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Conjugate Additions of Amines to Maleimides via Cooperative Catalysis

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Abstract. A cooperative system comprising of a lithium Lewis acid and amine base significantly enhances the rate of the conjugate addition of a wide array of amines to maleimides. This operationally simple, scalable method provides mono-addition products in high yields and purity. This conjugation was successfully applied to the kinase inhibitor crizotinib in a chemoselective ligation to create novel fluorescent probe.

Keywords: Amino-conjugate addition; bioconjugation; cooperative catalysis; crizotinib; maleimide; synergistic catalysis

Bioactive small molecules are the foundation of modern pharmaceutical sciences.[1] They can also serve as critical probes for elucidating complex biological pathways. Understanding how these important small molecules interact with biological systems has been aided through covalent conjugation of fluorophores and affinity labels.[2] Such conjugated small molecules are employed to label specific cellular components including tubulin, actin, as well as innumerable enzymes and proteins.[3] This strategy is effective when the covalent linkages are stable in vivo and do not adversely affect the overall pharmacology through perturbations in steric interactions, charge and/or solubility. The selective formation of N–C bonds fulfills most of the above criteria.[4] Highly useful systems, such as perfluorosulfones, isothiocyanates and *N*-hydroxy-succinimide esters provide stable conjugated products.[5] However, the latter two reagents generate thiourea and amide linkages possessing altered charge states to the native amines, which could potentially alter the pharmacology of the resulting bioconjugated products. In addition to the specifications of the structural components, the preparation of the conjugate must be high yielding, mild, atom economical, and chemoselective. The reaction between maleimides and 1° and 2° amines produces stable conjugates, but the rates for these additions are much slower by comparison, thereby potentially limiting their deployment and utility.[6]

To broaden the chemical toolbox, we sought to develop a rapid and selective conjugation strategy for the conjugate addition of amines to maleimides. A rate acceleration of this amino-conjugate addition could broaden access to a more diverse array of biologically active small molecule probes. Although reactions with nitrogen nucleophiles with various conjugate acceptors have been extensively studied,[7] very few systems involve maleimides.[8] With this specific electrophile, reactions are typically performed at elevated temperatures and/or extended times, potentially curtailing application in complex settings.[9] In many cases, mixtures of 1,2- and 1,4-addition products are also observed (vide infra). As a result of these reactivity and chemoselectivity limitations, amines are currently underutilized as nucleophiles with maleimides.

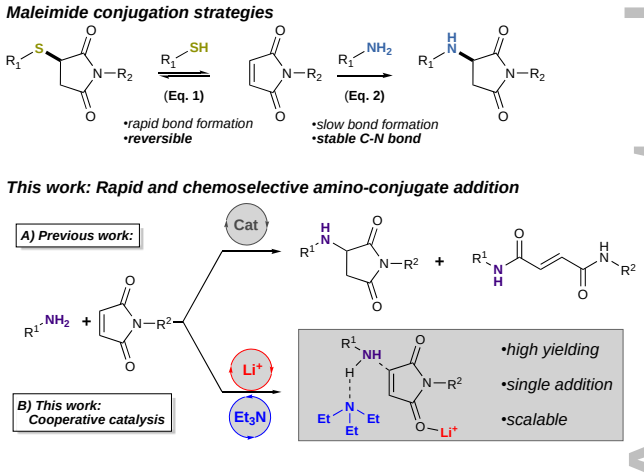


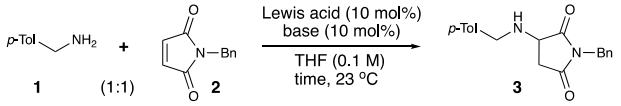
Figure. 1. General reaction design.

Our group has recently developed the first practical asymmetric amino-conjugate addition reaction between simple, unprotected alkyl amines and maleimides.[10] Our studies on this reaction were motivated by two overarching goals; the need for new and strategically simple methods to access chiral amines, as well as the potential to expand the existing toolbox of maleimide-tagged biological probes that are already commercially available and

predominantly marketed for use with more reactive sulfhydryl nucleophiles.[11] With these challenges in mind, we sought a general catalytic method for the efficient and rapid addition of amines to maleimides at ambient temperatures.[12] Given our interest in cooperative catalysis[13] and processes that involve dual activation of both electrophilic and nucleophilic reactants,[14] we initiated an investigation to engage maleimides with amines to afford conjugate addition products through a Lewis acid/Brønsted base strategy, in the context of enhancing the chemoselectivity of this process for general and operationally simple deployment with existing maleimide electrophiles when enantioselectivity or the use of chiral catalysts is not the primary goal of the application (Figure 1).

We began our studies with a reaction between equimolar quantities of *N*-tolylamine (**1**) and *N*-benzylmaleimide (**2**) focused on enhancing the reaction rate (Table 1). The rate constant of the uncatalyzed conjugate addition in THF at 0.1 M was approximately 4.40 x 10-4 $M^{-1}s^{-1}$ (entry 1).[15] A theoretical study by Yamabe on the conjugate addition of dimethylamine with maleimide derivatives suggested multiple molecules of reagent amine must be invoked in the reaction mechanism in order to rationalize the formation of aminosuccinimide products via an energetically feasible pathway.[16] The additional amine molecules are postulated to provide stabilization to the zwitterionic adduct formed after initial attack of the amine on maleimide via extended hydrogen-bonding interactions, enabling the subsequent rate-limiting proton transfer to generate product. We first investigated the possibility of employing an additional amine as a catalyst in an effort to improve the rate of conjugate addition. Using 10 mol % triethylamine (Et_3N) as a sole additive produced a negligible impact on the rate (entry 2). Based on the understanding that lithium ions can be activators of 1,4-conjugated systems,[17] we added 10 mol% lithium chloride (LiCl) and observed a significantly increased rate of the reaction (entry 3). Strikingly, when catalytic amounts of both Et_3N and LiCl were present, the rate significantly increased further (entry 4). While cooperative or synergistic effects of lithium salts and amines have been invoked previously for related transformations, the use of these descriptors has been widely accepted to mean "working together".[18] Based on our detailed kinetic analysis, we observed that the optimized conditions above are indeed cooperative. The combined effect of the two catalysts as depicted in Figure 2 (cooperative plot, red ◆◆◆) demonstrates that the rate enhancement observed is greater than the sum of the individual contributions of the catalysts depicted by the additive plot (blue ●●●, Figure 2 and Supporting Information).

Table 1. Optimization of the title reaction.



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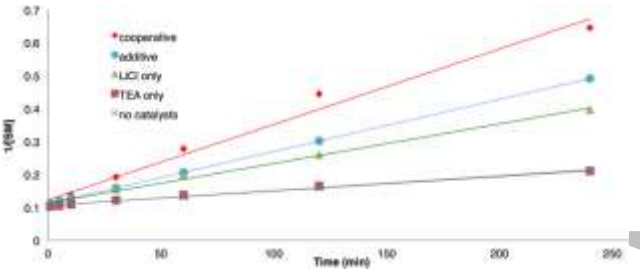
entry	Lewis acid	base	k ($M^{-1}s^{-1}$)[a]	$t_{1/2}$ (min)[b]
1	---	---	4.40 x 10^{-4}	378
2	---	Et_3N	4.50 x 10^{-4}	370
3	LiCl	---	1.18 x 10^{-3}	141
4	LiCl	Et_3N	2.29 x 10^{-3}	72 (97%)[c]
5	LiOTf	Et_3N	2.85 x 10^{-3}	58
6	$LiBF_4$	Et_3N	2.98 x 10^{-3}	55
7	$LiPF_6$	Et_3N	7.30 x 10^{-3}	22
8	LiOH	---	5.30 x 10^{-4}	314
9	Et_3N•HCl	---	5.00 x 10^{-4}	333

a) NMR yields with 1,3,5-trimethoxybenzene as an internal standard to calculate rate constants. b) $t_{1/2}$ values derived from rate constants c) Isolated yield after 4 h on 1 mmol scale.

Figure 2. Observation of cooperativity via detai analysis. Plot of 1/[starting material] vs. time. Lines with slopes greater than the calculated "additive" plot are considered cooperative (see SI).



In the presence of triethylamine, other lithium salts with weakly coordinating counter anions were surveyed. LiOTf and $LiBF_4$ were observed to moderately improve the reaction rate beyond that of LiCl (entries 5 and 6). Although $LiPF_6$ was observed to have the shortest t1/2 (entry 7), we mainly focused on LiCl for our purposes due to its low cost/mol, handling constraints, and reduced toxicity relative to $LiPF_6$. LiOH was investigated as a salt possessing both Lewis acidic and Brønsted basic properties, and we observed a similar profile to Et_3N alone (entry 8). Lastly, 10 mol% Et_3N•HCl was examined as the potential species responsible for the rate increase, however we observed a similar profile to Et_3N (entry 9). LiCl was selected as the Lewis acid to be paired with Et_3N moving forward due to its low molecular weight as well as its cost and ease of manipulation.[19]

We next investigated a range of primary amines for this cooperative process (Table 2). By employing a slight excess of the amine, we ensured the full consumption of maleimide **2** and improved the isolated yields. We also simplified the purification by filtering the excess amine and catalyst through a thin plug of silica gel, yielding pure conjugated product after concentration. Using this simplified protocol, aminosuccinimide products were furnished in excellent yields with primary amines (**4**—**7**). Products generated from branched amines were also well tolerated (**8**—**10**). When the steric size of the

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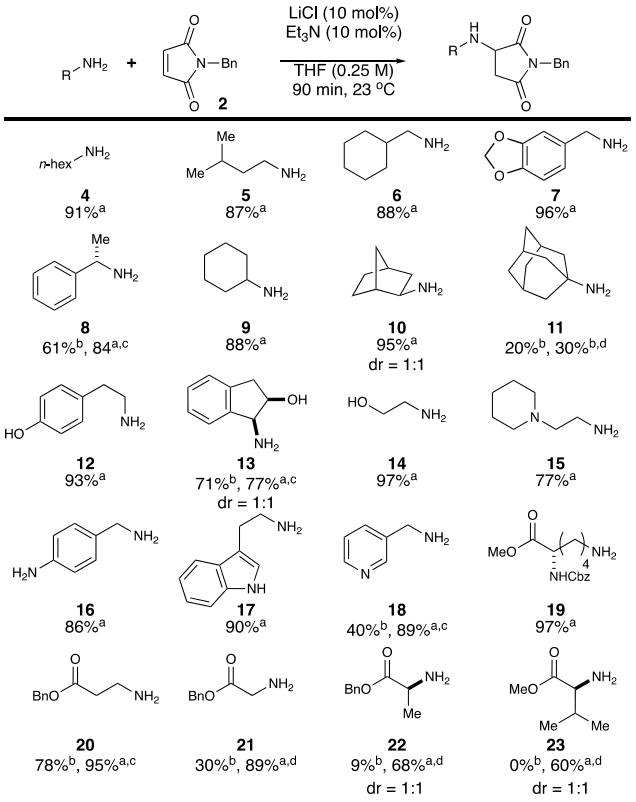
nucleophile was further increased to 1-adamantyl (**11**), the rate of the reaction slowed as judged by the yield after 90 min (20% yield). Other protic functional groups were well accommodated under these conditions, (**12**—**14**). Not surprisingly, nucleophiles containing two nitrogen atoms (**15**—**18**) selectively reacted at the more reactive amine position over others. For example, protected amino acid Cbz-Lys-OMe (**19**) was an excellent substrate. The reactivity of deactivated amines was increased with higher amounts of the cooperative Lewis acid/Brønsted base mixture (**20**—**23**).

Table 2. Scope of primary amine nucleophiles

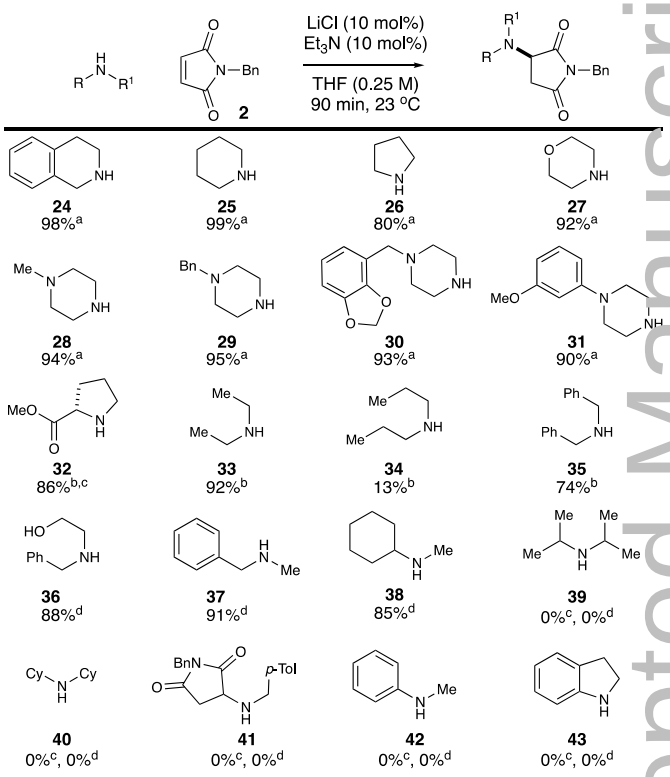


a) isolated yield. b) NMR yield. c) $LiPF_6$ (10 mol %), Et_3N (10 mol %). d) $LiPF_6$ (100 mol %), Et_3N (100 mol %).

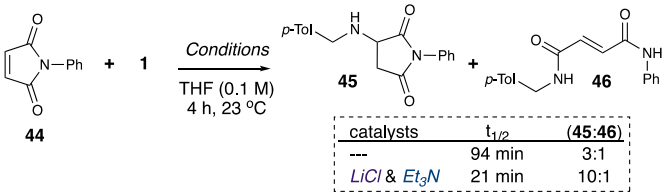
Secondary amines were also successful nucleophiles under the optimized set of reaction conditions (Table 3). Various cyclic secondary amines were excellent substrates (**24**—**31**). Proline methyl ester reacts rapidly under cooperative conditions with $LiPF_6$ (10 mol %) (**32**). Acyclic and secondary amines were not as successful nucleophiles since they required extended reaction times to react with maleimide **2** (**33**—**38**). Bulky acyclic secondary alkyl amines were inert under these conditions as well (**39**—**41**). These data are consistent with the observation that over-addition products are not generated under the reaction conditions. For example, secondary amine product **3** of the title reaction was re-subjected to the optimized conditions and did not produce **41**. Furthermore, secondary amines with aryl substituents (**42** and **43**) were unreactive under these conditions, which is consistent with the observation that aniline is unreactive (not shown).

The scope of conjugate acceptors through variation of the maleimide's nitrogen substituent was also investigated. More electron-deficient *N*-arylmaleimides react much faster with amines compared to the corresponding reaction with *N*-benzylmaleimide **2**. However, *N*-arylmaleimides suffer from competitive 1,2-additions.[12a, 12b] *N*-phenylmaleimide (**44**) in the absence of the cooperative catalysis conditions reacted with *N*-tolylamine **1** with a $t_{1/2}$ of 94 min, producing a reaction mixture that contained a ~3:1 ratio of 1,4- to 1,2-addition products (**45**:**46**, Scheme 1). In the presence of the cooperative catalysts, the observed $t_{1/2}$ was 21 min with an improved product ratio of 10:1.

Table 3. Scope of secondary amine nucleophiles



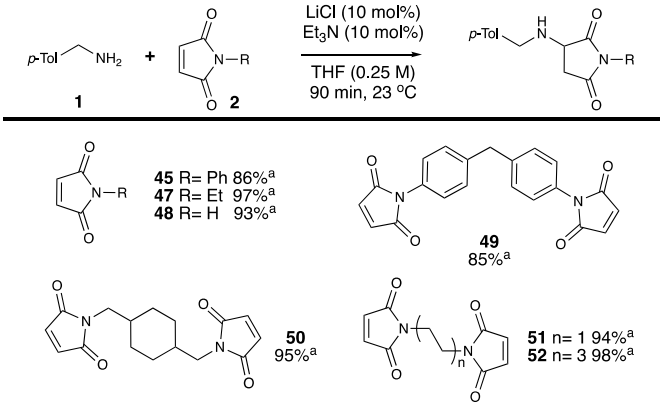
a) Isolated yield after 90 min on 1 mmol scale. b) Isolated yield after 15 h. c) As observed by NMR after 90 min. d) $LiPF_6$ (10 mol %),



Scheme 1. Improvement of 1,4-addition selectivity.

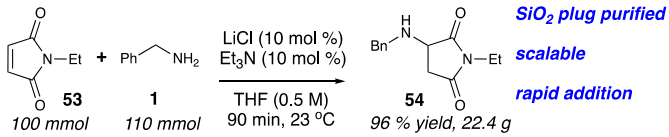
Other maleimides were also explored as a platform for future potential translation to materials/polymerization studies. For example, **44** underwent efficient 1,4-addition with *N*-benzylamine to afford the corresponding aminosuccinimide 45 in 86% yield. Other nitrogen-functionalized maleimides were suitable conjugate acceptors, providing the corresponding aminosuccinimide products of *N*-ethylmaleimide (**47**) and maleimide (**48**), in 97% and 93% yields, respectively (Table 4). As a potential entry into maleimide containing materials,[20][21] *N*-alkyl and *N*-aryl-bismaleimides were also investigated as electrophiles. The bismaleimides shown in Table 4 are commonly employed monomers in the synthesis of poly(imido) succinimides.[22] *N,N*-diaryl-bis-maleimide **49** provided its corresponding product in 85% yield, and was easily separated from the remaining 12% of 1,2-addition product formed. 1,4-bismethylenecylohexyl bis-maleimide **50** afforded its corresponding product in 95% yield as a 1:1 mixture of *cis*- and *trans*- diastereomers. Equally suitable bismaleimides were ethylenediamine (**51**) and hexamethylenediamine-derived bismaleimide (**52**), generating their corresponding conjugated products excellent yields.

Table 4. Differentially substituted maleimides as electrophiles



a) isolated yields after 90 min

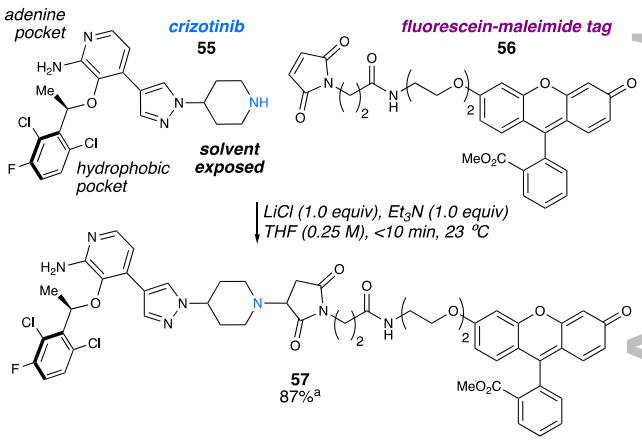
To further highlight the ease and utility of these conditions, we conducted the reaction on 100 mmol scale with *N*-ethylmaleimide **53** (the same starting material that provided **47**) and *N*-benzylamine. At 10 mol % loading of each catalyst, the reaction reached full conversion in 90 min with no observed exotherm. The large-scale reaction was purified by filtration through a diatomaceous earth-supported pad of silica gel to yield 22.4 g of pure conjugated product **54** in 96% yield.



Scheme 2. Scale-up of amine conjugate addition

After exploring the scope of our conjugate addition with a variety of amines and maleimides, we extended this method to a more complex reaction partner with potential biological/medical application. Crizotinib (**55**) was approved in 2011 for the treatment of late-stage lung cancer, anaplastic large cell lymphoma, and neuroblastoma.[23] Although **55** strongly binds ALK, it has also been found to bind ROS proto-oncogene 1-encoded kinase (ROS1), c-MET, and proto-oncogene-encoded kinase of the hepatocyte growth factor receptor (HGFR).[24] Based on the ALK-57 bound X-ray structure, **55** is observed to bind to the ATP pocket through the aminopyridine portion and to possess a hydrophobic interaction through its halogenated benzyloxy moiety. The piperidine moiety was observed to protrude out of the binding pocket, into the solvent exposed region, making this moiety an excellent handle for conjugation. We predicted that conjugation to the fluorescein-maleimide tag **56** would selectively occur at the piperidine nitrogen of **55** based on the chemoselectivity parameters we previously established (see Tables 2 and 3).

Using a full equivalent of LiCl (5 wt %) and Et_3N (10 wt %), we were able to selectively synthesize **57** in 87% yield in less than 10 min (Scheme 3). A higher catalyst loading was employed to expedite the reaction and highlight the expendability of these simple catalysts without detriment to yield or selectivity. Gratifyingly, **57** maintained activity against A549, human non-small cell lung cancer cells in vitro with an IC_{50} value of 9.0 □M. Although less potent compared to **57** (IC_{50}= 1.0 □M), **57** produced high quality confocal fluorescent images indicating the conjugate material localizing on the periphery of the cell and outside the nucleus (Figure 4). Further studies to understand this observation with our new crizotinib-fluorophore probe are ongoing.



Scheme 3. Conjugation of Crizotinib to a Maleimide-

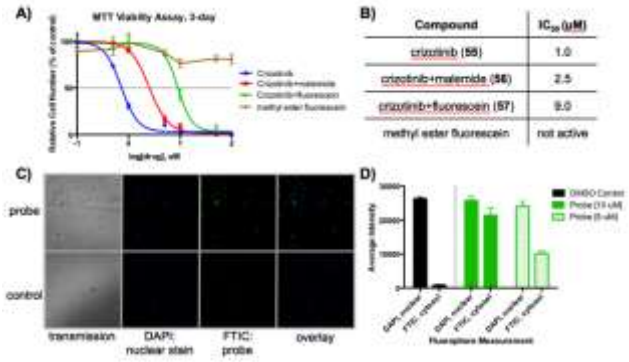


Figure 3. Preliminary characterization of crizotinib-fluorophore probe. A) 3-day viability assay data validated effect of fluorophore/malemide on drug, crizotinib results were comparable to previous literature values.[25]. B) IC_{50} values indicate that (**56**) and fluorescein methyl ester do not greatly effect the activity of the original drugs, mostly likely due to loss of solubility seen to the naked eye. C) Example images of condition optimization of probe incubation with A549 cells with and without probe **57** [24hr, 10uM probe] collected via ImageXpress. D) Fluorophore incorporation into cell to ensure limited autofluorescence background signal of A549 cell line in the FTIC range.

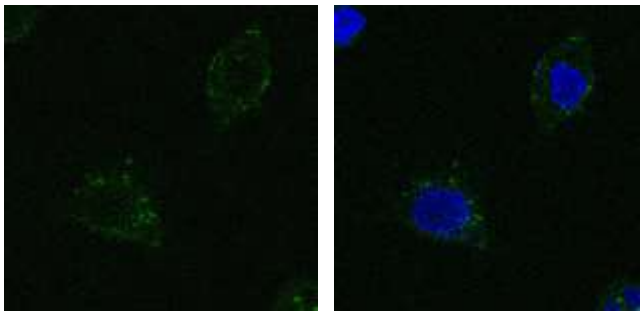


Figure 4. Confocal images (86 x 86 mm) of 10 □M (**57**). IC_{50} values: **55**: 1.0 □M, **57**: 9.0 □M.

In conclusion, the combination of a Lewis acid and Brønsted base is not unproductive based on canonical interactions, but instead catalyzes the conjugate addition reaction of amines and maleimides. Significant improvements in the rate and selectivity of the reaction were observed under these conditions. For electronically and sterically deactivated amines, greater amounts of cooperative catalysts can be employed in to increase reactivity. These mild conditions have been successfully deployed to generate biologically active chemical probes from an FDA approved drug. With this cooperative catalysis platform, the current array of commercial functionalized-maleimide tags (exclusively marketed toward sulfhydryl systems) could potentially be employed with amine-containing pharmacophores to shed light on biological effects such as cell permeability, trafficking, and localization. Additional investigations into further exploring this late stage pharmaceutical conjugation approach are underway.

Experimental Section

All reactions were carried out under ambient atmosphere in air-dried glassware with magnetic stirring. THF, toluene, and DMF were purified by passage through a bed of activated alumina. Reagents were purified prior to use unless otherwise stated following the guidelines of Perrin and Armarego.[26] *N*-tolylamine was distilled from CaH_2. Purification of reaction products was carried out by flash chromatography using EM Reagent silica gel 60 (230-400 mesh). See the Supporting Information for details regarding characterization of all new products.

Preparation of stock solution A:

A 20 mL vial was charged with THF (10 mL), followed by LiCl (21.2 mg, 0.2 mmol) and triethylamine (69.6 uL, 0.2 mmol). The resulting mixture was stirred until all LiCl crystals were fully dissolved and the stock solution became homogenous.

Preparation of stock solution B:

In a 20 mL vial, *N*-benzylmaleimide (940 mg, 2 mmol) was dissolved in THF (10 mL). The stock solution was stirred until homogenous.

General procedure for the synthesis of aminosuccinimides:

To a 20 mL screw-cap vial equipped with a magnetic stir bar was added 500 μL of **stock solution A**, followed by 0.11 mmol (1.1 equivalents) of the primary or secondary amine nucleophile at 23 ºC. 500 μL of **stock solution B** was added to the reaction mixture, and the resulting solution was stirred at ambient temperature for 90 minutes. The crude reaction mixture was subsequently filtered through a 1 cm pad of SiO_2 supported on a 0.5 cm plug of diatomaceous earth. The filter cake was flushed with EtOAc (3 x 1 mL). The combined organic filtrates were concentrated *in vacuo* on a rotary evaporator. The concentrated material was triturated with 1 mL of CH_2Cl_2 and re-concentrated *in vacuo* three additional times to yield the product aminosuccinimides in the reported yields (See Supporting Information for details).

Cell assay procedures

A549 cells were obtained from Northwestern University's Developmental Therapeutics Core. Cells were grown in Roswell Park Memorial Institute (RPMI) 1640 media (GIBCO, Life Technologies, Grand Island, NY) and supplemented with 10% FBS (Whittaker, Walkersville, MD), 1% HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer and 1% penicillin-streptomycin.[25c] Cells were maintained at 37 °C in a humidified atmosphere of 5% carbon dioxide, with media changes occurring three times per week. Cells were also maintained in the exponential growth phase, and routinely certified as mycoplasma free.

Cell viability assay:

MTT (dimethylthiazol-diphenyltetrazolium bromide) growth inhibition assays: Three-day growth inhibition assays were performed in clear Greiner TC microtiter plates as previously described.[25c] First, 3000 A549 cells per 100 μL of cell culture media were plated into each well and were incubated for 24 hours. The synthetic analogs and controls, as well as crizotinib (obtained from Combi-Blocks, Inc.; positive control), were suspended in culture media and added to the wells to give a final volume of 200 μL per well. Crizotinib and all of the synthetic probes were suspended in DMSO and stored at −20 °C prior to use. The final DMSO concentration did not exceed 0.5% in any experiment. The treated cells were then incubated for an

additional 3 days, at which time MTT was added to each well (20 μL of a stock solution containing 5 mg/mL MTT in PBS) and the cells were incubated at 37 °C. Four hours later, the cells were lysed by addition of 200 μL of DMSO to each well, and the optical density at 540 nm was measured on a EnSpire multimode plate reader (PerkinElmer, Waltham, MA, USA). The absorbance values were expressed as a ratio of treated to untreated cells. The concentration required to inhibit the growth of tumor cells by 50% (IC_{50}) was used to evaluate the effect of the drug and analogs. Assays were performed in triplicate and repeated (n = 2). Mean and standard error (SE) were calculated. (See Figures 3A and B and the Supporting Information for detailed procedures for the fluorescence microscopy imaging of treated cells).

Acknowledgements

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