumin (BSA), and 5.0 μM ATP. Compounds were tested in 20 dilution (1.5 fold) dose curves spanning a concentration range of 60–0.027 nM, 2.0 nM Mer, 1.0 μM peptide substrate, and 5.0 μM ATP, final. To 40 μL of enzyme (4 nM Mer), 8.0 μL of compound in 10% DMSO was added and allowed to incubate for 10 min followed by addition of 32 μL of substrate mix (containing 2.5 μM peptide substrate and 12.5 μM ATP).

Kinetic reads were taken from 7.0 to 209 min, 12 reads total, on an EZ Reader, using upstream voltage = −2250 V, downstream voltage = −500 V and pressure = −1.0 psi (sip time 0.2 s, buffer 30 s). The steady-state velocity was analyzed by linear regression of the peptide as percent-conversion/min, and a plot of nM compound (**UNC1062**) vs. velocity was fit to the Morison equation using GraphPad Prism 5.0, $Y = V_o^*(1 - ((((E_t + X + (K_i^*(1 + (S/K_m))))) - (((E_t + X + (K_i^*(1 + (S/K_m))))^2) - 4*E_t*X)^{0.5}))/(2*E_t)))$. The total enzyme concentration (E_t) was fixed to 0.855 nM as determined from previous titration studies (data not shown) [17b] (Y = velocity, X = inhibitor concentration).

For **UNC1062**, the Morrison K_i was 0.33 ± 0.083 nM (n = 3, error reported as S.D.).

Table 3
Assay conditions for MCE assays currently used.

Kinase	Peptide substrate	Kinase (nM)	ATP (uM)
Mer	5-FAM-EFPIYDFLPAKKK-$CONH_2$	2.0	5.0
Axl	5-FAM-KKKKEEIYFFF-$CONH_2$	120	65
Tyro	5-FAM-EFPIYDFLPAKKK-$CONH_2$	10	21

5.5. hERG binding assay

Assay buffer: Hank's balanced salt solution, 20 mM HEPES, 2.5 mM probenecid, pH 7.4, membrane potential sensitive dye (Molecular Devices).

To screen for drugs that block or open HERG K+ channels, we employ a fluorescence-based membrane potential assay (Molecular Devices). HEK293 cells stably expressing recombinant human HERG (provided by Dr. J. Overholt, Case Western Reserve University, Cleveland, OH; cells originally from Drs. A. Brown and E. Ficker, MetroHealth Medical Center, Cleveland, OH) are seeded in poly-L-lysine-coated 96-well plates (45,000 cells/100 μL DMEM supplemented with 10% fetal bovine serum/well) one day prior to assay. The next day, the medium is removed and replaced with 30 μL/well of assay buffer containing membrane potential dye (Molecular Devices) (the lyophilized dye is reconstituted with 15 mL of assay buffer). After a 15 min incubation at 37 °C, 30 μL/well of 2× dilutions of terfenadine (a known HERG blocker used as a reference compound) or test compound (final assay concentration ranging from 0.1 nM to 10 μM) are added to the cells (each concentration assayed in triplicate). Baseline fluorescence (excitation 530 nM, emission 565 nM) is measured over 15 min, then 140 μL of depolarization solution (143 mM KCl in distilled water) containing test or reference compound (1×) is added to the cells and fluorescence is recorded for 3 min. Raw fluorescence data are analyzed by GraphPad Prism 4.0.

5.6. Cell based assays for Mer kinase inhibition

697, BT-12, A549, or Colo699 cells were cultured in the presence of **UNC1062** or vehicle only for 1.0 h (697, BT-12) or 72 h (A549, Colo699). Pervanadate solution was prepared fresh by combining 20 mM sodium orthovanadate in 0.9× PBS in a 1:1 ratio with 0.3% (w/w) hydrogen peroxide in PBS for 15–20 min at room temperature. Cultures were treated with 120 μM pervanadate prior to collection for preparation of whole cell lysates, immunoprecipitation of Mer, and analysis by western blot.

697, A549, and Colo699 cells were treated with pervanadate for 3.0, 5.0, and 1.0 min respectively. Cell lysates were prepared in 50 mM HEPES pH 7.5, 150 mM NaCl, 10 mM EDTA, 10% glycerol, and 1% Triton X-100, supplemented with protease inhibitors (Roche Molecular Biochemicals, #11836153001). Mer protein was immunoprecipitated with a monoclonal anti-Mer antibody (R&D Systems, #MAB8912) and Protein G agarose beads (InVitrogen). Phospho-Mer was detected by western blot using a polyclonal anti-phospho-Mer antibody raised against a peptide derived from the tri-phosphorylated activation loop of Mer. Nitrocellulose membranes were stripped and total Mer protein was detected using a second anti-Mer antibody (Epitomics Inc., #1633-1). For 697 cells, relative phosphorylated and total Mer protein levels were determined by densitometry and IC_{50} values were determined by non-linear regression.

Confluent BT-12 cultures were treated with pervanadate for 15 min. Cell lysates were prepared in 20 mM HEPES pH 7.5, 500 mM NaCl, 5.0 mM EDTA, 10% glycerol, and 1% Triton X-100, supplemented with protease inhibitors (10 μg/mL leupeptin, 10 μg/mL phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin) and phosphatase inhibitors (50 mM sodium fluoride and 1.0 mM sodium orthovanadate) and Mer protein was immunoprecipitated using a custom polyclonal rabbit anti-Mer antisera raised against a peptide

derived from the N-terminus of Mer and Protein A agarose beads (Santa Cruz Biotechnology). Phosphotyrosine-containing proteins were detected by western blot with a monoclonal HRP-conjugated anti-phosphotyrosine antibody (Santa Cruz Biotechnology, #sc-508). Antibodies were stripped from membranes and total Mer levels were determined using a custom polyclonal rabbit anti-Mer raised against a peptide derived from the catalytic domain of Mer.

5.7. Soft agar colony formation assays

BT-12 cells (10,000 cells) were cultured in 2.0 mL of 0.35% soft agar containing 0.5× RPMI medium, 7.5% FBS, and the indicated concentrations of **UNC1062** or DMSO vehicle only and overlaid with 0.5 mL of 1× RPMI medium containing 15% FBS and **UNC1062** or DMSO vehicle only. Medium and **UNC1062** or vehicle were refreshed 2 times per week. Colonies were stained with thiazolyl blue tetrazolium bromide (Sigma Aldrich, #M5655) and counted after 3 weeks.

A549 or Colo699 cells (15,000 cells) were cultured in 1.5 mL of 0.35% soft agar containing 1× RPMI medium and 10% FBS and overlaid with 2.0 mL of 1× RPMI medium containing 10% FBS and the indicated concentrations of **UNC1062** or DMSO vehicle only. Medium and **UNC1062** or vehicle were refreshed 3 times per week. Colonies were stained with nitrotetrazolium blue chloride (Sigma Aldrich, #N6876) and counted after 2 weeks.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2013.03.035. These data include MOL files and InChiKeys of the most important compounds described in this article.

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