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Inhibition of the PCAF histone acetyl transferase and cell proliferation by isothiazolones

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ABSTRACT

Small molecule HAT inhibitors are useful tools to unravel the role of histone acetyl transferases (HATs) in the cell and have relevance for oncology. We present a systematic investigation of the inhibition of the HAT p300/CBP Associated Factor (PCAF) by isothiazolones with different substitutions. 5-chloro-isothiazolones proved to be the most potent inhibitors of PCAF. The growth inhibition of 4 different cell lines was studied and the growth of two cell lines (A2780 and HEK 293) was inhibited at micromolar concentrations by 5-chloroisothiazolones. Furthermore, the 5-chloroisothiazolone preservative Kathon™ CG that is used in cosmetics inhibited PCAF and the growth of cell lines A2780 and HEK 293, which indicates that this preservative should be applied with care.

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1. Introduction

Posttranslational modifications of histone proteins play a crucial role in gene-specific transcription regulation in eukaryotes.[1] These histone modifications occur in distinct patterns that mediate specific interactions with multiprotein complexes, which initiate or inhibit gene transcription via the so-called 'histone-code'.[2–4] There is increasing evidence that the histone code plays a crucial role in normal and aberrant cell function and differentiation. Small molecule modulators of histone modifying enzymes are useful tools to unravel the functions of these enzymes and might ultimately lead to therapeutic applications.

The histone acetyl transferases (HATs) form a disparate group of enzymes that mediate acetyl transfer to histones or other proteins.[5,6] The GNAT (Gcn5 related *N*-acetyltransferase) family HATs include the closely related enzymes PCAF (p300/CBP associated factor) and GCN5 (general control of amino-acid synthesis 5).[7] PCAF acetylates histone H3 on lysine 14 and less efficiently histone H4 on lysine 8.[8] The GNAT family HATs have been recognized as potential anticancer and antiviral targets.[6,9] The HAT GCN5 plays a key role in EGF mediated gene transcription, which is relevant for cancer therapy.[10] Furthermore, GCN5 is crucial for cell cycle progression.[11] Deregulation of the activity of GNAT and p300 family HATs plays an important role in a number of human cancers.[12–14]

Despite the potential therapeutic relevance of the GNAT family HATs, very few small molecule inhibitors for GCN5 and PCAF have been described so far. Lau et al. described bisubstrate inhibitors for p300 and PCAF by coupling the histone H3 peptide to CoA.[15] However, this provided compounds with poor membrane permeability. The natural products Curcumin,[16] Garcinol[17] and Anacardic acid[18] show HAT inhibitory activity, however their potency is low. GCN5 and PCAF are inhibited by α-methylene-γ-butyrolactones[19] and isothiazolones,[20] that covalently capture the active site thiol of these enzymes.

The isothiazolones provide an interesting starting point for structure based design of PCAF and GCN5 inhibitors. The isothiazolone functionality is readily available via organic synthesis[21] and can easily be decorated with diverse substitutions to enhance binding to the enzyme active site. The crystal structure and the catalytic mechanism of GCN5[22] and PCAF[23] provide inspiration for inhibitor design.[24,25] The crystal structure of the enzyme GCN5 in complex with a bisubstrate inhibitor[26] shows that the pyrophosphate and the pantothenic acid arm make extensive hydrogen bonding interactions with the enzyme. The same is observed in the crystal structure of the enzyme PCAF in complex with CoA (PDB entry 1CM0).[23] This suggests that hydrogen bonding interactions are relevant for binding to the enzyme active site. Thus, inhibitors with hydrogen bond donor/acceptors may show improved binding to the enzyme active site.

In this study we investigated structure activity relationships for inhibition of the enzyme PCAF by isothiazolones. The (5-chloro)isothiazolone scaffold was used to target the active site thiol and the substitution was explored to enhance binding by providing specific interactions with the enzyme. A series of 19 compounds was synthesized and tested for inhibition of PCAF activity. 5-Chloroisothiazolones showed the most potent inhibition of PCAF. 5-Chloroisothiazolone **2e** showed a slightly increased

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potency compared to the other 5-chloroisothiazolones. Cell lines A2780 and HEK 293 showed growth inhibition upon treatment with micromolar concentrations N-aliphatic substituted 5-chloroisothiazolones, whereas the cell lines WiDr and HEP G2 were much less affected.

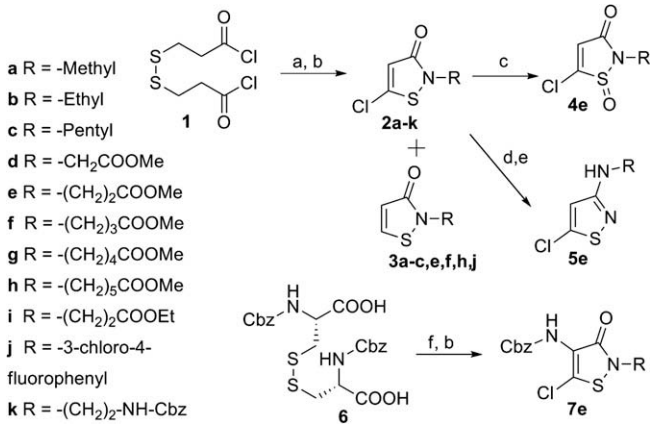
2. Results and discussion

2.1. Chemistry

A collection of N-functionalized isothiazolones was synthesized using procedures shown in Scheme 1.[21,27–29] Different amines were reacted with 3,3′-dithiodipropanoyl chloride **1** to give the dithiodipropionic amides in moderate to high yields. The dithiodipropionic amides were treated with sulfuryl chloride (3 equiv) at 0 °C in dichloromethane to give the 5-chloroisothiazolones **2** and isothiazolones **3** in ratios between 3:1 and 2:1, which were readily separated using column chromatography. 5-Chloroisothiazolones were obtained in yields between 50% and 70% and isothiazolones were obtained in yields around 20% from the same reaction mixture. Surprisingly, **2d** yielded only 23% of the 5-chloroisothiazolone and the corresponding isothiazolone could not be isolated from the same reaction mixture. 5-Chloroisothiazolone **2e** was oxidized by *m*CPBA to yield 5-chloroisothiazolone-1-oxide **4e**.[28] Treatment of 5-chloroisothiazolone **2e** with $POCl_3$ and ammonia provided 5-chloroisothiazol-3-amine **5e**.[21] 4-acylamino-isothiazolone **7e** was obtained using a modified procedure published by Nádel et al.[29] Cbz protected L-Cystin **6** was coupled to an amine HCl-salt using DCC, HOBt and triethylamine as a base followed by treatment with sulfuryl chloride to yield the 4-carbamoyl-5-chloroisothiazolone **7e**.

2.2. Structure–activity relationships for PCAF inhibition

Structure–activity relationships for inhibition of the HAT PCAF by a series of (5-chloro)isothiazolones were investigated in order to explore the binding properties of these compounds (Table 1). Binding studies were performed using a procedure published by Trievel et al.[22] The enzyme activity was measured by detection of CoA-SH by the fluorescent dye 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM). The CoA-SH concentrations measured with no inhibitor present were around 50 μM. Enzyme inhibition was measured by determination of the residual enzyme activity after 15 min incubation with the inhibitor. The inhibitor concentrations were maximal 10 μM, so inhibitory effects of more than 20% at 10 μM inhibitor concentration cannot be explained by direct reaction of the inhibitors with CoA-SH, but result from inhibition of the enzyme. Compounds that showed more than 50% inhibition at 10 μM ($n = 3$) were subjected to IC_{50} determination ($n = 3$). A representative example is shown in Figure 1A.



Scheme 1. Synthesis of a focused compound collection. Reagents and conditions: (a) R-NH_2, Et_3N, CH_2Cl_2, (b) SO_2Cl_2, CH_2Cl_2, (c) *m*CPBA, CH_2Cl_2, (d) $POCl_3$, (e) NH_3, CH_3CN, (f) DCC, HOBt, Et_3N, R-$NH_3^+Cl^-$, CH_2Cl_2.

Table 1
Inhibition of the HAT PCAF and proliferation of cell lines by (5-chloro)isothiazolones

	PCAF inhibition IC_{50} (μM)	A2780 GI_{50} (μM)	WiDr GI_{50} (μM)	HEPG2 GI_{50} (μM)	HEK 293 GI_{50} (μM)
2a/**3a** 3:1	3.0 ± 0.6	2.0 ± 0.2	5.0 ± 0.4	6.0 ± 1.2	1.4 ± 0.1
2b	3.0 ± 0.3	>10	>10	>10	5.6 ± 0.4
2c	2.9 ± 0.3	2.3 ± 0.2	>10	6.4 ± 0.2	1.7 ± 0.1
2d	2.9 ± 0.7	>10	>10	>10	>10
2e	1.8 ± 0.2	3.3 ± 0.3	7.5 ± 0.3	10 ± 1	2.8 ± 0.3
2f	2.5 ± 0.3	3.0 ± 0.4	>10	7.0 ± 0.8	1.5 ± 0.6
2g	2.8 ± 0.3	5.2 ± 0.1	>10	8.0 ± 0.4	0.7 ± 0.1
2h	3.2 ± 0.2	7.0 ± 0.8	5.6 ± 0.5	9.4 ± 0.3	2.9 ± 0.8
2i	2.0 ± 0.2	3.0 ± 0.1	>10	>10	>10
2j	2.5 ± 0.1	>10	>10	>10	>10
2k	2.6 ± 0.1	8.1 ± 0.7	>10	>10	3.0 ± 0.4
3b	>10	>10	>10	>10	>10
3c	>10	>10	>10	>10	>10
3e	>10	>10	>10	>10	>10
3f	>10	>10	>10	>10	>10
3h	>10	>10	>10	>10	>10
3j	4.2 ± 0.6	>10	>10	>10	>10
4e	5.6 ± 0.2	>10	>10	>10	>10
5e	>10	>10	8.6 ± 1.0	2.0 ± ± 0.3	4.7 ± 0.4
7e	4.9 ± 0.9	5.8 ± 0.6	>10	>10	10

Inhibition concentration 50% (IC_{50}) determination $n = 3$, growth inhibition 50% (GI_{50}) determinations $n = 8$, standard deviations from the non-linear curve fitting are reported.

The compounds were designed to evaluate the PCAF inhibition of 5-chloroisothiazolones and isothiazolones with different N-substitutions. The 5-chloroisothiazolone core with only an *N*-ethyl substitution **2b** shows a potent PCAF inhibition. The PCAF inhibition of **2b** is equal to the commercially available preservative Kathon™ CG **2a**/**3a**, which is a mixture of *N*-methyl-5-chloroisothiazolone and *N*-methylisothiazolone 3:1. An *N*-pentyl substitution **2c** did not improve binding compared to **2b**. A series of N-substitutions with a methyl ester at different numbers of carbon atoms distance to the (5-chloro-)isothiazolone **2d**–**h** was studied. Compound **2e** showed a slightly increased potency compared to the other 5-chloroisothiazolones, which suggests that the N-substitution provides additional interaction with PCAF. Based on compound **2e** a molecular modeling was performed to suggest a binding pose to aid future optimizations of the PCAF inhibition (Fig. 2).

There is a pronounced difference in potency between N-aliphatic substituted 5-chloroisothiazolones **2** and isothiazolones **3**. 5-Chloroisothiazolones **2b–i,k** inhibit PCAF with IC_{50} values around 2–3 μM, whereas isothiazolones **3b,c,e,f,h**, showed less than 50% inhibition at 10 μM. The difference in potency for the N-aromatic substituted 5-chloroisothiazolone **2j** and isothiazolone **3j** is much less pronounced. The same in observed in the data provided by Stimson et al.[20] in which the difference in PCAF inhibitory potency between N-aromatic 5-chloroisothiazolones and isothiazolones is a factor 2 or less.

Compounds with modifications directly on the isothiazolone scaffold were synthesized and the consequences for PCAF inhibition were studied. 5-Chloroisothiazolone-1-oxide **4e** inhibited the PCAF activity at micromolar concentrations. The 5-chloroisothiazol-3-amine **5e** did not inhibit PCAF. Substitution of the 5-chloroisothiazolone scaffold in the 4-position with an amino acyl substituent **7e** reduced the PCAF inhibition compared to compound **2e**. Nevertheless, compound **7e** provides an interesting starting point for further optimization of the PCAF inhibition by variation of the 4-amino acyl substituent.

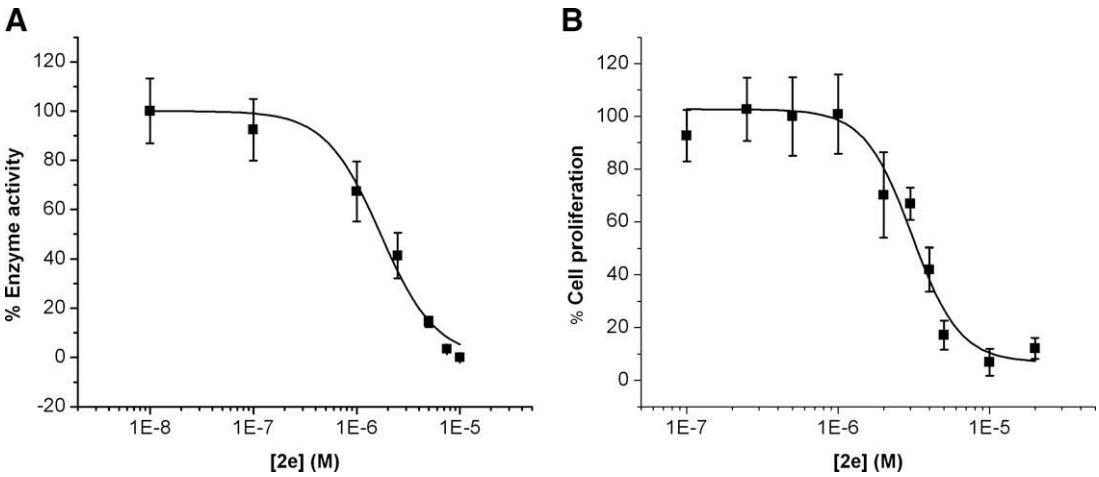


Figure 1. Panel A shows the concentration dependence for inhibition of the PCAF enzyme activity by 5-chloroisothiazolone **2e** ($n = 3$, standard deviation shown) and panel B shows the concentration dependence for growth inhibition of the cell line A2780 by **2e** ($n = 8$, standard deviation shown).

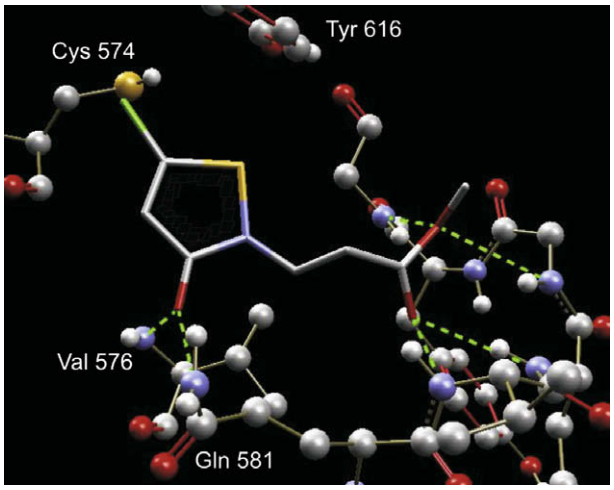


Figure 2. Binding pose for **2e** in the PCAF active site (PDB entry 1CM0) that was proposed by a molecular modeling study. Cys 574 is the active site cysteine. Hydrogen bonds are formed to valine 576 and Glutamine 581 and to the pyrophosphate binding pocket formed by the backbone amides of Glutamine 581, Valine 582, Lysine 583, Glycine 584 and Tyrosine 585.

Reports on the reactivity of *N*-methylisothiazolones and *N*-methyl-5-chloroisothiazolones indicate a high reactivity of 5-chloroisothiazolones. 5-chloroisothiazolones are prone to nucleophilic substitution in the 5-position and reductive cleavage of the N–S bond.[30] In contrast, for isothiazolones only reductive cleavage of the N–S bond by sulfides is observed and no nucleophilic addition in the 5-position.[30] This study supports the idea that the potent inhibition of 5-chloroisothiazolones is due to their reactivity. Interestingly, isothiazolone **3j** with a N-aromatic substitution shows an IC_{50} of 4.2 μM for PCAF inhibition. This indicates that N-aromatic isothiazolones are either more prone to nucleophilic addition in the 5-position of the isothiazolone or that they are more prone to reductive cleavage of the N–S bond, compared to N-aliphatic substituted isothiazolones.

2.3. Molecular modeling

Modeling studies were performed to propose a binding pose for inhibitor **2e** to aid future optimization of the inhibitors. The crystal structure of PCAF complexed with CoA (PDB entry code 1CM0) was used for docking studies.[23] The molecular modeling study suggested a binding pose in which the methyl ester is hydrogen bonded to a pocket in which the pyrophosphate is bound in the PCAF crystal structure 1CMO (Fig. 2).[23] The pyrophosphate binding pocket is formed by the backbone of amides of Glutamine 581, Valine 582, Lysine 583, Glycine 584 and Tyrosine 585. The carbonyl from the 5-chloroisothiazolone is hydrogen bonded to Glutamine 581 and Valine 576. Docking of 5-chloroisothiazolone-1-oxide **4e** provided a similar binding pose as observed for **2e.** Docking of **7e** provided also a binding pose comparable to **2e**. These results suggests that the PCAF inhibitory potency of **2e**, **4e** and **7e** might be improved by variations in the substitution in the 4-position of the isothiazolone core. In conclusion, compounds **2e**, **4e** and **7e** provide starting points to design inhibitors with an improved PCAF inhibitory potency.

2.4. Growth inhibition of cell lines

Inhibition of cancer cell lines was studied in order to explore the relevance of (5-chloro)isothiazolones as research tools to study the biological mechanisms underlying cell proliferation.[31] The growth inhibition of the human cancer cell lines A2780 (ovarian), WiDr (colon) and HEP G2 (liver) and the human embryonic cell line 293 (kidney) was studied. Growth inhibition was determined using a crystal violet assay. The compounds were screened at 10 μM inhibitor concentration for growth inhibition ($n = 8$) and compounds with more than 50% growth inhibition were subjected to growth inhibition 50% determination (GI_{50}) ($n = 8$) (Table 1). A representative example is shown in Figure 1B. The N-aliphatic substituted 5-chloroisothiazolones are the most potent compounds for growth inhibition of cell lines A2780 and HEK 293. The growth of cell lines WiDr and HEP G2 were not inhibited or required higher concentrations for inhibition compared to cell lines A2780 and HEK 293, which might be due to differences in the growth rate or differences in cellular biochemistry. Western blots on histone acetylation in cell lines that are treated with optimized inhibitors are required to proof a connection between the PCAF HAT inhibition and the cellular activity.

It is remarkable that compounds **2j** and **3j** do not inhibit the growth of the four cell lines that were studied. This indicates that N-aromatic isothiazolones like **2j** are less potent inhibitors of cell proliferation. Compound **5e** with a 5-chloroisothiazol-3-amine scaffold showed no inhibitory effect on PCAF but shows a potent inhibition of WiDr, HEP G2 and HEK 293 cell lines. This is the first

time that a biological effect is described for a compound with an 5-chloroisothiazol-3-amine scaffold.

The commercially available preservative Kathon™ CG **2a/3a** showed a potent PCAF inhibition and a potent growth inhibition of the four cell lines studied (Table 1). This is remarkable because the mixture has been used as a preservative in rinse-off cosmetics such as shampoos and conditioners for more than 25 years. Kathon™ CG is claimed to be safe and effective, however skin sensitization have been reported.[30,32] The growth inhibition of cell lines shows that Kathon™ CG **2a/3a** is cell-permeable and acts on intracellular targets. The inhibition of PCAF indicates that this preservative might modify the epigentics regulation of gene transcription. Taking these data together we conclude that Kathon™ CG should be applied with care.

3. Conclusion

In conclusion, N-aliphatic substituted 5-chloroisothiazolones inhibit the enzyme PCAF with an IC_{50} around 2–3 μM, whereas N-aliphatic substituted isothiazolones show less than 50% inhibition at 10 μM. The difference in PCAF inhibitory potency between N-aromatic substituted 5-chloroisothiazolones and isothiazolones is much less pronounced. N-Aromatic and N-aliphatic substituted 5-chloroisothiazolones showed a comparable potency for PCAF inhibition. N-Aliphatic substituted 5-chloroisothiazolones inhibited the growth of the cell lines A2780 and HEK 293 in the low micromolar range, whereas growth inhibition of WiDr and HEP G2 required higher concentrations or was not observed. N-Aromatic substituted 5-choroisothiazolones, N-aromatic and N-aliphatic substituted isothiazolones showed less than 50% inhibition of the cell proliferation at 10 μM. Finally, it has been shown that the preservative Kathon™ CG inhibits the HAT PCAF and inhibits the cell growth of A2780, WiDr, HEP G2 and HEK 293 cell lines.

4. Experimental

4.1. Organic synthesis

General: Chemicals were obtained from commercial sources (Sigma–Aldrich, Acros Organics) and used without previous purification, except dichloromethane that was distilled over CaH_2 before use. Thin-layer chromatography (TLC) was performed on aluminum sheets of Silica Gel 60 F254. Spots were visualized under ultraviolet light, with I_2 vapour, $KMnO_4$ solution or ninhydrin solution. Column chromatography was performed with MP Ecochrom Silica Gel 32–63, 60 Å. ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini-200 (50.32 MHz). Chemical shift values are reported as part per million (δ) relative to residual solvent peaks ($CHCl_3$, $^1H = 7.26$, ^{13}C $\delta = 77.16$ or CD_3OD, $^1H = 3.31$, ^{13}C $\delta = 49.00$). The coupling constants (J) are reported in Hertz (Hz). ^{13}C spectra were recorded using the attached proton test (APT) pulse sequence. Electrospray ionization mass spectra (ESI-MS) were recorded on a Applied Biosystems/SCIEX API3000-triple quadrupole mass spectrometer. High-resolution mass spectra (HR-MS) were recorded using a flow injection method on a LTQ-Orbitrap XL mass spectrometer (Thermo Electron, Bremen, Germany) with a resolution of 60,000 at m/z 400. Protonated testosterone (lock mass $m/z = 289.2162$) was used for internal recalibration in real time. Melting points were determined on an Electrothermal digital melting point apparatus and are uncorrected.

4.2. General procedure 1

The dithiobispropanamide (8 mmol) was dissolved in dry CH_2Cl_2 (50 mL) at 0 °C. SO_2Cl_2 (24 mmol) was added dropwise to the solution, and the mixture was stirred at 0 °C for 2 h. Subsequently, the mixture was washed with water (2 times 200 mL) and brine (1 time 200 mL), dried with Na_2SO_4 and filtered. The solution was concentrated under reduced pressure and purified using column chromatography.

4.2.1. 5-Chloro-2-ethylisothiazol-3(2*H*)-one (2b)

The product was obtained using procedure **1** starting from **10b**. Purification was performed using column chromatography with hexane/EtOAc 3:1 (v/v) as eluent. Yield 46%. Brown gum. $R_f = 0.44$ (CH_2Cl_2/MeOH 9:1). ^1H NMR (200 MHz, $CDCl_3$): δ 1.32 (t, $J = 7.2$ Hz, 3H), 3.81 (q, $J = 7.2$ Hz, 2H), 6.36 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 15.0, 39.4, 114.8, 146.3, 167.0. HPLC: purity >99%, retention time 6.6 min (column C8 mobile phase H_2O/CH_3CN/TFA 60:40:1). HRMS (m/z) 163.9933 $[M+H]^+$, calcd 163.9937 C_5H_7ClNOS.

4.2.2. 2-Ethylisothiazol-3(2*H*)-one (3b)

After elution of **2b** product **3b** was eluted from the column using EtOAc eluent. Yield 10%. Brown gum. $R_f = 0.38$ (CH_2Cl_2/MeOH 9:1). ^1H NMR (200 MHz, $CDCl_3$) δ 1.60 (m, 3H), 3.88 (m, 2H), 6.36 (m, 1H) 8.20 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 15.3, 39.8, 114.9, 140.4, 168.9. GC–MS purity >99%, retention time 2.69 min, (EI) $[M^+]$ m/z 129. HRMS (m/z) 130.0323 $[M+H]^+$, calcd 130.03266 C_5H_8NOS.

4.2.3. 5-Chloro-2-pentylisothiazol-3(2*H*)-one (2c)

The product was obtained using procedure **1** starting from **10c**. Purification was performed using column chromatography with hexane/EtOAc 8:1 (v/v) as eluent. An extra column chromatography step was required to obtain the pure product. Yield 55%. Orange oil. $R_f = 0.53$ (CH_2Cl_2/MeOH 94:6). ^1H NMR (200 MHz, $CDCl_3$) δ 0.88 (t, $J = 6.8$ Hz, 3H), 1.24-1.38 (m, 4H), 1.59-1.73 (m, 2H), 3.71 (t, $J = 7.3$ Hz, 2H), 6.25 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.1, 22.4, 28.8, 29.6, 44.0, 115.0, 145.8, 167.1. GC-MS RT 5.2 min, purity >99%, MS(EI) (m/z) 205, calcd 205. HRMS (m/z) 206.0402 $[M+H]^+$, calcd 206.04064 $C_8H_{13}ClNOS$.

4.2.4. 2-pentylisothiazol-3(2H)-one (3c)

After elution of **2c** product **3c** was eluted from the column using hexane/EtOAc 1:1 (v/v) as eluent. An extra column chromatography step was required to obtain the pure product. Yield 25%. Brown gum. $R_f = 0.29$ (CH_2Cl_2/MeOH 94:6). ^1H NMR (200 MHz, $CDCl_3$) δ 0.89 (t, $J = 6.5$ Hz, 3H), 1.24-1.37 (m, 4H), 1.51-1.79 (m, 2H), 3.79 (t, $J = 7.3$ Hz, 2H), 6.32 (d, $J = 6.2$ Hz, 1H), 8.1 (d, $J = 6.2$ Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.2, 22.6, 28.8, 29.7, 44.5, 114.7, 139.7, 169.1. GC-MS RT 4.8 min, purity >95%, MS(EI) (m/z) 171, calcd 171. HRMS (m/z) 172.0792 $[M+H]^+$, calcd 172.0796 $C_8H_{14}NOS$.

4.2.5. Methyl 2-(5-chloro-3-oxoisothiazol-2(3*H*)-yl)ethanoate (2d)

The product was obtained using procedure **1** starting from the **10d**. Purification was performed using column chromatography with hexane/EtOAc 3:2 (v/v) as eluent. Yield 23%. Dark brown gum. $R_f = 0.76$ (CH_2Cl_2/MeOH 9:1). ^1H NMR (200 MHz, $CDCl_3$) δ 3.78 (s, 3H), 4.48 (s, 2H), 6.32 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 44.1, 53.0, 112.9, 148.2, 167.3, 168.0. GC–MS rt 4.88 min, MS (EI) gave anomalous results. MS (ESI) (m/z) 208.0 $[M+H]^+$, calcd 208.0. HRMS (m/z) 207.9831 $[M+H]^+$, calcd 207.9835 $C_6H_7ClNO_3S$.

4.2.6. Methyl 3-(5-chloro-3-oxoisothiazol-2(3*H*)-yl)propanoate (2e)

The product was obtained using procedure **4** starting from **10e**. Purification was performed using column chromatography with hexane/EtOAc 1:2 (v/v) as eluent. Yield 49%. Orange oil. $R_f = 0.58$ (CH_2Cl_2/MeOH 10:1). ^1H NMR (200 MHz, $CDCl_3$) δ 2.67 (t, $J = 6.2$

Hz, 2H), 3.66 (s, 3H), 3.95 (t, J = 6.2 Hz, 2H), 6.19 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 33.9, 39.8, 52.3, 114.3, 147.0, 167.1, 171.8. GC–MS rt 5.97 min, purity >99%, MS(EI) (m/z) 221 [M], calcd 221. HRMS (m/z) 221.9987 $[M+H]^+$, calcd 221.9992 $C_7H_9ClNO_3S$, (m/z) 243.9805 $[M+Na]^+$, calcd 243.9811 $C_7H_8ClNNaO_3S$. HPLC purity >99%%, rt 5.0 min, (column C8 mobile phase H_20/CH_3CN/TFA 60:40:1).

4.2.7. Methyl 3-(3-oxoisothiazol-2(3*H*)-yl)propanoate (3e)

After elution of **2e** product **3e** was eluted from the column using hexane/EtOAc 1:20 (v/v) as eluent. Yield 23%. Brown oil. R_f = 0.49 (CH_2Cl_2/MeOH 10:1). ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.76 (t, J = 6.5 Hz, 2H), 3.70 (s, 3H), 4.09 (t, J = 6.5 Hz, 2H), 6.31 (d, J = 6.4 Hz, 1H), 8.10 (d, J = 6.2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 33.9, 40.2, 52.2, 114.2, 140.4, 169.1, 171.6. GC–MS rt 6.36 min, purity >99%, MS (EI) (m/z) 187 [M], calcd 187. HRMS (m/z) 188.0378 $[M+H]^+$, calcd 188.0381 $C_7H_9ClNO_3S$, (m/z) 210.01961 $[M+Na]^+$, calcd 210.0201 $C_7H_9NNaO_3S$.

4.2.8. Methyl 4-(5-chloro-3-oxoisothiazol-2(3*H*)-yl)butanoate (2f)

The product was obtained using procedure **1** starting from **10f**. Purification was performed using column chromatography with hexane/EtOAc 1:1 (v/v) as eluent. Yield 67%. Brown solid. Mp = 47.9 °C. R_f = 0.83 (CH_2Cl_2/MeOH 9:1). ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.92–2.06 (m, 2H), 2.38 (t, J = 7.2 Hz, 2H), 3.66 (s, 3H), 3.78 (t, J = 7.1 Hz, 2H), 6.19 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 24.9, 30.7, 42.9, 51.9, 114.8, 146.1, 167.1, 173.0. GC–MS rt 6.69 min, purity >99%, MS(EI) (m/z) 235 $[M]^+$, calcd 235. HRMS (m/z) 236.0143 $[M+H]^+$, calcd 236.0148 $C_8H_{11}ClNO_3S$, (m/z) 257.9962 $[M+Na]^+$, calcd 257.9968 $C_8H_{10}ClNNaO_3S$.

4.2.9. Methyl 4-(3-oxoisothiazol-2(3*H*)-yl)butanoate (3f)

After elution of **2f** product **3f** was eluted from the column using hexane/EtOAc 1:7 (v/v) as eluent. Yield 9%. Brown oil. R_f = 0.78 (CH_2Cl_2/MeOH 9:1). ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.02 (t, J = 7.1 Hz, 2H), 2.37 (t, J = 7.2 Hz, 2H), 3.66 (s, 3H), 3.87 (t, J = 7.1 Hz, 1H), 6.28 (d, J = 6.2 Hz, 1H), 8.1 (d, J = 6.2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 25.1, 30.8, 43.1, 51.9, 114.8, 139.2, 173.2, 180.3. GC–MS rt time 6.36 min, purity >99%, MS (EI) (m/z) 201 $[M]^+$, calcd 201. HRMS (m/z) 202.0534 $[M+H]^+$, calcd 202.0538 $C_8H_{12}NO_3S$, (m/z) 224.0352 $[M+Na]^+$, calcd 224.03573 $C_8H_{11}NNaO_3S$.

4.2.10. Methyl 4-(5-chloro-3-oxoisothiazol-2(3*H*)-yl)pentanoate (2g)

The product was obtained using procedure **1** starting from **10g**. Purification was performed using column chromatography with hexane/EtOAc 3:1 (v/v) as eluent. Yield 62%. Brown oil. R_f = 0.52 (CH_2Cl_2/MeOH 94:6). ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.60–1.90 (m, 4H), 2.35 (t, J = 7.2 Hz, 2H), 3.65 (s, 3H), 3.75 (t, J = 7.1 Hz, 2H), 6.32 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 21.9, 29.2, 33.5, 43.7, 51.9, 114.9, 146.4, 167.3, 173.6. GC–MS rt 7.58 min, purity >99%, MS (EI) (m/z) 249, calcd 249. HRMS (m/z) 250.0300 $[M+H]^+$, calcd 250.0305 $C_9H_{13}ClNO_3S$, (m/z) 272.0119 $[M+Na]^+$, calcd 272.0124 $C_9H_{12}ClNNaO_3S$.

4.2.11. Methyl 4-(3-oxoisothiazol-2(3*H*)-yl)pentanoate

After elution of **2g** the product was eluted from the column using hexane/EtOAc 1:8 (v/v) as eluent. Yield 12%. Brown oil. R_f = 0.39 (CH_2Cl_2/MeOH 94:6). The compound was not pure enough for biochemical characterization and was thus excluded from the study.

4.2.12. Methyl 6-(5-chloro-3-oxoisothiazol-2(3*H*)-yl)hexanoate (2h)

The product was obtained using procedure **1** starting from **10h**. Purification was performed using column chromatography with hexane/EtOAc 3:1 (v/v) as eluent. Yield 61%. Yellow oil. R_f = 0.56 (EtOAc/hexane 6:1). ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.30–1.42 (m, 2H), 1.56–1.71 (m, 4H), 2.29 (t, J = 7.2 Hz, 2H), 3.63 (s, 3H), 3.71 (t, J = 7.2 Hz, 2H), 6.24 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 24.4, 26.0, 29.4, 33.8, 43.5, 51.6, 114.8, 145.7, 166.9, 173.9. GC-MS RT 8.36 min, purity >99%, MS (EI) (m/z) 263, calcd 263. HRMS (m/z) 264.0456 $[M+H]^+$, calcd 264.04612 $C_{10}H_{15}ClNO_3S$.

4.2.13. Methyl 6-(5-chloro-3-oxoisothiazol-2(3*H*)-yl)hexanoate (3h)

After elution of **2h** product **3h** was eluted from the column using hexane/EtOAc 1:5 (v/v) as eluent. Yield 25%. Yellow oil. R_f = 0.30 (EtOAc/hexane 6:1). ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.30–1.74 (m, 6H), 2.29 (t, J = 7.2 Hz, 2H), 3.63 (s, 3H), 3.76 (t, J = 7.2 Hz, 2H), 6.23 (d, J = 6.5 Hz, 1H), 8.04 (d, J = 6.5 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 24.5, 26.1, 29.6, 33.9, 43.8, 51.7, 114.8, 139.0, 169.0, 174.0. GC–MS rt 7.98 min, purity >99%, MS (EI) (m/z) 229, calcd 229. HRMS (m/z) 230.0846 $[M+H]^+$, calcd 230.08509 $C_{10}H_{16}NO_3S$.

4.2.14. Ethyl 3-(5-chloro-3-oxoisothiazol-2(3*H*)-yl)propanoate (2i)

The product was obtained using procedure **1** starting from **10i**. Purification was performed using column chromatography with hexane/EtOAc 2:1 (v/v) as eluent. Yield 51%. Brown solid. Mp = 73.2 °C. R_f = 0.67 (CH_2Cl_2/MeOH 9:1). ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.26 (t, J = 7.2 Hz, 3H), 2.70 (t, J = 6.2 Hz, 2H), 4.00 (t, J = 6.2 Hz, 2H), 4.16 (q, J = 7.1, 7.3 Hz, 2H), 6.24 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.4, 34.2, 39.9, 61.4, 114.3, 147.1, 167.2, 171.4. GC–MS rt 6.43 min, purity >99%, MS (EI) (m/z) 235, calcd 235. HRMS (m/z) 236.0143 $[M+H]^+$, calcd 236.0148 $C_8H_{11}ClNO_3S$.

4.2.15. 5-Chloro-2-(3-chloro-4-fluorophenyl)isothiazol-3(2*H*)-one (2j)

The product was obtained using procedure **1** starting from **10j**. Purification was performed using column chromatography with ethyl acetate/hexanes 1:10 (v/v) as eluent. Yield 62%. White solid, Mp = 116.9 °C; R_f = 0.66 (ethyl acetate/cyclohexane 1:1). ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.36 (s, 1H), 7.10–7.21 (m, 1H), 7.43–7.36 (m, 1H), 7.63 (dd, J = 2.6, 6.5 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 114.8, 117.2, 117.6, 124.9, 125.0, 127.6, 146.5, 154.8, 159.8, 165.4. GC–MS purity >99%, retention time 7.8 min, MS (EI) (m/z) 263, calcd 263. HRMS (m/z) 263.9448 $[M+H]^+$, calcd 263.9453 $C_9H_5Cl_2FNOS$.

4.2.16. 2-(3-Chloro-4-fluorophenyl)isothiazol-3(2*H*)-one (3j)

After elution of **2j** product **3j** was eluted from the column using ethyl acetate/hexanes 1:2 as eluent. The product was obtained using. Yield 23%. Orange oil, R_f = 0.18 (ethyl acetate/cyclohexane 1:1). ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.33 (d, J = 6.3 Hz, 1H), 7.10–7.21 (m, 1H), 7.47–7.41 (m, 1H), 7.68 (dd, J = 2.6/6.5 Hz, 1H), 8.19 (d, J = 6.3 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 114.8, 117.0, 117.5, 125.0, 127.4, 140.1, 154.7, 159.7, 167.7. GC–MS purity >95%, retention time 7.7 min, MS (EI) (m/z) 229, calcd 229. HRMS (m/z) 229.9838 $[M+H]^+$, calcd 229.98427 $C_9H_6ClFNOS$.

4.2.17. Benzyl [2-(5-chloro-3-oxoisothiazol-2(3*H*)-yl)ethyl]carbamate (2k)

The product was obtained using procedure **1** starting from **10k**. Purification was performed using column chromatography with ethyl acetate/hexanes 3:1 (v/v) as eluent. Yield 29%. Yellow gum, R_f = 0.35 (ethyl acetate/hexane 3:1), ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.45–3.47 (m, 2H), 3.86 (t, J = 6 Hz, 2H), 5.01 (s, 2H), 6.25 (s, 1H), 7.35 (m, 5H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 41.1, 43.5, 66.9, 114.3, 128.1, 128.2, 128.6, 136.4, 152.4, 156.9, 160.8. HPLC purity >94%%, RT 12.3 min, (column C8 mobile phase H_20/CH_3CN/TFA

60/40/1). HRMS (*m*/*z*) 313.0414 $[M+H]^+$, calcd 313.0408 $C_{13}H_{14}ClN_2O_3S$. (*m*/*z*) 335.0233 $[M+Na]^+$, calcd 335.0228 $C_{13}H_{13}ClN_2NaO_3S$.

4.2.18. Methyl 3-(5-chloro-1-oxido-3-oxoisothiazol-2(3*H*)-yl)propanoate (4e)

Compound **2e** (1 mmol) was dissolved in CH_2Cl_2 (15 mL) and *m*-chloroperbenzoic acid was added in several portions. The mixture was stirred at room temperature for 16 h. Several crystals $Na_2S_2O_3{\cdot}5H_2O$ and H_2O (5 mL) were added and the mixture was stirred for 10 min. A saturated solution of $NaHCO_3$ (50 mL) was added, this mixture was stirred for 5 min and the phases were separated. The organic layer was washed with saturated $NaHCO_3$ (50 mL), brine (50 mL) and dried with Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography with ethyl acetate/hexanes 1:2 as eluent. Yield 50%, yellow oil, $R_f = 0.72$ (ethylacetate/hexane 10:1). ^1H NMR (200 MHz, CD_3OD) δ 2.70–2.77 (m, 2H), 3.68 (s, 3H), 3.99 (t, $J = 6.8$ Hz, 2H), 6.98 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 34.5, 38.3, 52.4, 125.4, 158.6, 166.5, 173.7. GC–MS rt 6.1 min, purity >99%, MS (ESI) (*m*/*z*) 206, calcd 206 (M−OMe). HRMS (*m*/*z*) 237.9936 $[M+H]^+$, calcd 237.9941 $C_7H_9ClNO_4S$.

4.2.19. Methyl 3-[(5-chloroisothiazol-3-yl)amino]propanoate (5e)

Compound **2e** (0.74 mmol) was dissolved in $POCl_3$ (1.5 mL) and stirred overnight at room temperature. The next morning an orange-brown precipitate was observed. Isopropylether (20 mL) was added and the precipitate intensified. The suspension was centrifuged (4000 rpm, 10 min), the supernatant was discarded and the residue washed with isopropylether. Subsequently, the residue was suspended in CH_3CN (10 mL), cooled to 0 °C and NH_3 gas was bubbled through the solution for 30 min. The red-white suspension turned into a brown solution with a white precipitate. The suspension was filtered and the filtrate was concentrated under reduced pressure and purified using column chromatography ethyl acetate/hexanes 1:6 as eluent. Yield 44% Yellow solid, Mp = 73.2 °C, $R_f = 0.33$ (ethylacetate/hexane 3:1), ^1H NMR (200 MHz, $CDCl_3$) δ 2.64 (t, $J = 6.2$ Hz, 2H), 3.61 (t, $J = 5.9$ Hz, 2H), 3.68 (s, 3H), 6.34 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 33.9, 38.5, 51.9, 112.3, 151.8 163.6, 173.2. GC–MS rt 5.9 min, purity >99%, MS (EI) (*m*/*z*) 220, calcd 220. HRMS (*m*/*z*) 221.0146 $[M+H]^+$, calcd 221.0152 $C_7H_{10}ClN_2O_2S$.

4.2.20. Methyl 3-[4-{[(benzyloxy)carbonyl]amino}-5-chloro-3-oxoisothiazol-2(3*H*)-yl]propanoate (7e)

The product was obtained using procedure **1** starting from **9**. Purification was performed using column chromatography with ethyl acetate/hexanes 1:1 (v/v) as eluent. Yield 70%. Off-white gum. $R_f = 0.38$ (ethyl acetate/cyclohexane 2:1). ^1H NMR (200 MHz, $CDCl_3$) δ 2.67 (m, 2H), 3.70 (s, 3H), 3.96 (m, 2H), 5.15 (s, 2H), 7.34 (m, 5H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 33.6, 40.5, 52.2, 67.9, 119.1, 128.3, 128.4, 128.6, 135.7 153.2, 163.0 171.6. HPLC purity >99%. HRMS (*m*/*z*) 371.0463 $[M+H]^+$, calcd 371.0469 $C_{15}H_{16}ClN_2O_5S$.

4.3. Modeling

Molecular dockings were performed using the program Molegro Virtural Docker 2007 (MVD) from Molegro ApS, Aarhus, Denmark. The crystal structure from PCAF (PDB entry 1CM0) chain B,[23] was prepared for dockings using the default settings in MVD. The water molecules were removed before docking. The ligands were geometry optimized using HYPERCHEM 7.5 Professional (Hypercube, Inc) using molecular mechanics with the Amber force field and Polar-Ribiere (conjugate gradient) algorithm. The optimized ligands were prepared for docking using the default settings in MVD.[33] The 5-chloroisothiazolones, isothiazolones and 5-chloro-isothiazolones-1-oxide were docked with a distance constraint of 6 Å between the Cys 574 thiol and the carbon atom 5 in the isothiazolone scaffold. Docking solutions were calculated within a sphere of 15 Å from the Cys 574 thiol. Docking solutions were selected based on the MOLDOCKSCORE[33] and the docking solutions were evaluated manually and the amino acid side chain in a radius of 10 Å from the ligand were energy minimized, followed by energy minimization of the ligand.

4.4. Enzyme inhibition studies

A fluorescent histone acetyl transferase assay described by Trievel et al.[22] was used for enzyme inhibition studies. The human recombinant histone acetyl transferases PCAF (p300/CREB-binding protein Associated Factor) was purchased from Biomol International. The histone H3 peptide (Ac-QTARKSTGGKAPRKQLATK-NH_2) was purchased from Pepscan (Lelystad, NL) (purity >98%, *m*/*z* 1034.9 $[M+2H]^{2+}$ calcd 1035.2).The other chemicals were purchased from Sigma–Aldrich. Detail on the enzyme inhibition studies can be found in the Supplementary data.

4.5. Cell growth inhibition

4.5.1. Cell culture

The growth inhibition of the human cancer cell lines A2780 (ovarian), WiDr (colon) and HEP G2 (liver) and the human embryonic cell line 293 (kidney) was studied. The cells were maintained in Dulbecco's modified Eagles Medium with 10% heat-inactivated fetal calf serum, 50 IU/mL penicillin and 50 mg/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator. All cell culture reagents were purchased from invitrogen.

4.5.2. Cell proliferation

Cell proliferation was measured by a Crystal Violet assay. For this assay, cells were seeded at 5000 cells per well into 96-well plates, grown for 24 h, and treated for an additional 48 h with the different inhibitors. After this, the medium was aspirated and the cells were fixed with 50 μL 1% crystal violet in 70% ethanol for 30 min. Subsequently, the cells were washed with water and the staining was solubilized by addition of 100 μL 1% SDS in water. The plates were read at 550 nm. A blank extinction value in which no cells were seeded was subtracted from all determinations and cell growth with no inhibitor present was set to 100%. All concentrations were tested in 8-fold on one plate and the GI_{50} values of most potent inhibitors were measured again on a new plate.

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Supplementary data

The synthesis of the precursors of the final products and their characterizations can be found in the supporting information numbered **8b**–**f**, **9**, **10b**–**k**. Details on the enzyme inhibition studies can also be found. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.12.008.

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