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Inhibition of the CRM1-mediated nucleocytoplasmic transport by *N*-azolylacrylates: Structure–activity relationship and mechanism of action

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ABSTRACT

CRM1-mediated nucleocytoplasmic transport plays an important role in many cellular processes and diseases. To investigate the structural basis required for the inhibition of the CRM1-mediated nuclear export we have synthesized analogs of a previously identified small molecule lead compound and monitored their activity against the Rev function of the human immunodeficiency virus. Microscopy studies show that the active congeners of this series inhibit the nucleocytoplasmic transport of Rev and the co-localization between Rev and CRM1 in living cells. Mechanism of action studies show their interaction with the Cys528 residue of CRM1 involving a Michael-addition type of reaction. However, structure–activity relationship demonstrates strict constraints to the structure of the inhibitors, and shows that activity is not solely correlated to Michael-addition suggesting a more complex mechanism of action. Our results are suggestive for the existence of a well-defined interaction at the CRM1-NES binding site. In addition, the most selective congener inhibited the HIV-1 production in latently infected cells. These specific CRM1 inhibitors are of interest as tool for analyzing the mechanisms of post-transcriptional control of gene expression and provide insight in the design of new agents.

1. Introduction

The eukaryotic cell nucleus is separated from the cytoplasm by a double nuclear membrane. The transport of macromolecules across this nuclear membrane is mediated by nuclear pore complexes (NPCs), which are large proteinaceous assemblies. Molecular transport between the nucleus and cytoplasm through the nuclear pore complexes (NPCs) is mediated by transport receptors, called karyopherins. Importins mediate import of molecules in the nucleus while exportins take care of the nuclear exit of molecules from the nucleus.[1] One well-characterized exportin is CRM1, which exports proteins carrying a leucine-rich nuclear export signal (NES).[2–5] The *crm1* gene was originally discovered in *Saccharomyces cerevisiae* to be involved in chromosomal region maintenance.[6] Evidence that CRM1 is a transport receptor came from inhibition studies with leptomycin B (LMB). Leptomycin B was first identified in *Streptomyces* sp. as an unsaturated, branched-chain fatty acid with antifungal activity.[7] This drug was shown to have antitumor activity,[8] to inhibit the G_1 and G_2 phases of the cell cycle[9] and other cellular functions. These pleotropic effects led to the study of the mechanisms by which leptomycin B influences intracellular processes. Resistance selection in fission yeast revealed that leptomycin B targets CRM1 (exportin 1), a nuclear protein, which was thought to be involved in the control of gene expression.[10] A few years later it was shown that one of the functions of CRM1 is to export proteins from the nucleus[11] by binding to a specific leucine-rich nuclear export signal (NES) of exported proteins and to introduce them to the export machinery of the nuclear pores.[12] CRM1 has also an important function in mitosis.[13–15] Crm1 has been identified as a mitotic effector of Ran-GTP at kinetochores, where it has a role in microtubule attachment.[14] Leptomycin B forms a covalent complex via its α,β-unsaturated δ-lactone with the sulfhydryl group of a conserved cysteine residue in CRM1, inhibiting the interaction with the nuclear export signal of cargo proteins.[16,17] LMB was also found to inhibit the nuclear export of the human immunodeficiency virus (HIV) Rev protein, and consequently CRM1 was suggested to be the nuclear export receptor for Rev.[18] The HIV Rev protein is required for the transport of viral mRNA to the cytoplasm,[19,20] a process that is essential for virus replication.[21] Rev contains a leucine-rich NES essential for interaction with CRM1 and nuclear export.[22,23] Similar NESs were also found in other proteins.[24–30] In CRM1, residues Asp716 and Lys810 have been shown important for NES binding.[31] Recently a structural model of CRM1 based on X-ray crystallography, homology modeling and electron microscopy was proposed.[32] Evidence indicates

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that the central conserved region of CRM1 (residues 415–600) mediates NES recognition.

In this context, several compounds have been pursued as specific inhibitors of the CRM1-mediated transport pathway. LMB has been shown to efficiently disrupt the CRM1–NES interaction by covalently binding to residue Cys528 of CRM1.[33] The much smaller low-molecular weight heterocyclic compound PKF050-638[34] also inhibits CRM1-mediated export. Interestingly PKF050-638 meets strict structural requirements for its activity, since its stereoisomer was less active. Thorough NMR-analyses on this compound PKF050-638 **1a**, including ^{1}H NMR, ^{13}C NMR, DEPT, HMBC, HMQC and COSY revealed the published lead-structure to be the inaccurate stereoisomeric form. The correct regioisomeric structure **1b** that is assigned now (see Fig. 1) implies that an intramolecular Michael addition of the amine onto the acrylate is no longer possible, as it would have been in the former structure.

To pinpoint the structural features responsible for the activity of this lead compound and to explore its structural variations, we have now synthesized a series of analogs and evaluated their activities for inhibition of CRM1-mediated transport in different assays and defined their molecular mechanism of action. It was suggested that this class of compounds exert their activity through a Michael addition on the target cysteine. However, here we demonstrate that this is not the only determinant as even chemically very related compounds display a strong differential activity. The data presented reveal the structural basis required for CRM1 inhibition and provide insight into the development of new agents.

2. Results

2.1. Inhibition of CRM1-mediated nucleocytoplasmic Rev transport by 3-substituted acrylic acid ethyl esters

The steady state subcellular localization of wild-type HIV-1 Rev is mainly nucleolar but it shuttles continuously between the nucleus and the cytoplasm.[20,35,36] Nuclear localization of Rev is mediated by a short stretch of basic amino acids characterized by eight arginine residues located near the amino-terminus of Rev that serves both as nuclear localization signal (NLS) and as RNA binding domain.[19,37,38] Mutations in this domain alter the steady state subcellular localization of Rev, for example, the mutant RevM5 is found mainly in the cytoplasm of the cell.[19,39] The nuclear export of Rev is mediated by its nuclear export signal, which is a leucine-rich domain between Leu75 and Leu84 located near the carboxy-terminus of the protein.[22] The cellular nucleocytoplasmic transport receptor for the leucine-rich NES of Rev has been identified earlier as CRM1.[2,3,40] Inhibition of the CRM1-mediated export can therefore be easily visualized by monitoring the subcellular redistribution of Rev. A GFP fusion protein of a RevM5 mutant was found predominantly in the cytoplasm of HeLa cells (Fig. 2A) but is still able to enter the nucleus. Consequently, inhibition of the CRM1-mediated RevM5-GFP export to the cytoplasm of the cell will cause a nuclear accumulation of RevM5-GFP. Indeed, treatment of RevM5-GFP expressing cells with 25 nM LMB, a well-characterized inhibitor of the CRM1-mediated Rev export,[16,18] caused an accumulation of RevM5-GFP in the nucleus of the cells (Fig. 2B).

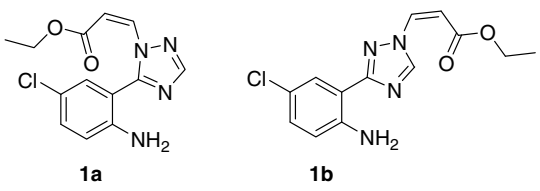


Figure 1. Erroneous (**1a**) and revised (**1b**) structure of PKF050-638.

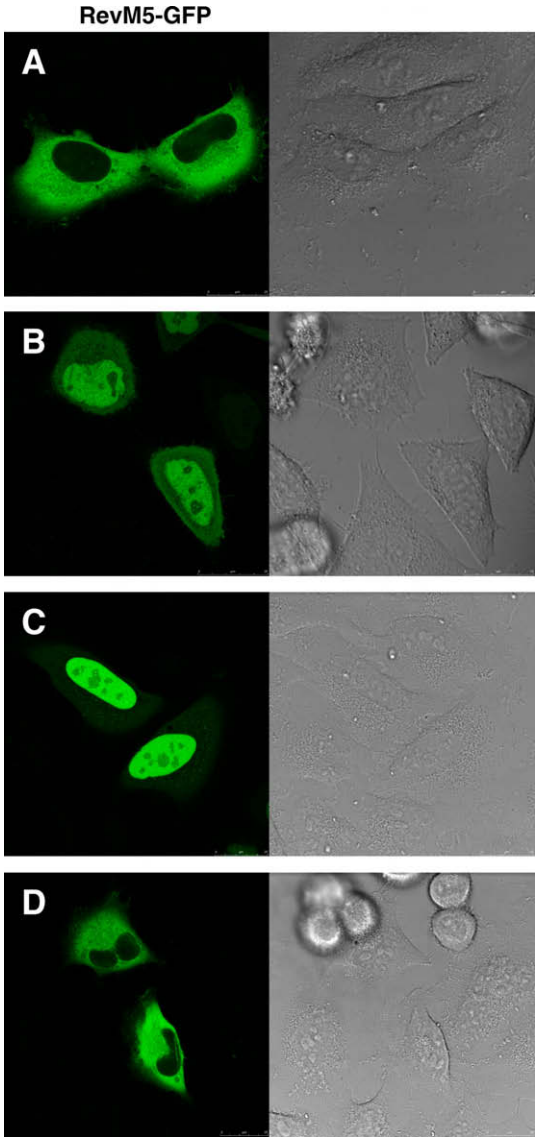
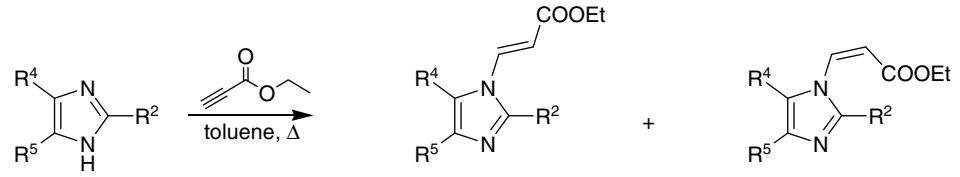


Figure 2. Effect of 3-(5-phenyltetrazol-2-yl)acrylic acid ethyl esters on the localization of RevM5-GFP in living cells. HeLa cells expressing RevM5-GFP were incubated with test compound. Two hours after addition of the compounds, the cells were analyzed by fluorescence microscopy. The left panels are the fluorescent images and the right panels the corresponding differential interference contrast images. RevM5-GFP before addition of compound is localized in the cytoplasm (A). Addition of 25 nM LMB (B) or 10 μg/ml (36 μM) **7d**-*cis* (C) resulted in a predominant nuclear localization of the RevM5-GFP, while 10 μg/ml **6d** (D) had no effect on the localization of RevM5-GFP.

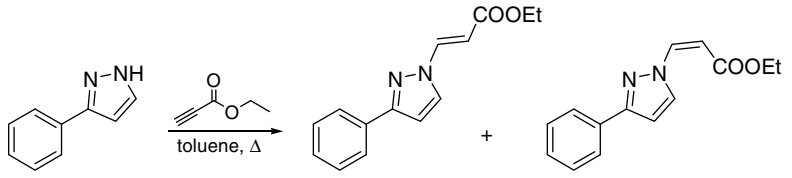
Previously we have shown that the small molecule compound PKF050-638 is specifically interfering with the CRM1-mediated nucleocytoplasmic transport mechanism. We have now synthesized analogs of PKF050-638 (see Fig. 3) and tested their ability to inhibit the nucleocytoplasmic Rev transport assessing the RevM5-GFP subcellular redistribution. For example, upon addition of 10 μg/ml **7d**-*cis* to the cells, RevM5-GFP redistributed to the nucleus, indicating that **7d**-*cis* is an inhibitor of the Rev nucleocytoplasmic transport (Fig. 2C). Interestingly, **6d** which has a similar core structure to **7d**-*cis* but is lacking the α,β-unsaturated carbonyl moiety has no effect on the Rev transport (Fig. 2D).



2a: $R^2 = R^4 = R^5 = H$
2b: $R^2 = Ph$; $R^4 = R^5 = H$
2c: $R^4 = Ph$; $R^2 = R^5 = H$

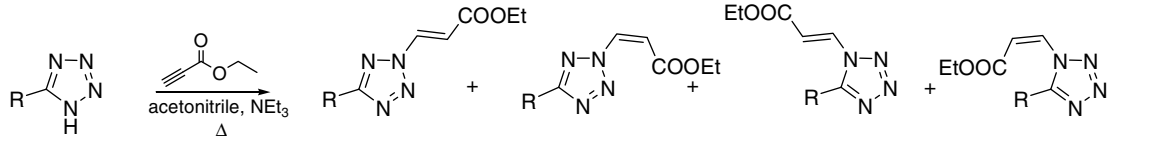
3a-*trans*: $R^2 = R^4 = R^5 = H$ (20 %) **3a-*cis***: $R^2 = R^4 = R^5 = H$ (12 %)
3b-*trans*: $R^2 = Ph$; $R^4 = R^5 = H$ (31 %) **3b-*cis***: $R^2 = Ph$; $R^4 = R^5 = H$ (22 %)
3c-*trans*: $R^5 = Ph$; $R^2 = R^4 = H$ (19 %) **3c-*cis***: $R^5 = Ph$; $R^2 = R^4 = H$ (8 %)

Scheme 1: condensation reaction of imidazole **2** with ethyl propiolate



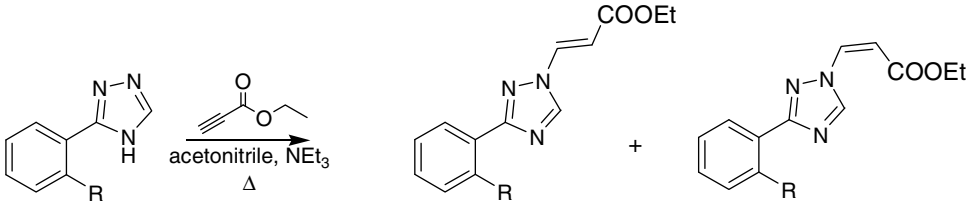
4 **5-*trans*** (46 %) **5-*cis*** (10 %)

Scheme 2: condensation reaction of pyrazole **4** with ethyl propiolate



6a: R= C_6H_5	**7a-*trans*** (51 %)	**7a-*cis*** (33 %)	**8a-*trans*** (8 %)	**8a-*cis*** (6%)
6b: R= 2-NH_2-4-Cl-C_6H_3	**7b-*trans*** (7 %)	**7b-*cis*** (13 %)	/	/
6c: R= 2-Cl-C_6H_4	**7c-*trans*** (30 %)	**7c-*cis*** (9 %)	/	**8c-*cis*** (21 %)
6d: R= 3-Cl-C_6H_4	**7d-*trans*** (43 %)	**7d-*cis*** (15 %)	**8d-*trans*** (6 %)	**8d-*cis*** (5 %)
6e: R= 4-Cl-C_6H_4	/	**7e-*cis*** (33 %)	**8e-*trans*** (23 %)	**8e-*cis*** (10 %)
6f: R= 3-F-C_6H_4	**7f-*trans*** (57 %)	**7f-*cis*** (17 %)	**8f-*trans*** (5 %)	**8f-*cis*** (5 %)
6g: R= 3-Br-C_6H_4	**7g-*trans*** (28 %)	**7g-*cis*** (24 %)	**8g-*trans*** (2 %)	**8g-*cis*** (7 %)
6h: R= 3-I-C_6H_4	**7h-*trans*** (37 %)	**7h-*cis*** (22 %)	**8h-*trans*** (10 %)	**8h-*cis*** (15 %)
6i: R= H	**7i-*trans*** (15 %)	**7i-*cis*** (2 %)	**8i-*trans*** (30 %)	/

Scheme 3: condensation reaction of 5-aryltetrazoles **6** with ethyl propiolate



9a: R = H
9b: R = NH_2

10a-*trans*: R = H (18 %) **10a-*cis***: R = H (7 %)
10b-*trans*: R = NH_2 (19 %) **10b-*cis***: R = NH_2 (3 %)

Scheme 4: condensation reaction of 3-aryl-1,2,4-triazole **9** with ethyl propiolate

Figure 3. Overview of the structures of the arylazole acrylic acid ethyl esters.

2.2. Inhibition of the Rev/CRM1 interaction in live cells

To study the mechanism of action of the new compounds in more detail we examined the direct effect of **7d**-*cis* on the interaction of Rev with CRM1 in living cells. Therefore, HeLa cells were co-transfected with constructs expressing BFP-tagged Rev protein[41] and EYFP-tagged CRM1 (Fig. 4A). CRM1-EYFP was localized at the nuclear rim as well as within the nucleus (Fig. 4A[a]), in agreement with published data[11,17,42,43], whereas Rev localized predominantly to the nucleoli (Fig. 4A[b]). The predominant association of CRM1-EYFP with the nuclear membrane is consistent with its interaction with nucleoporins.[11,44,45] When Rev-BFP and CRM1-EYFP where co-expressed in the same cell, a significant fraction of CRM1-EYFP was found in the Rev-containing nucleoli (Fig. 4A[b]). This redistribution of CRM1 by Rev suggests interaction between the two proteins. Therefore, this assay is an excellent approach to demonstrate Rev/CRM1 interaction inside living cells. Upon treatment with 10 μg/ml **7d**-*cis*, the Rev-dependent nucleolar localization of CRM1-EYFP was abolished after 120 min (Fig. 4A[d]). Moreover, the compound did not affect the nucleolar distribution of Rev. As a control, LMB and compound **6d** were included in this assay. LMB has been described to obstruct the Rev/CRM1 interaction in vitro as well as in living cells.[16,31,33,42] Indeed, also LMB abolished the Rev-

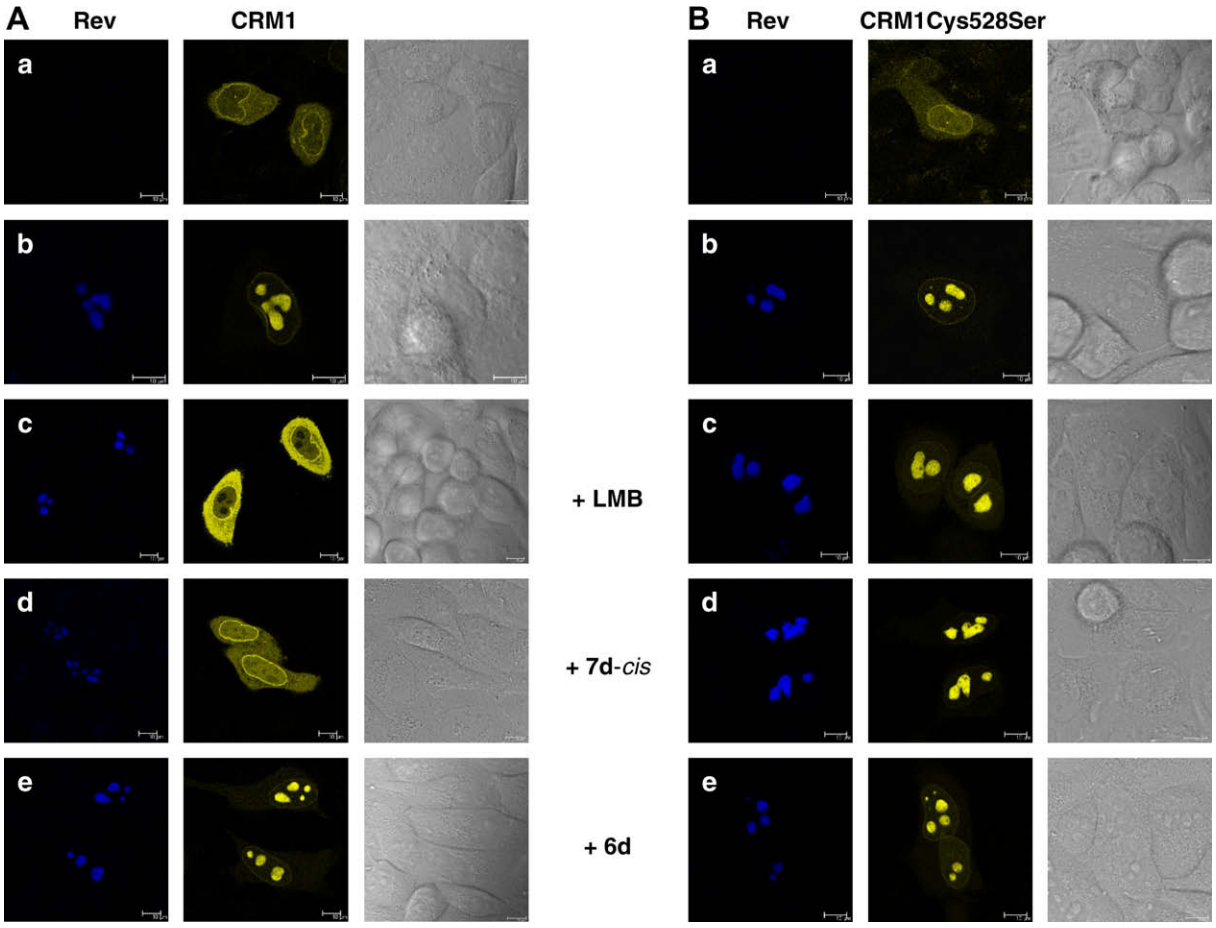


Figure 4. 3-(5-Phenyltetrazol-2-yl)acrylic acid ethyl esters interfere with the co-localization of Rev-BFP and CRM1-EYFP in living cells but not with the co-localization of the Rev-BFP and EYFP-CRM1Cys528 mutant. (A) HeLa cells expressing Rev-BFP or/and CRM1-EYFP were analyzed by fluorescence microscopy. Rev-BFP localizes in the nucleoli of the cells (b, left panel), while CRM1-EYFP is found at the nuclear rim (a, middle panel). In cells co-expressing both Rev-BFP and CRM1-EYFP, CRM1 colocalizes with Rev-BFP in the nucleoli (b, middle panel). Two hours after addition of compound the co-localization of CRM1-EYFP with Rev-BFP was analyzed (c: LMB; d: **7d**-*cis*; e: **6d**). (B) Similar as in (A) but with a mutant CRM1-EYFP with a serine at position 528 instead of cysteine.

dependent nucleolar localization of CRM1-EYFP (Fig. 4A^{c}). In contrast, **6d** a compound lacking the α,β-unsaturated carbonyl moiety, had no effect on the Rev/CRM1 co-localization demonstrating that this compound does not interfere with the Rev/CRM1 interaction (Fig. 4A^{e}). Taken together, our results suggest that these compounds are specifically interfering with the Rev/CRM1 interaction in living cells.

2.3. The 3-substituted acrylic acid ethyl esters target Cys528 of CRM1

LMB has been described to hinder the NES-mediated nuclear export by covalently modifying CRM1 at Cys528 by a Michael-type addition.[16,33] To determine whether these new compounds similarly target the CRM1 Cys528 residue, HeLa cells were co-transfected with constructs expressing the BFP-tagged Rev protein and an EYFP-tagged CRM1 mutant expressing a Cys528Ser substitution (Fig. 4B). Similar to the wild-type, the EYFP-CRM1Cys528Ser mutant was mainly localized at the nuclear rim (Fig. 4B^{a}). When Rev-BFP and EYFP-CRM1Cys528Ser where co-expressed in the same cell, a significant fraction of EYFP-CRM1Cys528Ser was found in the Rev-containing nucleoli (Fig. 4B^{b}) suggesting that also the Cys528Ser mutant interacts with Rev. Treatment with 25 nM LMB (Fig. 4B^{c}), or 10 μg/ml **7d**-*cis* (Fig. 5B^{d}), had no effect on the Rev/CRM1Cys528Ser co-localization, suggesting that similarly to LMB, **7d**-*cis* is specifically targeting Cys528 of the CRM1 protein. As expected, **6d** had no effect on the Rev/CRM1Cys528Ser co-localization (Fig. 4B^{e}).

2.4. Structure–activity relationship for inhibition of CRM1-mediated nuclear export

Taking advantage of our ability to evaluate inhibition of the CRM1-mediated nucleocytoplasmic transport using the RevM5-GFP subcellular redistribution, a broad series of analogs (Fig. 3) of PKF050-638 were analyzed in order to explore the structural features required for activity. Therefore, HEK 293T cells were transfected with a construct expressing RevM5-GFP, and incubated in the presence of different concentrations of test compound. Activity was scored by fluorescence microscopy two hours after addition of compound (Table 1). Conditions where RevM5-GFP was predominantly nuclear were scored as positive, and conditions were RevM5-GFP was localized in the cytoplasm were scored negative. From the results in Table 1 we can conclude there are a number of structural requirements to be met for activity. All active compounds possess an α,β-unsaturated carbonyl moiety and the *cis* enantiomer is usually more active than the *trans*. Furthermore, the presence of a phenyl moiety is crucial for activity. Interestingly, the position of the α,β-unsaturated carbonyl group on the imidazole or tetrazole appears to be important. Indeed, activity is drastically impaired if the ethyl acrylic acid moiety is positioned on the nitrogen that is directly adjacent to the carbon carrying the phenyl

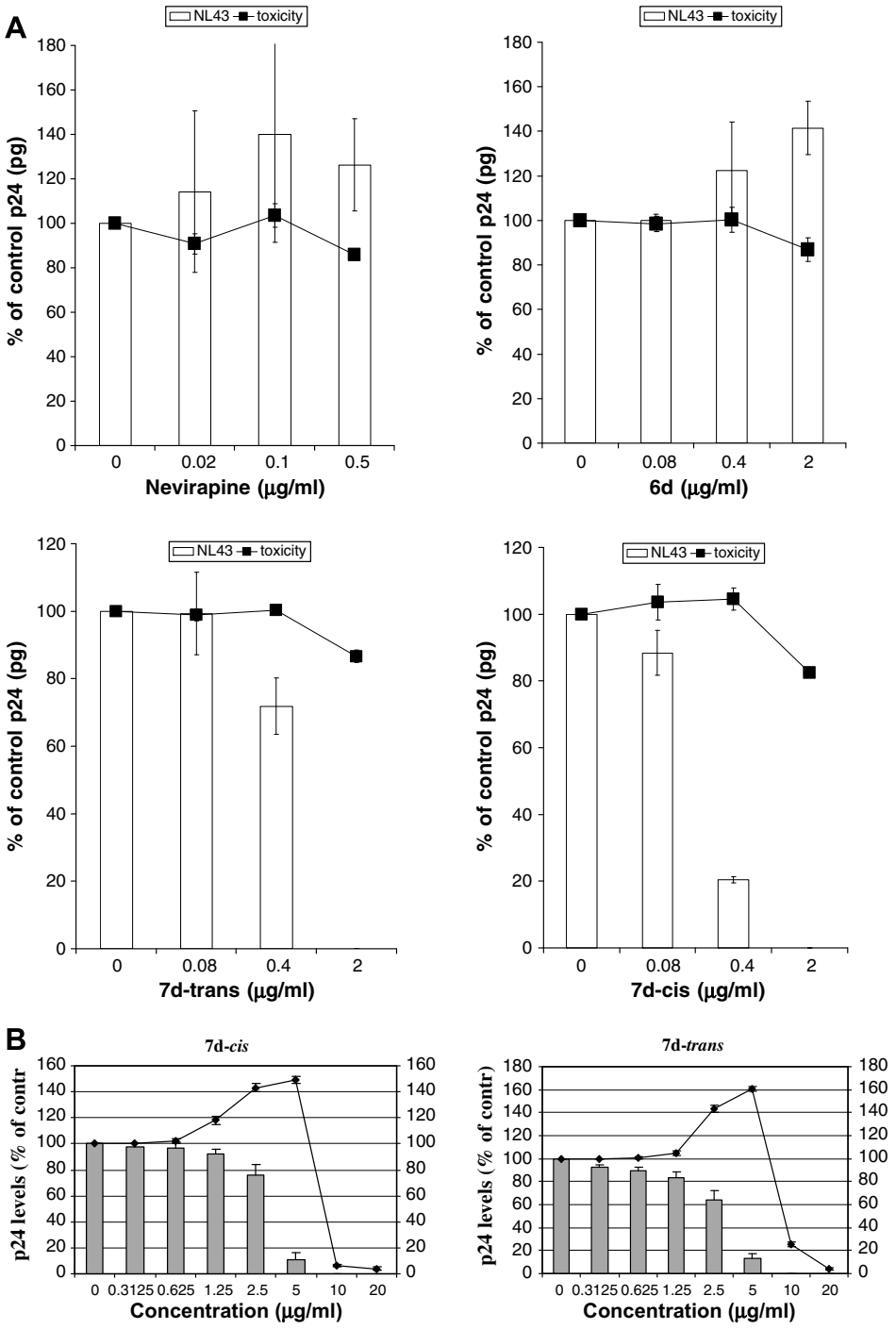


Figure 5. Inhibitory effects of 3-(5-phenyltetrazol-2-yl)acrylic acid ethyl esters on the release of infectious HIV-1 progeny (virus yield), p24 production (bars), in the supernatants of (A) transfected 293T cells or (B) chronically HIV-1(III_B)-infected OM10.1 cells. The cytotoxicities (rectangles ■) of the test compounds were determined by the MTT-viability staining method.

ring (compare activities of **3b** with **3c** and **8** with **7**). However, no significant difference in activity was observed between the compounds containing an imidazole, triazole or tetrazole nucleus (compare **3c**, **7a** and **10a**). Surprising is that the compounds with a pyrazole ring are inactive. Furthermore, chlorine substitution at position 2 or 3 of the phenyl ring increases the activity while substitution at position 4 had no effect. There is no apparent difference of activity within the homologous series of halogen derivatives (F, Cl, Br, I). Interestingly, addition of an amino-group at position 2 of the 5-Cl-phenyl slightly increases the activity (compare **7d** with **7b**). These results are suggestive for the existence of a well-defined interaction in the CRM1-NES binding site.

2.5. Inhibition of latent HIV infection

Since HIV exploits the CRM1-mediated nucleocytoplasmic transport pathway for its RNA transport, the effect of these derivatives on HIV-1 virus expression was assessed. Therefore HEK 293T cells were transfected with the molecular clone NL_{4-3} in the presence of different concentrations of **7d**-*cis* and **7d**-*trans* and viral production was

Table 1
Inhibition of the nuclear export of RevM5-GFP by *N*-azolylacrylates

Compound	Concentration (μg/ml)				
	10	2	0.4	0.08	0
3b-*trans*	−	−	−	−	−
3b-*cis* and **3b**-*trans* (mixture)	−	−	−	−	−
3c-*cis*	+	+/−	−	−	−
3c-*trans*	+/−	−	−	−	−
5-*cis*	−	−	−	−	−
5-*trans*	−	−	−	−	−
6a	−	−	−	−	−
6b	−	−	−	−	−
6c	−	−	−	−	−
6d	−	−	−	−	−
6e	−	−	−	−	−
6f	−	−	−	−	−
6g	−	−	−	−	−
6h	−	−	−	−	−
7a-*cis*	+	+/−	−	−	−
7a-*trans*	+/−	−	−	−	−
7b-*cis*	+	+	+	−	−
7b-*trans*	+	+	−	−	−
7c-*cis*	+	+	+/−	−	−
7c-*trans*	+	−	−	−	−
7d-*cis*	+	+	+/−	−	−
7d-*trans*	+	+/−	−	−	−
7e-*cis*	+	+/−	−	−	−
7e-*trans*	+	−	−	−	−
7f-*cis*	+	+	+/−	−	−
7f-*trans*	+	−	−	−	−
7g-*cis*	+	+	+/−	−	−
7g-*trans*	+	−/+	−	−	−
7h-*cis*	+	+	+/−	−	−
7h-*trans*	+	+/−	−	−	−
7i-*cis*	−	−	−	−	−
7i-*trans*	−	−	−	−	−
8c-*cis*	−/+	−	−	−	−
8d-*cis*	+	−	−	−	−
8d-*trans*	+/−	−	−	−	−
8e-*cis*	+	−	−	−	−
8e-*trans*	−	−	−	−	−
8f-*cis*	−	−	−	−	−
8f-*trans*	+/−	−	−	−	−
8g-*cis*	−	−	−	−	−
8g-*trans*	−/+	−	−	−	−
8h-*cis*	−	−	−	−	−
8h-*trans*	+/−	−	−	−	−
8i-*trans*	−	−	−	−	−
10b-*cis*	+	−/+	−	−	−
10b-*trans*	−	−	−	−	−

Unchanged cytoplasmic localization of RevM5-GFP was scored as (−), while nuclear accumulation of RevM5-GFP was scored as (+).

monitored by quantifying the virion-associated p24 Ag in the supernatants. Both compounds effectively inhibited the virus production with a 50% effective concentration (EC_{50}) of 0.2 and 0.6 μg/ml, respectively, and showing minimal cytotoxicity at 2 μg/ml (Fig. 5A). Both nevirapine, an inhibitor of the HIV reverse transcription process and **6d**, lacking the α,β-unsaturated carbonyl moiety, were included as controls. These compounds were unable to inhibit the virus expression from the molecular clone.

To confirm these results the effect of **7d**-*cis* and **7d**-*trans* was monitored in chronically infected OM10.1 monocytes (Fig. 5B). Culture of these cells leads to little virus expression unless the cells are stimulated with an agonist such as TNF-α. More than a ~1000-fold increase in p24 levels was noted with TNF-α at 1 ng/ml. In OM10.1 cells, addition of 5 μg/ml **7d**-*cis* or **7d**-*trans* inhibited the TNF-α-induced release of virus by as much as 94%, with no detectable concomitant cytotoxicity at this concentration, whereas at 10 and 20 μg/ml both compounds were toxic for the cells. These results are also pointing toward a post-integrational mechanism of action.

3. Discussion

Earlier studies using a low-molecular weight inhibitor of the CRM1-mediated pathway indicated a structural requirement for activity.[34] Indeed, in all assays the *trans* stereoisomer of this compound was much less active than the *cis* congener. In order to pinpoint the structural features required for inhibition of the CRM1–cargo interaction, analogs of this compound were developed and structure–activity relationship was performed. This information is essential to unravel the CRM1–cargo interaction and is important for the development of selective compounds against diseases involving CRM1-mediated nucleocytoplasmic transport such as the HIV-1 replication or diseases induced by mislocalized proteins such as breast cancer and leukemia (for review see 46).

We have synthesized a series of phenylazol acrylic acid ethyl esters and monitored their activity against the CRM1-mediated nucleocytoplasmic transport of the HIV-1 Rev protein. Mechanism of action studies suggests that this class of molecules inhibit the CRM1-cargo interaction. Indeed, the most potent congener was able to inhibit HIV-1 expression from latently infected cells. As expected, these compounds are cytotoxic because the CRM1-mediated nucleocytoplasmic process is a general pathway used by many cellular cargos. However the compounds exert limited selectivity toward HIV. One explanation could be that the affinity of Rev for CRM1 is lower than the cellular NES-baring cargos and therefore more susceptible for inhibition. Earlier studies have shown that the HIV-1 Rev has a rather weak affinity for CRM1.[47] It can be argued though that even partial inhibition of Rev function may dramatically affect the pathogenic potential of HIV.

It has been suggested earlier that LMB covalently modifies CRM1 at Cys528 by a Michael-type addition.[33] Similarly, we show that the compounds described here have the potential to covalently interact with a cysteine residue. Indeed, the Ser528 CRM1 mutant resisted to the action of the phenylazol acrylic acid ethyl esters.

Structure–activity relationship studies demonstrate that the acrylic acid ethyl ester is essential for activity of the compounds in cell culture; indeed, the 5-phenyl-1*H*-tetrazole is inactive in all assays. However, we cannot exclude the possibility that 5-phenyl-1*H*-tetrazole is inactive because it is not able to penetrate the cells. Also, the propionic acid ethyl ester (data not shown) is inactive in cells demonstrating the double bond is essential for activity and participates in the Michael addition. Moreover, chlorine substitution at position 2 or 3 on the phenyl ring contributes to a better selectivity of the compounds. Substitution with other halogen atoms did not markedly affect activity. Halogen substitution at position 4 of the phenyl had no beneficial effect on the activity. The effect on the biological properties of the chlorine atom introduced at different positions can not be explained by differences in the reactivity of the obtained compounds in the Michael-addition reaction or to differences in lipophilicity or electronic effects. In that case one would expect a similar activity for the 2-chloro and the 4-chloro derivative whereas here, both the 2-chloro and 3-chloro derivatives display greater selectivity. Thus the change in activity must therefore be attributed to steric effects. Indeed, we were unsuccessful in demonstrating interaction of these compounds with glutathione (carrying a cysteine available for Michael addition) suggesting that recognition within the CRM1 structure is necessary before subsequent covalent binding is generated or that activation of the target cysteine by a change in electrons in the carbonyl group is necessary.

The mechanism of action of this series of compounds is similar to that of the lead compound described in previous publication, which is not surprising. But the structure–activity relationship study described here delineates the minimal structural requirements for activity. Moreover, we demonstrate in detail that these compounds target the cysteine not just through a Michael addition. It is noticeable that all active compounds carry the unsaturated

carbonyl group, however, this is not the only determinant for activity as not all, even chemically related compounds bearing the conjugated unsaturated carbonyl system, display activity. Indeed, it is interesting to note that the compound bearing the pyrazole ring is inactive in all assays, although its general structure is similar to the compound containing the imidazole nucleus. Also, the presence of the phenyl ring is essential and substitutions on the phenyl ring change activity. Moreover, the position of the unsaturated carbonyl group is an important determinant for activity.

Our results demonstrate that the inhibitory activity of the compounds is not just a simple Michael addition but suggest more elements are into play. This study promotes a better understanding, at the molecular level, of the interaction of these compounds with the nucleocytoplasmic transport machinery. Interestingly, the availability of these compounds through straightforward chemical synthesis could make them a useful tool for studying CRM1-mediated export pathways.

4. Experimental

4.1. Chemical synthesis

For the design of the molecules, we have varied the central 1,2,4-triazole part to other azoles, including pyrazole, imidazole, 1,2,4-triazole and tetrazole rings and also varied the substitution pattern on the phenyl ring. As a general method, the arylazole was reacted with ethyl propiolate to afford the Michael-addition products.

Firstly we synthesized diazoles, having one nitrogen less than the lead compound. The condensation reaction of imidazole **2a** and its 2- and 4-substituted phenyl derivatives (**2b**, **2c**) with ethyl propiolate in toluene at reflux temperature resulted in the formation of both *cis*- and *trans*-isomers of the imidazolyl-*N*-acrylates **3** and **4**. In the case of 4-phenylimidazole **2c**, the acrylate ester substituent is directed specifically to *N*-1, probably to avoid sterical hindrance (Fig. 3, Scheme 1).

The isomeric diazole 3-phenylpyrazole **4** reacted under the same circumstances with ethyl propiolate to afford the *cis*- and *trans*-isomers of the corresponding 3-phenylpyrazolyl-*N*1-acrylates **5**. The 1,2-disubstituted regioisomers apparently did not form (Fig. 3, Scheme 2).

Finally, we prepared the tetrazole analog of our lead compound, now increasing the number of nitrogens in the azole ring. A series of 5-aryltetrazoles is easily accessible from the corresponding benzonitriles, so at this point it seemed logical to investigate the effect of the substitution pattern of the phenyl ring on the yield and the regioselectivity of the condensation with ethyl propiolate. The general procedure for the synthesis of 5-aryltetrazoles is the reaction of a benzonitrile with sodium azide under acidic catalysis.

In the following step when the condensation reaction of 5-aryltetrazole with ethyl propiolate was carried out in toluene, no reaction product was obtained, probably due to the lower nucleophilic character of the tetrazole ring as compared to the diazoles. The reaction conditions were altered and the best results were obtained when the condensation was carried out in basic (triethylamine) medium and under reflux conditions in acetonitrile. In the reaction with the different 5-aryltetrazoles we theoretically could expect the formation of four products, namely the *cis* and *trans* isomer of each regio isomer. We have indeed separated most of the theoretically possible products by column chromatography or HPLC. The isolated amounts of each isomer and the regio selectivity vary greatly with the substituent on the aryl ring although in most cases the *trans*-isomers are more abundant than the corresponding *cis*-adducts (Fig. 3, Scheme 3).

Finally, analogs closely related to the lead compound PKF050-638, albeit without the chloro-substituent on the arylring and without any substituent on the arylring were synthesized. The starting materials, 3-phenyl-4*H*-1,2,4-triazole and its 2′-amino analog, were prepared from the corresponding benzonitrile. The condensation reaction was performed under the same conditions as for the tetrazoles. However, we obtained only the 1,3-disubstituted *cis* and *trans*-regioisomers, in accordance with revised structure **1b** of PKF050-638 (Fig. 3, Scheme 4).

All synthesized compounds were purified by column chromatography on silica gel and fully characterized by the usual techniques including ^{1}H NMR, ^{13}C NMR and mass spectroscopy.

The chemicals used for synthetic procedures were of reagent grade quality. They were obtained from commercial sources and used as received. NMR spectra were acquired on commercial instruments (Bruker Avance 300 MHz or Bruker AMX 400 MHz) and chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (J) in Hertz (Hz) referenced to tetramethylsilane (TMS) (^{1}H) or the carbon signal of deuterated solvents (^{13}C). Detailed ^{13}C NMR peak assignments were obtained by careful analysis of DEPT, HMQC and HMBC NMR spectra. Mass spectra were run using a Kratos MS50TC instrument for exact mass measurement (performed in the EI mode at a resolution of 10,000).

4.2. General method A (use of toluene)

A mixture of imidazole (0.23 g, 3.4 mmol) and ethyl propiolate (0.34 ml, 3.4 mmol) in toluene under nitrogen was heated overnight at reflux. Toluene was removed under reduced pressure to leave a solid. The mixture was separated by column chromatography (silica, eluent dichloromethane).

4.2.1. Ethyl (*E*)-3-(imidazol-1-yl)-propenoate (3a-*trans*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.34 (t, J = 7, 3H), 4.27 (q, J = 7, J = 14, 2H), 6.05 (d, J = 14, 1H), 7.17 (s, 1H), 7.22 (s, 1H), 7.76 (s, 1H), 7.87 (d, J = 14, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.37 (CH_3), 61.10 (CH_2), 107.19 (CH), 120.84 (CH), 137.96 (CH), 132.51 (CH), 139.91 (CH), 166.01 C; HR-MS: calcd for ($C_8H_{10}O_2N_2$): m/z 166.0742. Found: 166.0625.

4.2.2. Ethyl (2*Z*)-3-(3-imidazol-1-yl)-2-propenoate (3a-*cis*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.34 (t, J = 7, 3H), 4.27 (q, J = 7, J = 14, 2H), 5.50 (d, J = 10, 1H), 5.90 (d, J = 10, 1H), 7.10 (s, 1H), 7.84 (s, 1H), 8.07 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.37 (CH_3), 61.10 (CH_2), 106.22 (CH), 116.27 (CH), 130.37 (CH), 131.99 (CH), 136.48 (CH), 166.01 C; HR-MS: calcd for ($C_8H_{10}O_2N_2$): m/z 166.0742. Found: 166.0625.

4.2.3. Ethyl (*E*)-3-(2-phenyl-1*H*-imidazol-1-yl)-2-propenoate (3b-*trans*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.26 (t, J = 7, 3H), 4.23 (q, J = 7, J = 14, 2H), 6.04 (d, J = 14, 1H), 7.23 (d, J = 1, 1H), 7.34 (d, J = 1, 1H), 7.50 (m, 3H), 7.56 (m, 2H), 7.97 (d, J = 14, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.41 (CH_3), 61.01 (CH_2), 107.83 (CH), 116.73 (CH), 128.12 (C), 129.09 (2× CH), 129.77 (2× CH), 130.03 (CH), 130.99 (CH), 137.32 (CH), 140.08 (C), 166.20 (C); HR-MS: calcd for ($C_{14}H_{14}O_2N_2$): m/z 242.10553. Found: 242.10515.

4.2.4. Ethyl (*Z*)-3-(2-phenyl-1*H*-imidazol-1-yl)-2-propenoate (3b-*cis*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.25 (t, J = 7, 3H), 4.24 (q, J = 7, J = 14, 2H), 5.57 (d, J = 10, 1H), 6.92 (d, J = 10, 1H), 7.16 (d, J = 2, 1H), 7.45 (m, 3H), 7.62 (m, 2H), 7.93 (d, J = 2, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.07 (CH_3), 60.62 (CH_2), 108.29 (CH), 121.87 (CH), 128.48 (C), 128.54 (CH), 128.70 (2× CH), 129.40 (2× CH), 129.61 (CH), 133.33 (CH), 149.17 (C), 163.97 (C); HR-MS calcd for ($C_{14}H_{14}O_2N_2$): m/z 242.10553. Found: 242.10548.

4.2.5. Ethyl (*E*)-3-(4-phenyl-1*H*-imidazol-1-yl)-propenoate (3c-*trans*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.33 (t, J = 7, 3H), 4.27 (q, J = 7, J = 14, 2H), 6.10 (d, J = 15, 1H), 7.30 (d, J = 7, 1H), 7.39 (t,

J = 7, 2H), 7.48 (s, 1H), 7.87 (d, J = 14, 1H), 7.79 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.37 (CH_3), 61.01 (CH_2), 106.89 (CH), 111.00 (CH), 125.38 (2× CH), 127.97 (CH), 128.85 (2× CH), 128.89 (C), 132.75 (C), 138.36 (CH), 144.62 (CH), 166.05 C; HR-MS: calcd for ($C_{14}H_{14}O_2N_2$): m/z 242.1055. Found: 242.1057.

4.2.6. Ethyl (*Z*)-3-(4-phenyl-1*H*-imidazol-1-yl)-2-propenoate (3c-*cis*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.34 (t, J = 7, 3H), 4.25 (q, J = 7, J = 14, 2H), 5.51 (d, J = 11, 1H), 6.90 (d, J = 11, 1H), 7.28 (d, J = 7, 1H), 7.40 (t, J = 7, 2H), 7.83 (d, J = 7, 2H), 8.05 (s, 1H), 8.29 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.20 (CH_3), 60.89 (CH_2), 105.70 (CH), 116.03 (CH), 128.28 (2× CH), 127.51 (CH), 128.67 (2× CH), 132.29 (C), 133.08 (C), 140.28 (CH), 152.87 (CH), 164,43 C; HR-MS: calcd for ($C_{14}H_{14}O_2N_2$): m/z 242.1055. Found: 242.1071.

4.2.7. Ethyl (*E*)-3-(3-phenyl-1*H*-pyrazol-1-yl)-propenoate (5-*trans*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.31 (t, J = 7, 3H), 4.23 (q, J = 7, J = 14, 2H), 5.41 (d, J = 11, 1H), 6.72 (d, J = 3, 1H), 7.32 (d, J = 11, 1H), 7.39 (m, 3H), 7.83 (m, 2H), 9.14 (d, J = 3, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.35 (CH_3), 60.71 (CH_2), 102.11 (CH), 106.55 (CH), 126.23 (CH), 128.82 (CH), 128.86 (CH), 132.17 (C), 134.67 (CH), 137.93 (CH) 163.70 (C), 165.22 C; HR-MS calcd for ($C_{14}H_{14}O_2N_2$): m/z 242.10553. Found: 242.10671.

4.2.8. Ethyl (*Z*)-3-(3-phenyl-1*H*-pyrazol-1-yl)-propenoate (5-*cis*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.31 (t, J = 7, 3H), 4.25 (q, J = 7, J=14, 2H), 6.43 (d, J = 14, 1H), 6.70 (d, J = 3, 1H), 7.36 (m, 3H), 7.60 (d, J = 3, 1H), 7.84 (m, 2H), 7.95 (d, J = 14, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.35 (CH_3), 60.55 (CH_2), 105.52 (CH), 106.62 (CH), 126.14 (CH), 128.76 (CH), 128.88 (CH), 131.70 (CH), 132.02 (C), 139.51 (CH), 155.08 (C), 166.71 C; HR-MS calcd for ($C_{14}H_{14}O_2N_2$): m/z 242.10553. Found: 242.1054.

4.3. General method B (use of base)

A mixture of 5-phenyltetrazole (0.50 g, 3.4 mmol), ethyl propiolate (0.52 ml, 5.1 mmol) and some drops of triethylamine in acetonitrile under nitrogen was heated overnight at reflux. Acetonitrile was removed under reduced pressure to leave a yellow oil. The mixture was separated by column chromatography (silica, eluent dichloromethane).

4.3.1. Ethyl (*E*)-3-(5-phenyl-2*H*-tetrazol-2-yl)-propenoate (7a-*trans*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.38 (t, J = 7, 3H), 4.34 (q, J = 7, J = 14, 2H), 6.93 (d, J = 14, 1H), 7.52 (m, 3H), 8.20 (m, 2H), 8.41 (d, J = 14, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 13.68 (CH_3), 61.03 (CH_2), 113.45 (CH), 125.96 (C), 126.92 (2× CH), 128.66 (2× CH), 130.75 (C), 134.72 (CH), 164.21 C, 165.25 (C); HR-MS: calcd for ($C_{12}H_{12}O_2N_4$): m/z 244.0960. Found: $C_{12}H_{12}O_2N_4$–CH_3CH_2COO: 171.0540.

4.3.2. Ethyl (*Z*)-3-(5-phenyl-2*H*-tetrazol-2-yl)-propenoate (7a-*cis*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.30 (t, J = 7, 3H), 4.34 (q, J = 7, J = 14, 2H), 6.10 (d, J = 10, 1H), 7.14 (m, 2H), 7.50 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.09 (CH_3), 61.87 (CH_2), 115.54 (CH), 126.65 (CH), 126.72 (C), 127.27 (2× CH), 129.11 (2× CH), 130.98 (CH), 164.19 C, 165.23 (C); HR-MS: calcd for ($C_{12}H_{12}O_2N_4$): m/z 244.0960. Found: $C_{12}H_{12}O_2N_4$–CH_3CH_2COO: 171.0540.

4.3.3. Ethyl (*E*)-3-(5-phenyl-1*H*-tetrazol-1-yl)-propenoate (8a-*trans*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.30 (t, J = 7, 3H), 4.30 (q, J = 7, J = 14, 2H), 6.98 (d, J = 14, 1H), 7.65 (m, 5H), 7.92 (d, J = 14, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.29 (CH_3), 61.67 (CH_2), 116.07 (CH), 129.12 (C), 129.54 (2× CH), 129.77 (2× CH), 131.89 (C), 132.30 (CH), 154.61 (C), 164.19 C; HR-MS: calcd for ($C_{12}H_{12}O_2N_4$): m/z 244.0960. Found: $C_{12}H_{12}O_2N_4$–CH_3CH_2COO: 171.0547.

4.3.4. Ethyl (*Z*)-3-(5-phenyl-1*H*-tetrazol-1-yl)-propenoate (8a-*cis*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.28 (t, J = 7, 3H), 4.10 (q, J = 7, J = 14, 2H), 6.25 (d, J = 9, 1H), 7.18 (d, J = 10, 1H), 7.58 (m, 3H), 7.79 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 13.89 (CH_3), 61.77 (CH_2), 121.87 (CH), 123.25 (C), 127.91 (CH), 128.98 (2× CH), 129.04 (2× CH), 131.89 (CH), 154.08 (C), 162.85 C; HR-MS: calcd for ($C_{12}H_{12}O_2N_4$): m/z 244.0960. Found: $C_{12}H_{12}O_2N_4$–CH_3CH_2COO: 171.0544.

4.3.5. Ethyl (*E*)-3-[5-(2-amino-5-chlorophenyl)-2H-tetrazol-2-yl]-propenoate (7b-*trans*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.38 (t, J = 7, 3H), 4.34 (q, J = 7, J = 14, 2H), 5.47 (s, NH_2), 6.47 (d, J = 9, 1H), 6.90 (d, J = 15, d), 7.22 (dd, J = 9, J = 2, 1H), 8.18 (d, J = 2, 2H), 8.41 (d, J = 15, 2H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.31 (CH_3), 61.88 (CH_2), 109.96 (C), 115.05 (CH), 118.12 (CH), 122.29 (C), 128.54 (CH), 132.12 (CH), 134.97 (CH), 144.95 (C), 164.03 (C), 164.70 C; HR-MS: calcd for ($C_{12}H_{12}O_2N_5Cl$): m/z 293.0680. Found: 293.0678.

4.3.6. Ethyl (*Z*)-3-[5-(2-amino-5-chlorophenyl)-2*H*-tetrazol-2-yl]-propenoate (7b-*cis*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.31 (t, J = 7, 3H), 4.32 (q, J = 7, J = 14, 2H), 5.42 (s, NH_2), 6.08 (d, J = 10, 1H), 6.72 (d, J = 9, d), 7.20 (dd, J = 9, J = 2, 1H), 7.53 (d, J = 10, 2H), 8.14 (d, J = 2, 2H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.13 (CH_3), 61.66 (CH_2), 109.97 (C), 114.29 (CH), 118.00 (CH), 122.11 (C), 126.58 (CH), 128.38 (CH), 131.81 (CH), 144.77 (C), 163.95 (C), 164.69 C; HR-MS: calcd for ($C_{12}H_{12}O_2N_5Cl$): m/z 293.0680. Found: 293.0678.

4.3.7. Ethyl (*E*)-3-[5-(2-chlorophenyl)-2*H*-tetrazol-2-yl]-propenoate (7c-*trans*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.35 (t, J = 7, 3H), 4.34 (q, J = 7, J = 14, 2H), 6.96 (d, J = 15, 1H), 7.45 (m, 2 H), 7.57 (dd, J = 2, J = 7, 1H), 8.05 (dd, J = 3, J = 6, 1H), 8.45 (d, J = 15, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.32 (CH_3), 61.66 (CH_2), 114.59 (CH), 125.47 (C), 127.18 (CH), 131.26 (CH), 131.62 (CH), 131.90 (CH), 133.48 (C), 135.25 (C), 164.10 C, 164.74 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4Cl$): m/z 278.0571. Found: $C_{12}H_{11}O_2N_4Cl$–C_2H_6: 250.0504.

4.3.8. Ethyl (*Z*)-3-[5-(2-chlorophenyl)-2*H*-tetrazol-2-yl]-propenoate (7c-*cis*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.28 (t, J = 7, 3H), 4.34 (q, J = 7, J = 14, 2H), 6.13 (d, J = 10, 1H), 7.42 (m, 2H), 7.53 (m, 2H), 8.02 (dd, J = 2, J = 6, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.07 (CH_3), 61.96 (CH_2), 115.94 (CH), 125.78 (C), 126.39 (CH), 127.12 (CH), 131.14 (CH), 131.60 (CH), 131.66 (CH), 133.36 (C), 163.46 (C), 164.04 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4Cl$): m/z 278.0571. Found: $C_{12}H_{11}O_2N_4Cl$–C_2H_6: 250.0508.

4.3.9. Ethyl (*Z*)-3-[5-(2-chlorophenyl)-1*H*-tetrazol-1-yl]-propenoate (8c-*cis*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.19 (t, J = 7, 3H), 4.12 (q, J = 7, J = 14, 2H), 6.10 (d, J = 9, 1H), 7.07 (d, J = 9, 1H), 7.50 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 13.98 (CH_3), 61.78 (CH_2), 121.21 (CH), 123.22 (C), 127.33 (CH), 127.48 (CH), 130.47 (CH), 132.60 (CH), 133.03 (CH), 133.57 (C), 152.80 (C), 162.79 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4Cl$): m/z 278.0571. Found: $C_{12}H_{11}O_2N_4Cl$–CH_3CH_2COO: 205.0070.

4.3.10. Ethyl (*E*)-3-[5-(3-chlorophenyl)-2H-tetrazol-2-yl]-propenoate (7d-*trans*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.14 (t, J = 7, 3H), 4.34 (q, J = 7, J = 14, 2H), 6.92 (d, J = 14, 1H), 7.45 (m, 2H), 8.06 (dt, J = 2, J = 7, 1H), 8.16 (s. 1H), 8.38 (d, J = 14, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 14.26 (CH_3), 61.56 (CH_2), 114.35 (CH), 125.38 (CH), 127.00 (CH), 127.93 (C), 130.44 (CH), 131.22 (CH), 135.04 (CH), 135.19 (C), 164.58 C, 165.59 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4Cl$): m/z 278.0570. Found: MH: 279.0691.

4.3.11. Ethyl (*Z*)-3-[5-(3-chlorophenyl)-2*H*-tetrazol-2-yl]-propenoate (7d-*cis*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.31 (t, J = 7, 3H), 4.35 (q, J = 7, J = 14, 2H), 6.13 (d, J = 10, 1H), 7.43 (m, 2H), 7.52 (d, J = 10, 1H), 8.03 (d, J = 16, 1H), 8.13 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.04 (CH_3), 61.90 (CH_2), 115.95 (CH), 125.20 (CH), 126.51 (CH), 127.18 (CH), 129.11 (2× CH), 128.27 (C), 130.41 (CH), 130.96 (CH), 135.09 (C), 164.04 C, 165.04 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4Cl$): m/z 278.0570. Found: MH: 279.0077.

4.3.12. Ethyl (*E*)-3-[5-(3-chlorophenyl)-1*H*-tetrazol-1-yl]-propenoate (8d-*trans*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.35 (t, J = 7, 3H), 4.30 (q, J = 7, J = 14, 2H), 6.98 (d, J = 14, 1H), 7.56 (m, 2H), 7.64 (m, 1H), 7.74 (s, 1H), 7.90 (d, J = 14, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 14.30 (CH_3), 61.82 (CH_2), 116.62 (CH), 124.32 (C), 127.49 (CH), 129.63 (CH), 131.09 (C), 131.42 (CH), 132.52 (C), 136.05 (C), 153.42 (C), 164.78 C; HR-MS calcd for ($C_{12}H_{11}O_2N_4Cl$): m/z 278.0570. Found: 278.0583.

4.3.13. Ethyl (*Z*)-3-[5-(3-chlorophenyl)-1*H*-tetrazol-1-yl]-propenoate (8d-*cis*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): m/z 1.16 (t, J = 7, 3H), 4.10 (q, J = 7, J = 14, 2H), 6.30 (d, J = 9, 1H), 7.20 (d, J = 10, 1H), 7.52 (m, 2H), 7.69 (d, J = 8, 1H), 7.81 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 13.93 (CH_3), 61.91 (CH_2), 122.77 (CH), 125.12 (C), 127.04 (CH), 127.95 (CH), 130.75 (CH), 135.57 (C), 153.02 (C), 162.62 C; HR-MS calcd for ($C_{12}H_{11}O_2N_4Cl$): m/z 278.0571. Found: 278.0563.

4.3.14. Ethyl (*Z*)-3-[5-(4-chlorophenyl)-2*H*-tetrazol-2-yl]-propenoate (7e-*cis*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.30 (t, J = 7, 3H), 4.34 (q, J = 7, J = 14, 2H), 6.10 (d, J = 10, 1H), 7.50 (m, 3H), 8.10 (d, J = 8, 2H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.13 (CH_3), 61.99 (CH_2), 115.78 (CH), 126.18 (C), 128.55 (2× CH), 129.52 (2× CH), 137.20 (C), 147.65 (CH), 164.12 C, 164.40 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4Cl$): m/z 278.0570. Found: MH: 279.0588.

4.3.15. Ethyl (*E*)-3-[5-(4-chlorophenyl)-1*H*-tetrazol-1-yl]-propenoate (8e-*trans*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.35 (t, J = 7, 3H), 4.30 (q, J = 7, J = 14, 2H), 6.97 (d, J = 14, 1H), 7.90 (d, J = 14, 1H), 7.53 (d, J = 8, 2H), 7.76 (d, J = 8, 2H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.29 (CH_3), 61.81 (CH_2), 116.49 (CH), 131.03 (CH), 121.05 (C), 130.22 (2× CH), 130.77 (2× CH), 153.71 (C), 164,80 C; HR-MS calcd for ($C_{12}H_{11}O_2N_4Cl$): m/z 278.0570. Found: 278.0562.

4.3.16. Ethyl (*Z*)-3-[5-(4-chlorophenyl)-1*H*-tetrazol-1-yl]-propenoate (8e-*cis*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.14 (t, J = 7, 3H), 4.07 (q, J = 7, J = 14, 2H), 6.29 (d, J = 9, 1H), 7.22 (d, J = 9, 1H), 7.52 (d, J = 8, 2H), 7.76 (d, J = 8, 2H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 13.89 (CH_3), 61.84 (CH_2), 121.81 (CH), 122.61 (C), 128.09 (CH), 129.76 (2× CH), 130.16 (2× CH), 153.28 (C), 162.57 C; HR-MS calcd for ($C_{12}H_{11}O_2N_4Cl$): m/z 278.0570. Found: MH: 279.1633.

4.3.17. Ethyl (*E*)-3-[5-(3-fluorophenyl)-2*H*-tetrazol-2-yl]-propenoate (7f-*trans*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.38 (t, J = 7, 3H), 4.33 (q, J = 7, J = 14, 2H), 6.93 (d, J = 14 1H), 7.22 (td, J = 3, J = 8, 1H), 7.50 (td, J = 5, J = 8, 1H), 7.90 (dt, J = 8, J = 2, 1H), 8.00 (d, J = 8, 1H), 8.40 (d, J = 14, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.30 (CH_3), 61.67 (CH_2), 114.27 and 114.58 ($^2J_{CF}$ = 23, CH), 114.46 (CH), 118.31 and 118.43 ($^2J_{CF}$ = 21, CH), 123.17 (CH), 128.32 and 128.44 ($^3J_{CF}$ = 9, C), 130.90 and 131.03 ($^3J_{CF}$ = 9, CH), 135.14 (CH), 161.49 and 164.84 ($^1J_{CF}$ = 251, C), 164.70 (C), 164.77 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4F$): m/z 262.0866. Found: $C_{12}H_{11}O_2N_4F$ - CH_3CH_2COO: 189.0478.

4.3.18. Ethyl (*Z*)-3-[5-(3-fluorophenyl)-2*H*-tetrazol-2-yl]-propenoate (7f-*cis*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.31 (t, J = 7, 3H), 4.34 (q, J = 7, J = 14, 2H), 6.13 (d, J = 10, 1H), 7.20 (td, J = 3, J = 8, 1H), 7.48 (m, 1H), 7.53 (d, J = 10, 1H), 7.86 (m, 1H), 7.97 (d, J = 8, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.13 (CH_3), 61.86 (CH_2), 114.14 and 114.45 ($^2J_{CF}$ = 23, CH), 115.97 (CH), 117.92 and 118.19 ($^2J_{CF}$ = 20, CH), 122.97 and 123.02 ($^4J_{CF}$ = 3, CH), 126.60 (CH), 128.64 and 128.76 ($^3J_{CF}$ = 9, C), 130.86 and 130.98 ($^3J_{CF}$ = 9, CH), 161.51 and 164.75 ($^1J_{CF}$ = 243, C), 164.13 (C), 164.31 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4F$): m/z 262,0866. Found: $C_{12}H_{11}O_2N_4F$–CH_3CH_2COO: 189.0353.

4.3.19. Ethyl (*E*)-3-[5-(3-fluorophenyl)-1*H*-tetrazol-1-yl]-propenoate (8f-*trans*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.34 (t, J = 7, 3H), 4.31 (q, J = 7, J = 14, 2H), 6.98 (d, J = 15, 1H), 7.50 (m, 4H), 7.94 (d, J = 15, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.41 (CH_3), 60.68 (CH_2), 104.45 (CH), 116.64 and 117.72 ($^2J_{CF}$ = 25, CH), 119.47 and 119.75 ($^2J_{CF}$ = 21, CH), 125.23, 131.68 and 131.80 ($^3J_{CF}$ = 9, CH), 131.40 and 131.47 ($^3J_{CF}$ = 5, CH), 137.20 (CH), 164.18 and 166.23 ($^1J_{CF}$ = 154, CH), 164.70 (C), 164.79 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4F$): m/z 262,0866. Found: $C_{12}H_{11}O_2N_4F$–CH_3CH_2COO: 189.0461.

4.3.20. Ethyl (*Z*)-3-[5-(3-fluorophenyl)-1*H*-tetrazol-1-yl]-propenoate (8f-*cis*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.15 (t, J = 7, 3H), 4.10 (q, J = 7, J = 14, 2H), 6.32 (d, J = 9, 1H), 7.21 (d, J = 9, 1H), 7.23 (m, 1H), 7.58 (m, 3H); HR-MS calcd for ($C_{12}H_{11}O_2N_4F$): m/z 262,0866. Found: $C_{12}H_{11}O_2N_4F$–CH_3CH_2COO: 189.0433.

4.3.21. Ethyl (*E*)-3-[5-(3-bromophenyl)-2*H*-tetrazol-2-yl]-propenoate (7g-*trans*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.38 (t, J = 7, 3H), 4.34 (q, J = 7, J = 14, 2H), 6.91 (d, J = 14, 1H), 7.37 (t, J = 8, 1H), 7.62 (d, J = 8, 1H), 8.11 (d, J = 8, 1H), 8.34 (s, 1H), 8.32 (d, J = 14, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.27 (CH_3), 61.60 (CH_2), 114.39 (CH), 123.20 (C), 125.85 (CH), 128.19 (C), 130.23 (CH), 130.69 (CH), 134.16 (CH), 135.04 (CH), 164.44 (C), 165.59 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4Br$): m/z 322.0065. Found: $C_{12}H_{11}O_2N_4Br$–CH_3CH_2COO: 248.9666.

4.3.22. Ethyl (*Z*)-3-[5-(3-bromophenyl)-2*H*-tetrazol-2-yl]-propenoate (7g-*cis*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.32 (t, J = 7, 3H), 4.35 (q, J = 7, J = 14, 2H), 6.13 (d, J = 10, 1H), 7.37 (t, J = 8, 1H), 7.52 (d, J = 10, 1H), 7.62 (d, J = 8, 1H), 8.07 (d, J = 8, 1H), 8.29 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.04 (CH_3), 61.90 (CH_2), 115.94 (CH), 123.10 (C), 125.66 (CH), 126.51 (CH), 128.49 (C), 130.10 (CH), 130.65 (CH), 133.88 (CH), 163.82 (C), 164.04 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4Br$): m/z 322.0065. Found: $C_{12}H_{11}O_2N_4Br$–CH_3CH_2COO: 248.9596.

4.3.23. Ethyl (*E*)-3-[5-(3-bromophenyl)-1*H*-tetrazol-1-yl]-propenoate (8g-*trans*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.34 (t, J = 7, 3H), 4.29 (q, J = 7, J = 14, 2H), 6.98 (d, J = 14, 1H), 7.52 (m, 1H), 7.55 (d, J = 7,1H), 7.80 (d, J = 7 1H), 7.89 (s, 1H), 7.91 (d, J = 14, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.30 (CH_3), 61.82 (CH_2), 116.62 (CH), 123.87 (CH), 124.54 (C), 127.92 (CH), 131.21 (CH), 131.39 (C), 132.49 (CH), 135.44 (CH), 153.29 (C), 164.29 C; HR-MS calcd for ($C_{12}H_{11}O_2N_4Br$): m/z 322.0065. Found: $C_{12}H_{11}O_2N_4Br$–CH_3CH_2COO: 248.9200.

4.3.24. Ethyl (*Z*)-3-[5-(3-bromophenyl)-1*H*-tetrazol-1-yl]-propenoate (8g-*cis*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.14 (t, J = 7, 3H), 4.11 (q, J = 7, J = 14, 2H), 6.31 (d, J = 8, 1H), 7.20 (d, J = 9, 1H), 7.30 (m, 2H), 7.43 (t, J = 8, 1H), 7.96 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 13.93 (CH_3), 61.94 (CH_2), 122.74 (CH), 123,44 (CH), 125.33 (C), 127.46 (C), 127.89 (CH), 130.93 (CH), 131.79 (CH), 134.96 (CH), 152.62 (C), 162.62 C; HR-MS calcd for ($C_{12}H_{11}O_2N_4Br$): m/z 322.0065. Found: $C_{12}H_{11}O_2N_4Br$: 322.0059.

4.3.25. Ethyl (*E*)-3-[5-(3-iodophenyl)-2*H*-tetrazol-2-yl]-propenoate (7h-*trans*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.39 (t, J = 7, 3H), 4.34 (q, J = 7, J = 14, 2H), 6.93 (d, J = 14, 1H), 7.27 (t, J = 8, 1H), 7.48 (d, J = 8, 1H), 8.17 (d, J = 8, 1H), 8.56 (s, 1H), 8.40 (d, J = 14, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.32 (CH_3), 61.66 (CH_2), 94.68 (C), 114.51 (CH), 126.54 (CH), 128.27 (C), 130.83 (CH), 135.13 (CH), 136.16 (CH), 140.18 (CH), 164.37 (C), 164.71 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4I$): m/z 322.0065. Found: $C_{12}H_{11}O_2N_4Br$–C_6H_4: 341.9715.

4.3.26. Ethyl (*Z*)-3-[5-(3-iodophenyl)-2*H*-tetrazol-2-yl]-propenoate (7h-*cis*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.32 (t, 3H), 4.36 (q, 2H), 6.12 (d, J = 10, 1H), 7.24 (t, 1H), 7.50 (d, J = 10, 1H), 7.82 (d, J = 8, 1H), 8.00 (d, J = 8, 1H), 8.52 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.17 (CH_3), 61.02 (CH_2), 94.61 (C), 115.99 (CH), 126.36 (CH), 126.53 (CH), 128.61 (C), 130.80 (CH), 136.10 (C), 139.91 (CH), 163.79 (C), 164.13 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4I$): m/z 322.0065. Found: $C_{12}H_{11}O_2N_4Br$–C_6H_4: 341.9788.

4.3.27. Ethyl (*E*)-3-[5-(3-iodophenyl)-1*H*-tetrazol-1-yl]-propenoate (8h-*trans*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.32 (t, J = 7, 3H), 4.36 (q, J = 7, J = 14, 2H), 6.12 (d, J = 14, 1H), 7.24 (t, J = 8, 1H), 7.50 (d, J = 14, 1H), 7.82 (d, J = 8, 1H), 8.12 (d, J = 8, 1H), 8.52 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.47 (CH_3), 61.86 (CH_2), 95.13 (C), 116.57 (CH), 128.46 (CH), 131.17 (CH), 131.62 (C), 134.58 (CH), 138.25 (CH) 140.86 (CH), 150.66 (C), 165.23 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4I$): m/z 322.0065. Found: $C_{12}H_{11}O_2N_4Br$: 369.9924.

4.3.28. Ethyl (*Z*)-3-[5-(3-iodophenyl)-1*H*-tetrazol-1-yl]-propenoate (8h-*cis*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.32 (t, J = 7, 3H), 4.21 (q, J = 7, J = 14, 2H), 6.28 (d, J = 9, 1H), 7.76 (d, J = 9, 1H), 7.29 (m, 1H), 7.76 (d, J = 8, 1H), 7.92 (d, J = 7, 1H), 8.16 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.29 (CH_3), 61.96 (CH_2), 94.74 (C), 122.61 (CH), 125.38 (C), 127.79 (CH), 128.04 (CH), 130.91 (CH), 137.19 (CH), 140.86 (CH), 152.74 (C), 162.64 (C); HR-MS calcd for ($C_{12}H_{11}O_2N_4I$): m/z 322.0065. Found: $C_{12}H_{11}O_2N_4Br$: 369.9914.

4.3.29. Ethyl (*E*)-3-(tetrazol-2-yl)-propenoate (7i-*trans*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.37 (t, J = 7, 3H), 4.33 (q, J = 7, J = 14, 2H), 6.93 (d, J = 14, 1H), 8.41 (d, J = 14, 1H), 8.65 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.24 (CH_3), 61.59 (CH_2), 114.90 (CH), 134.47 (CH), 153.56 (CH), 164.49 C.

4.3.30. Ethyl (*Z*)-3-(tetrazol-2-yl)-propenoate (7i-*cis*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.29 (t, J = 7, 3H), 4.30 (q, J = 7, J = 14, 2H), 6.14 (d, J = 10, 1H), 7.53 (d, J = 10, 1H), 8.60 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.04 (CH_3), 61.90 (CH_2), 116.45 (CH), 126.72 (CH), 152.91 (CH), 163.91 C.

4.3.31. Ethyl (*Z*)-3-(tetrazol-1-yl]-propenoate (8i-*trans*). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.36 (t, J = 7, 3H), 4.32 (q, J = 7, J = 14, 2H), 6.80 (d, J = 14, 1H), 8.20 (d, J = 10, 1H), 8.98 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.29 (CH_3), 61.90 (CH_2), 114.81 (CH), 137.95 (CH), 154.14 (CH), 164.46 C.

4.3.32. Ethyl (*Z*)-3-(3-phenyl-1,2,4-triazol-1-yl)-propenoate (10a-*cis*). ^{1}H NMR (300 MHz, CDCl3, δ): 1.33 (t, J = 7, 3H), 4.27 (q, J = 7, J = 14, 2H), 5.57 (d, J = 11, 1H), 7.29 (d, J = 11, 1H), 7.44 (m, 3H), 8.13 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 14.25 (CH_3), 61.26 (CH_2), 106.95 (CH), 126.90 (2× CH), 128.78 (2× CH), 130.07 (CH), 133.73 (CH), 147.71 (CH), 162.57 C, 164.62 (C); HR-MS calcd for ($C_{14}H_{14}O_2N_2$): m/z 243.1009. Found: 243.0999.

4.3.33. Ethyl (*E*)-3-(3-(2-aminophenyl-1,2,4-triazol-1-yl)-2-propenoate (10b-*trans*). ^{1}H NMR (400 MHz, CDCl3, δ): 1.35 (t, J = 7, 3H), 4.30 (q, J = 7, J = 14, 2H), 5.90 (s, NH_2), 6.57 (d, J = 14, 1H), 6.78 (m, 2H), 7.39 (m, 1H), 8.00 (d, J = 14, 1H), 8.35 (m, 1H), 8.31 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 14.2 (CH_3), 61.1 (CH_2), 109.2 (CH), 112.3 (C), 116.4 (CH), 117.2 (CH), 129.3 (CH), 131.2 (CH), 134.8 (CH), 144.2 (CH). 146.3 (C), 164.4 (C), 165.8 C; HR-MS: calcd for ($C_{13}H_{14}O_2N_4$): m/z 258.1117. Found: 258.1114.

4.3.34. Ethyl (*Z*)-3-(3-(2-aminophenyl-1,2,4-triazol-1-yl)-2-propenoate (10b-*cis*). ^{1}H NMR (400 MHz, CDCl3, δ): 1.32 (t, J = 7, 3H), 4.27 (q, J = 7, J = 14, 2H), 5.60 (s, NH_2), 5.65 (d. J = 11, 1H), 6.75 (m, 2H), 7.20 (dq, J = 2, J = 8, 1H), 7.26 (d, J = 11, 1H), 8.14 (dd, J = 1, J = 8, 1H), 9.64 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 14.1 (CH_3), 61.1 (CH_2), 106.6 (CH), 112.3 (C), 116.3 (CH), 117.2 (CH), 129.2 (CH), 130.8 (CH), 133.1 (CH), 146.15 (C). 146.23 (CH), 162.6 (C), 164.5 C; HR-MS: calcd for ($C_{13}H_{14}O_2N_4$): m/z 258.1117. Found: 258.1128.

4.4. Cell culture, transfections, and plasmids

HeLa cells, human epithelial cells, and human embryonal kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Mediatech Inc., Herndon, VA) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 2 mM L-glutamine 0.1% sodium bicarbonate, and 20 μg/ml gentamicin. The OM10.1 cell line was obtained from the AIDS Research and Reference Reagent Program (ERC Bio Services, Rockville, MD).[48] Cells were grown in RPMI 1640 medium (Mediatech Inc., Herndon, VA) supplemented with 10% (vol/vol) fetal calf serum, 2 mM L-glutamine 0.1% sodium bicarbonate, and 20 μg/ml gentamicin.

pRevM5-GFP and pCRM1-GFP plasmids produce fusion proteins of RevM5 and hCRM1, respectively, fused to the enhanced version of the GFP (GFPsg25).[41] RevM5 has two point mutations (R38D and R39L) in its RNA-binding-site and is therefore unable to bind RRE RNA. pCRM1-GFP was a kind gift of Dr. Felber (National Cancer Institute, Frederick, MD). pRev-BFP expresses the Rev protein fused to the enhanced BFP emitting blue light.[41] pCRM1-EYFP was constructed by cloning the CRM1 gene from pCRM1-GFP into pEYFP-N1 vector (Clonetech, Mountain View, CA). The pEYFP-CRM1Cys528Ser plasmid was constructed by cloning the CRM1Cys528Ser from pXHCK1 (a kind gift from Dr. M. Yoshida, Chemical Genetics Laboratory, Discovery Research Institute, RIKEN, Japan)[33] into pEYFP-N1 (Clonetech, Mountain View, CA).

For transfection of HeLa cells, cells were plated onto glass bottom microwell dishes (MatTek Corporation, Ashland, MA) at 0.1×10^6 cells/plate and until 50% confluent. Cells were washed with PBS and transfected with 1 μg of plasmid DNA using SuperFect® transfection reagent (Qiagen, Valencia, CA) according to the manufacturers manual and incubated overnight.

4.5. RevM5-GFP transport assay

The inhibition of the CRM1-mediated Rev transport was monitored as follows. HEK 293T cells were plated in 96-well trays and transfected with pRevM5-GFP using the calcium phosphate transfection method. One day after transfection, test compounds were added to the cells at different concentrations. Inhibition of the CRM1-mediated nucleocytoplasmic transport was measured by verifying the cellular distribution of RevM5-GFP two hours after addition of the compounds. Using fluorescence microscopy, conditions where the RevM5-GFP was found mainly in the nucleus were scored positive, conditions where the RevM5-GFP localization was cytoplasmic were scored as negative. Cytoxicity of the test compounds was measured according to a tetrazolium-based viability assay (CellTiter96 Aqueous Cell Proliferation Assay, Promega, Belgium).

4.6. Microscopy of living cells

One day after transfection of HeLa cells, cells were washed with PBS and treated with the appropriate concentrations of test compound dissolved in complete DMEM. Cells were analyzed at 30, 120, 180 min, and 24 h after treatment. The cells were examined with an SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with an AOBS, using a HCX PL APO 63.0× (NA:1.20) water objective. BFP was monitored with a Mai-Tai HP multi-photon laser (Newport, Spectra Physics) at 760 nm for excitation and emission was detected between 390 and 480 nm. GFP and YFP were excited with an Ar laser using, respectively, the 488 nm and the 514 nm laser line. Emission was detected between 450 and 490 nm for GFP and between 520 and 600 nm for YFP.

4.7. HIV-1 induction and inhibition in cell lines

OM10.1 chronically infected cells in logarithmic growth were washed twice (to remove cell-free virus), resuspended at 120,000 cells/well in fresh medium containing the test compounds at the appropriate concentrations and stimulated with 1 ng/ml TNF-α. After 48 h of incubation at 37 °C, the supernatants of the cell cultures were collected to determine viral progeny virus. Viral p24 production was assessed by an HIV-1 p24 enzyme-linked immunosorbent assay (PE, Brussels, Belgium). The same cell cultures were used for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability staining as previously described.[49]

Inhibition of HIV virus production was monitored in HEK 293T. Therefore 293T cells were transfected with NL_{4-3} plasmid, a molecular clone of HIV, using the calcium phosphate method and test compounds were added at different concentrations. Twenty-four hours after the addition of compounds, virus production in the supernatants of the cells was monitored by virus-associated p24 Ag by enzyme-linked immunosorbent assay.

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