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Original article

Reactivity of isothiazolones and isothiazolone-1-oxides in the inhibition of the PCAF histone acetyltransferase

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ABSTRACT

Development of small molecule inhibitors of the histone acetyltransferase p300/CBP associated factor (PCAF) is relevant for oncology. The inhibition of the enzyme PCAF and proliferation of the cancer cell line HEP G2 by a series of 5-chloroisothiazolones was compared to a series of 5-chloroisothiazolone-1-oxides. The PCAF inhibitory potency of 5-chloroisothiazolones and 5-chloroisothiazolone-1-oxides is influenced by substitution in the 4-position. A study on the reactivity of the HAT inhibitors towards thiols and thiolates indicates that 5-chloroisothiazolones reacted quickly with propane-1-thiolate to provide many products, whereas 5-chloroisothiazolone-1-oxides provide only one defined product. Growth inhibition studies indicate that 5-chloroisothiazolones inhibit proliferation of HEP G2 cells at concentrations between 8.6 and 24 μM, whereas 5-chloroisothiazolone-1-oxides required higher concentrations or showed no inhibition.

1. Introduction

Histone acetylation and deacetylation play a crucial role in regulation of gene transcription in eukaryotes [1]. These histone modifications are regulated by two classes of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs), which respectively catalyze the addition to and the removal of acetyl groups on specific lysine residues [2]. Small molecule inhibitors are useful tools to study the functions of these enzymes and have potential therapeutic applications [3,4].

HATs are grouped into different families according to their sequence similarity [5]. 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) [6]. The members of this family play a key role in endothelial growth factor (EGF) mediated gene transcription and in cell cycle progression and have therefore been recognized as potential anticancer targets [5,7–9]. Furthermore, deregulation of the activities of GNAT and p300 family HATs plays an important role in human immunodeficiency virus (HIV) and chronic obstructive pulmonary disease (COPD) [10,11].

Despite significant efforts, very few small molecule and cell-permeable inhibitors for GCN5 and PCAF have been described until now. Potent and selective bisubstrate inhibitors for p300 and PCAF have been reported but their lack of cell-permeability limits their application [12]. The natural products curcumin [13], garcinol [14] and anacardic acid [15] show HAT inhibitory activity, however their potency is low. A promising class of inhibitors is the isothiazolones that inhibit HATs with IC_{50}'s in the micromolar range [16–18].

The PCAF inhibition of isothiazolones is expected to derive from their reactivity towards the enzyme's active site thiol. The reaction of isothiazolones with a thiolate involves nucleophilic attack at the sulfur atom with concomitant cleavage of the S–N bond to give a ring opened product that can react further [19]. For 5-chloroisothiazolone-1-oxides the reactivity towards thiolates is unknown.

Previously, we have reported a significant difference in PCAF HAT inhibition between N-aliphatic substituted 5-chloroisothiazolones and isothiazolones [16]. The most potent derivative is denoted isothiazolone A (Fig. 1). We also found that PCAF is inhibited by the 5-chloroisothiazolone-1-oxide denoted isothiazolone oxide A (Fig. 1). Furthermore, we have demonstrated that 5-chloroisothiazolones inhibit the growth of cancer cell lines in the micromolar range, whereas isothiazolones showed no inhibition at 10 μM.

In this study we investigated synthetic modifications of 5-chloroisothiazolones and 5-chloroisothiazolone-1-oxides and studied their reactivity to thiols and thiolates using HPLC and NMR. We studied also their inhibition of the enzyme PCAF and inhibition of cell proliferation. Chlorine and methyl substituents were

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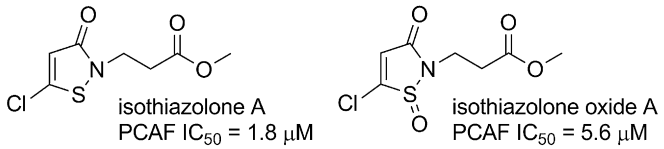


Fig. 1. Inhibitors of the HAT PCAF with an isothiazolone or isothiazolone-1-oxide core structure.

introduced in the 4-position of the 5-chloroisothiazolone and 5-chloroisothiazolone-1-oxide core in order to study the effect of substituents on the inhibition of the enzyme PCAF. Finally, the growth inhibition of HEP G2 liver cancer cells upon treatment with 5-chloroisothiazolones and 5-chloroisothiazolone-1-oxides was investigated to explore the toxicity of these molecules.

2. Chemistry

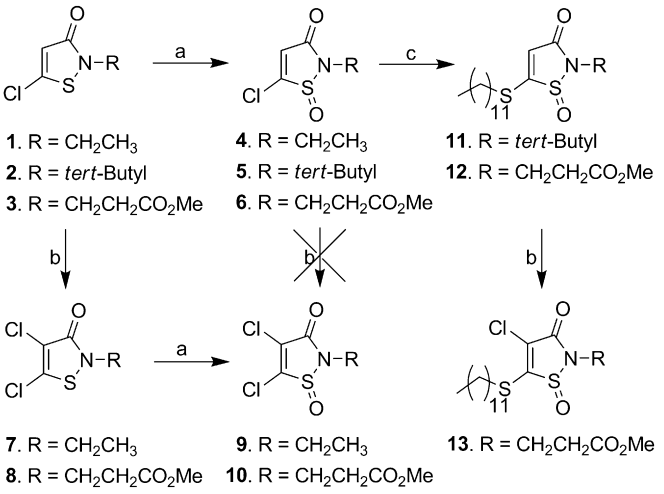
2.1. Synthesis of isothiazolones and isothiazolone-1-oxides

A series of 5-chloroisothiazolones and 5-chloroisothiazolone-1-oxides was synthesized in order to explore synthetic modifications of the isothiazolone ring and their effects on inhibition of the enzyme PCAF and cell proliferation [16,20]. Synthesis was performed according to previously published procedures. Amines were reacted with 3,3′-dithiobispropionyl chloride to yield the corresponding 3,3′-dithiobispropionic amides. The 3,3′-dithiobispropionic amides were treated with 3 equivalents sulfuryl chloride in dichloromethane. This provided reaction mixtures in which the 5-chloroisothiazolones were the main products (40–60%) and the isothiazolones the minor product (10–20%). According to the literature, 4,5-dichloroisothiazolones **7** and **8** can be obtained in one step from 3,3′-dithiobispropionic amides by treatment with 5 equivalents of sulfuryl chloride in yields between 10% and 40% [20]. We hypothesized that chlorination of the purified 5-chloroisothiazolones in the 4-position would provide the 4,5-dichloroisothiazolones with higher yields. Treatment of **1** and **3** with sulfuryl chloride gave the 4,5-dichloroisothiazolones **7** and **8** in yields of 55% and 90%, respectively (Scheme 1). Compound **2** was synthesized according to the procedure described by Yue et al. [21]. However, product **2** was obtained with a yield of 10% instead of the reported 30–70%. The lower yield may be explained by the steric hindrance for formation of the S–N bond when the N is *tert*-butyl substituted.

The 5-chloroisothiazolones were oxidized by *m*CPBA to yield the corresponding 5-chloroisothiazolone-1-oxides **4**–**6** as racemic mixtures in yields between 60% and 80% after purification. Combs et al. demonstrated that comparable S-oxides can be resolved using chiral HPLC and that the stereochemistry is stable [22]. However, attempts to separate the enantiomers of 5-chloroisothiazolone-1-oxides **6** using chiral HPLC failed.

Attempts were made to chlorinate the 5-chloroisothiazolone-1-oxides **4** and **6** in the 4-position using sulfuryl chloride. However, overnight treatment with 3 equivalents of sulfuryl chloride at room temperature did not show conversion of the starting materials into the desired products **9** and **10**. Apparently, it is not possible to chlorinate 5-chloroisothiazolone-1-oxides in the 4-position under these conditions. Compounds **9** and **10** were obtained via oxidation of **7** and **8** using *m*CPBA in yields around 80%.

Thiol substitution on the 5-chloroisothiazolone-1-oxides **5** and **6** was studied using dodecane-1-thiol as a reagent. The thiol (–SH) did not react with the 5-chloroisothiazolone-1-oxides, whereas a very quick reaction (<30 min) with the thiolate ($-S^-$) was observed. Products **11** and **12** were obtained by reaction of **5** and **6** with dodecane-1-thiol in the presence of triethylamine in yields around 60% after purification. Compound **12** was chlorinated in the 4-position using sulfuryl chloride as a reagent to give **13** in 58% yield.

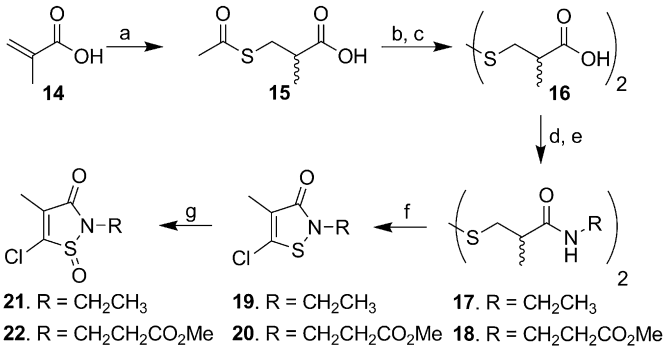


Scheme 1. Reagents and conditions: (a) mCPBA, CH_2Cl_2, (b) SO_2Cl_2, CH_2Cl_2, (c) CH_3–$(CH_2)_{11}$–SH, Et_3N, CH_2Cl_2.

5-Chloro-4-methylisothiazolones **19** and **20** were synthesized using the procedure shown in Scheme 2 [19,23]. Methacrylic acid (**14**) was subjected to Michael addition with thioacetic acid to give the 3-(acetylthio)-2-methylpropanoic acid (**15**) as a racemic mixture in 50% yield. Deacetylation was achieved by treatment with 6 N HCl, and the resulting thiol was oxidized using H_2O_2 to yield the 3,3′-dithiobis(2-methylpropanoic acid) (**16**) that was pure enough to continue to the next step. The diacid **16** was converted to the diacylchloride and coupled to different amines to give the crude 3,3′-dithiobispropionic amides **17** and **18** in yields around 30% over 3 steps. The crude 3,3′-dithiobispropionic amides were treated with 3 equivalents of sulfuryl chloride at 0 °C in dichloromethane to give the 5-chloro-4-methylisothiazolones **19** and **20** in yields of 38% and 52%, respectively. Compounds **19** and **20** were oxidized by mCPBA to the corresponding 1-oxide derivates **21** and **22** in yields around 75%.

2.2. Reactivity of isothiazolones and isothiazolone-1-oxides

The biological activity of isothiazolones and their derivatives originates from both their binding to proteins and their reactivity



Scheme 2. Reagents and conditions: (a) thioacetic acid, cyclohexane, reflux, (b) 6 N HCl in H_2O, reflux (c) aqueous 35% H_2O_2, (d) $SOCl_2$, reflux, (e) R–NH_2, Et_3N, CH_2Cl_2, (f) SO_2Cl_2, CH_2Cl_2 (g) mCPBA, CH_2Cl_2.

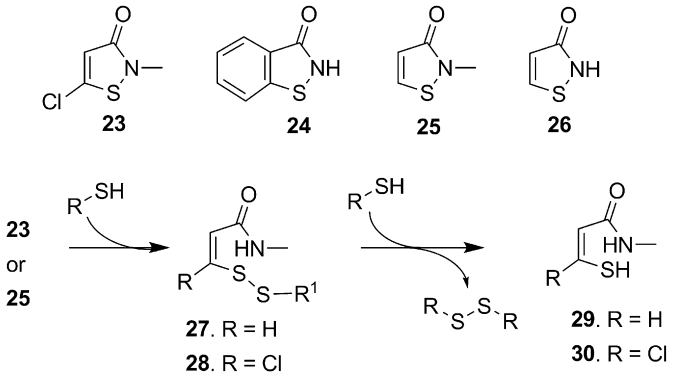


Fig. 2. Reactivity of the isothiazolone core.

towards thiolates [24,25]. It has been described that isothiazolones react with thiolates on the sulfur atom, with concomitant cleavage of the S–N bond. A study on N-methylisothiazolone **25**, with N-Acetyl-Cys as thiolate, showed formation of intermediate **27** (Fig. 2) [19]. The reaction between 5-chloro-N-methylchloroisothiazolone **23** is expected to proceed through the same mechanism, however intermediate **28** has not been identified. This might be due to the increased reactivity of intermediate **28** due to the presence of the chlorine atom. Another line of evidence for this inhibitory mechanism has been provided by a study on inhibition of $p56^{lck}$ tyrosine kinase by isothiazolones [25]. Morley et al. have studied the reactivity of (5-chloro)isothiazolones towards 2-methyl-2-propanethiol at pH 4 in aqueous environment. They have found kinetic rate constants in the order **23** > **24** > **25** > **26** (Fig. 2) [26].

The reactivity of 5-chloroisothiazolones and 5-chloroisothiazolone-1-oxides towards thiols and thiolates was studied using comparable methods as published previously [27]. HPLC analysis showed that **3** did not react with propane-1-thiol in acetonitrile as a solvent after 24 h treatment at room temperature (HPLC chromatograms are shown in the Supporting information). In contrast, treatment with sodium propane-1-thiolate in acetonitrile resulted in a quick conversion into many new products. Treatment with 0.1 equivalent thiolate resulted in 10% conversion of the starting material. Treatment with 0.5 equivalents resulted in 50% conversion and many new products were observed. After treatment with 1.0 equivalent thiolate the starting material disappeared and many new peaks were observed. These results show that 5-chloroisothiazolones do not react with thiols, whereas they react quickly with thiolates providing many products that could not be identified.

The thiol reactivity of 5-chloroisothiazolone-1-oxide **6** was investigated. No reaction was observed between **6** and propane-1-thiol after treatment overnight. On the contrary, **6** reacted quickly with sodium propan-1-thiolate in acetonitrile to provide one new product (HPLC chromatograms are shown in the Supporting information). Treatment with 0.1 equivalent resulted in one new product with a conversion of about 10%. Treatment with 0.5 equivalent resulted in about 50% conversion into one new product. After treatment with 1.0 equivalent, the starting material was completely converted into one new product. NMR data showed that this product derives from addition-elimination in the 5-position (NMR data can be found in the Supporting information). Treatment of 5-chloroisothiazolon-1-oxide with more than 1.0 equivalent of sodium propan-1-thiolate provided substitution in the 5-position, followed by substitution in the 4-position. These data show that 5-chloroisothiazolone-1-oxides do not react with thiols, whereas they react quickly with thiolates through addition-elimination in the 5-position.

3. Pharmacology

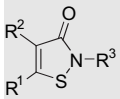
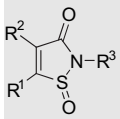
3.1. Histone acetyltransferase inhibition

Inhibition of the HAT PCAF by 5-chloroisothiazolones and 5-chloroisothiazolone-1-oxides was investigated in order to explore the binding properties of these compounds. The PCAF HAT inhibition studies were performed using a fluorescent assay as described previously [16,28]. 5-Chloroisothiazolones **1** and **3** showed PCAF inhibition with IC_{50} values of 3.0 and 1.8 μM, respectively (Table 1). The effects of substitutions in the 5-chloroisothiazolone 4-position on PCAF inhibition were studied. Introduction of a chlorine in the 4-position provided compounds **7** and **8** with an IC_{50} of 2.4 and 2.6 μM, whereas introduction of a methyl group in the 4-position provided compounds **19** and **20** with less than 50% inhibition at 10 μM.

The inhibitory properties of these molecules derive both from their binding to the enzyme active site and from their chemical reactivity. Chloro- or methyl substitution in the 4-position of 5-chloroisothiazolones could change the binding configuration to the active site of the enzyme and thus influence the inhibitory properties.

Substitution of the 5-chloroisothiazolones in their 4-position changes their reactivity. The methyl group is an electron donating

Table 1
Inhibition of HAT PCAF and proliferation of cell lines by 5-chloroisothiazolinone and isothiazolone-1-oxide.

	Compound	R^1	R^2	R^3	PCAF inhibition (IC_{50} μM)[a]	HEP G2 (IC_{50} μM)[b]
	**1**	Cl	H	Ethyl	3.0 ± 0.3	12 ± 0.5
	7	Cl	Cl	Ethyl	2.4 ± 0.1	12 ± 2
	19	Cl	Methyl	Ethyl	>10	9.1 ± 0.9
	3	Cl	H	$-(CH_2)_2CO_2Me$	1.8 ± 0.2	24 ± 3
	8	Cl	Cl	$-CH_2)_2CO_2Me$	2.6 ± 0.6	16 ± 3
	20	Cl	Methyl	$-(CH_2)_2CO_2Me$	>10	8.6 ± 1
	**4**	Cl	H	Ethyl	>10	>100
	9	Cl	Cl	Ethyl	>10	>100
	21	Cl	Methyl	Ethyl	>10	>100
	6	Cl	H	$-(CH_2)_2CO_2Me$	5.6 ± 0.2	>100
	10	Cl	Cl	$-(CH_2)_2CO_2Me$	>10	>100
	22	Cl	Methyl	$-(CH_2)_2CO_2Me$	>10	>100
	5	Cl	H	*tert*-Butyl	>10	27 ± 9
	11	$-S(CH)_{11}CH_3$	H	*tert*-Butyl	>10	>50
	12	$-S(CH)_{11}CH_3$	H	$-(CH_2)_2CO_2Me$	>10	>100
	13	$-S(CH)_{11}CH_3$	Cl	$-(CH_2)_2CO_2Me$	>10	>100

[a] Inhibition concentration 50% (IC_{50}) determination $n = 3$.
[b] Growth inhibition 50% determinations $n = 8$.

group (EDG) due to inductive effects, whereas the chlorine is a net electron-withdrawing group (EWG) due to inductive effects partially counteracted by an electron donating contribution through resonance. Introduction of an EDG like a methyl group (**20**) causes loss of potency, which suggests that an EDG in the 4-position adds electron density to the isothiazolone ring and thus stabilizes it for nucleophilic attack. On the contrary, the introduction of an EWG like a chlorine atom (**8**) does not affect the inhibitory properties. This datum suggests that the isothiazolone reactivity on the 1-position towards thiolates is already at its maximum and cannot be increased further by an EWG.

The effect of substitution in the 4-position of 5-chloroisothiazolone-1-oxides on inhibition of PCAF activity was studied. The compounds were tested as racemic mixtures. Compound **4**, with an N-ethyl substitution, showed less than 50% inhibition at 10 μM. Introduction of either chlorine (**9**) or methyl group (**21**) in the 4-position, provided compounds that did not show any inhibition at 10 μM. Compound **6** with a methyl ester in the N-substitution showed PCAF inhibition with an IC_{50} value of 5.6 μM. Introduction of either a chlorine (**10**) or a methyl group (**22**) provided compounds that showed less than 50% PCAF inhibition at 10 μM. These differences in binding could derive from changes in binding to the enzyme active site as well as from changes in reactivity.

The 5-dodecylthioisothiazolone-1-oxides **11** and **12** and 5-dodecylthio-4-chloroisothiazolone-1-oxide **13** showed no detectable PCAF inhibition at 10 μM. The lack of activity of these compounds might derive both from their binding properties to the enzyme active site as well as their reactivity towards thiolates.

3.2. Growth inhibition

Growth inhibition of cancer cells was studied in order to explore how modifications of the isothiazolone core influence the inhibition of cell proliferation. The growth inhibition of the human cancer cell line HEP G2 (liver cancer) was studied using a crystal violet assay as described previously [16]. The 5-chloroisothiazolones inhibited the growth of HEP G2 cells with IC_{50} values between 8.6 and 24 μM (Table 1). The differences in PCAF inhibitory potency are not reflected in the inhibition of cell proliferation. Other mechanisms might be involved in the inhibition of cell proliferation by these compounds like for example binding to other proteins or reactions with cellular glutathione. Western blot on inhibition of histone acetylation upon treatment of this cell line with the inhibitors is required to investigate if the molecules inhibit histone acetylation in cell-based assays.

5-Chloroisothiazolone-1-oxides showed less than 50% inhibition of cell proliferation at 100 μM, with the exception of compound **5**. In general, 5-chloroisothiazolone-1-oxides are less lipophilic then 5-chloroisothiazolones, which might hamper their cellular permeability. The partition coefficient (log *P*) for isothiazolone-1-oxide **6** was determined to be 0.08, whereas for isothiazolone **3** a log *P* value of 0.69 was measured. The low log *P* value of isothiazolone-1-oxide **6** might hamper the inhibition of cell proliferation.

4. Conclusion

A series of 5-chloroisothiazolones and 5-chloroisothiazolone-1-oxides with chlorine and methyl substitutions in the 4-position was synthesized. 5-Chloroisothiazolones were readily chlorinated in the 4-position by sulfuryl chloride, whereas the 5-chloroisothiazolone-1-oxides could not be chlorinated in the 4-position under the applied conditions. 5-Chloroisothiazolones do not react with thiols whereas they react quickly with thiolates. Upon reaction, multiple products were observed which hampers the elucidation of the reaction mechanism. 5-Chloroisothiazolone-1-oxides do not react with thiols, whereas they react quickly with thiolates in the 5-position. The reaction occurs via an addition-elimination mechanism to give 5-alkylthioisothiazolone-1-oxides. The resulting 5-alkylthioisothiazolone-1-oxide was chlorinated in the 4-position using sulfuryl chloride.

N-aliphatic substituted 5-chloroisothiazolones (**1** and **3**) showed PCAF inhibition at micromolar concentrations (2–3 μM). Chlorine substitution in the 4-position yielded products which retained PCAF inhibition, whereas methyl substitution provided compounds that showed no inhibition at 10 μM. 5-Chloroisothiazolone-1-oxide **6** with a methyl ester in the N-substitution showed an IC_{50} of 5.6 μM whereas **4** showed an IC_{50} higher than 10 μM. These data indicate that the methyl ester contributes to PCAF inhibition. Methyl or chlorine substitution in the 4-position and thiol substitution in the 5-position of 5-chloroisothiazolone-1-oxides provided compounds with IC_{50} values higher than 10 μM. 5-Chloroisothiazolones inhibited the growth of HEP G2 cancer cells in concentrations between 8.6 and 24 μM, whereas little or no growth inhibition was observed for 5-chloroisothiazolone-1-oxides.

5. Experimental protocols

5.1. General procedures

Chemicals were obtained from commercial suppliers (Sigma–Aldrich, Acros Organics) and in most cases used without further purification. Dichloromethane was dried by distillation over CaH_2 before use. Aluminum sheets of Silica Gel 60 F254 were used for Thin-layer chromatography (TLC). Spots were visualized under ultraviolet light or stained with $KMnO_4$ solution. MP Ecochrom Silica Gel 32–63 60 Å was used for column chromatography. Melting points were determined on an Electrothermal digital melting point apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini-200 (50.32 MHz) spectrometer. Chemical shift values are reported relative to residual solvent peaks (CD_3OD, ^1H $\delta = 3.31$, ^{13}C $\delta = 49.00$ or $CHCl_3$, ^1H $\delta = 7.26$, ^{13}C $\delta = 77.16$). The coupling constants (*J*) are reported in Hertz (Hz). ^{13}C spectra were recorded using the attached proton test (APT) pulse sequence. HPLC analyses were performed with a Waters 510 pump, equipped with an ISCO 2360 gradient programmer and a Waters 486 UV detector (254 nm). The column was a 25 cm × 4.6 mm (5 μm) Discovery C18, with a flow of 0.5 mL/min. Electrospray ionization mass spectra (ESI-MS) were recorded on an Applied Biosystems/SCIEX API3000-triple quadrupole mass spectrometer. High-resolution mass spectra (HR-MS) were recorded using a flow injection method on an LTQ-Orbitrap XL mass spectrometer (Thermo Electron, Bremen, Germany) with a resolution of 60,000 at *m*/*z* 400. Internal recalibration in real time was performed with protonated testosterone (lock mass *m*/*z* = 289.2162).

5.2. Synthetic procedure 1

The starting material (1.0 mmol) was dissolved in dichloromethane (10 mL), and *m*-chloroperbenzoic acid (70%) (1.2 mmol, 0.29 g) was added in several portions over 20 min. The mixture was stirred for 3 h at room temperature. A few milligrams of sodium metabisulfite were added to quench the excess *m*CPBA, and the mixture was stirred for 5 min. The solvent was evaporated under reduced pressure, and the residue was dissolved in EtOAc (15 mL), and extracted with saturated $NaHCO_3$ solution (3 × 15 mL) and brine (1 × 15 mL). The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under

reduced pressure. The crude product was purified by column chromatography.

5.3. Synthetic procedure 2

The starting material (1.0 mmol) was dissolved in dichloromethane (10 mL). Sulfuryl chloride (3.0 mmol, 0.24 mL) was added slowly and the solution was stirred overnight at room temperature. H_2O (1.0 mL) was added to the solution, and the mixture was stirred for 5 min. The mixture was extracted with water (3 × 10 mL) and brine (1 × 10 mL), dried over Na_2SO_4, and filtered. The solvent was evaporated under reduced pressure, and the product was purified by column chromatography.

5.4. Synthetic procedure 3

The dithiobispropanamide (1.0 mmol) was dissolved in dry dichloromethane (5 mL) and cooled to 0 °C. Sulfuryl chloride (3.0 mmol, 0.24 mL) was added dropwise, and the mixture was stirred for 2 h at room temperature. H_2O (1.0 mL) was added, and the mixture was stirred for 5 min. Subsequently, the mixture was extracted with water (3 × 10 mL) and brine (1 × 10 mL), dried over Na_2SO_4, and filtered. The solvent was evaporated under reduced pressure, and the product was purified by column chromatography.

5.5. 5-Chloro-2-ethylisothiazol-3(2H)-one-1-oxide (**4**)

The product was obtained using procedure 1 starting from **1** and purified using column chromatography with EtOAc/Hex 1:2 (v/v) as eluent. The compound was obtained as a yellow oil (100 mg, 56%): $R_f = 0.38$ (EtOAc/Hex, 1:2); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.33$ (t, $J = 7.1$ Hz, 3H), 3.65–3.89 (m, 2H), 6.67 ppm (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 14.8$, 36.8, 124.4, 156.9, 169.4 ppm; HPLC: purity >98%, RT = 9.6 min, mobile phase: H_2O:CH_3CN:TFA 60:40:0.1; HRMS: m/z $[M+H]^+$ calcd for $C_5H_7^{35}ClNO_2S$: 179.9988, found: 179.9876.

5.6. 2-Tert-butyl-5-chloroisothiazol-3(2H)-one 1-oxide (**5**)

The product was obtained using procedure 1 starting from **2** and purified using column chromatography with EtOAc/Hex 1:2 (v/v) as eluent. The compound was obtained as a colorless oil (62 mg, 30%): $R_f = 0.6$ (EtOAc/Hex, 1:1); ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.63 (s, 9H), 6.55 ppm (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$): δ 29.4, 59.7, 125.8, 136.3, 165.6 ppm; HPLC: purity 98%, RT = 11.1 min, mobile phase: H_2O:CH_3CN:TFA 50:50:0.1; MS (ES, 70 eV): m/z 207.9 $[M+H]^+$.

5.7. 4,5-Dichloro-2-ethylisothiazol-3(2H)-one (**7**)

The product was obtained using procedure 2 starting from **1** and purified using column chromatography with EtOAc/Hex 1:1 (v/v) as eluent. The compound was obtained as a yellow oil (109 mg, 55%): $R_f = 0.51$(EtOAc/Hex 1:2); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.32$ (t, $J = 7.2$ Hz, 3H), 3.86 ppm (q, $J = 7.0$ Hz, 2H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 14.7$, 40.4, 138.4, 161.8, 168.0 ppm; HPLC: purity 99%, RT = 12.2 min, mobile phase: H_2O:CH_3CN:TFA 60:40:0.1; HRMS: m/z $[M+H]^+$ calcd for $C_5H_6^{35}Cl_2NOS$: 197.9542, found: 197.9543.

5.8. Methyl 3-(4,5-dichloro-3-oxoisothiazol-2(3H)-yl) propanoate (**8**)

The product was obtained using procedure 2 starting from **3** and was purified using column chromatography with EtOAc/Hex 1:1 (v/v) as eluent. The compound was obtained as a yellow oil (233 mg, 91%): $R_f = 0.40$ (EtOAc/Hex 1:1); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 2.75$ (t, $J = 6.1$ Hz, 2H), 3.71 (s, 3H), 4.07 ppm (t, $J = 6.0$, Hz 2H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 33.6$, 41.0, 52.3, 114.5, 139.7, 162.1, 171.7 ppm; HPLC: purity >98%, RT = 10.5 min, mobile phase: H_2O:CH_3CN:TFA 50:50:0.1; HRMS: m/z $[M+H]^+$ calcd for $C_7H_8^{35}Cl_2NO_3S$: 255.9597, found: 255.9596.

5.9. 4,5-Dichloro-2-ethylisothiazol-3(2H)-one-1-oxide (**9**)

The product was obtained using procedure 1 starting from **7** and purified using column chromatography with EtOAc/Hexane 1:3 (v/v) as eluent. The compound was obtained as a pale yellow solid (165 mg, 77%): $R_f = 0.58$ (EtOAc/Hex, 1:2); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.32$–1.40 (m, 3H), 3.72–3.91 (m, 2H) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 14.7$, 38.0, 131.0, 148.7, 159.8 ppm; HPLC: purity 98%, RT = 12.2 min, mobile phase: H_2O:CH_3CN:TFA 50:50:0.1; HRMS: m/z $[M+H]^+$ calcd for $C_5H_6^{35}Cl_2NO_2S$: 213.9491, found: 213.9491.

5.10. Methyl 3-(4,5-dichloro-1-oxido-3-oxoisothiazol-2(3H)-yl)propanoate (**10**)

The product was obtained using procedure 1 starting from **8** and purified using column chromatography with EtOAc/Hex 1:2 (v/v) as eluent. The compound was obtained as a yellow oil (212.2 mg, 78%): $R_f = 0.48$ (EtOAc/Hex, 1:1); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 2.71$–2.81 (m, 2H), 3.70 (s, 3H), 4.05 ppm (t, $J = 6.5$ Hz, 2H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 33.4$, 38.2, 52.3, 130.6, 149.4, 160.3, 171.1 ppm; HPLC: purity 98%, RT = 11.5 min, mobile phase: H_2O:CH_3CN:TFA 50:50:0.1; HRMS: m/z $[M+H]^+$ calcd for $C_7H_8^{35}Cl_2NO_4S$: 271.9546, found: 271.9544.

5.11. 2-Tert-butyl-5-(dodecylthio)isothiazol-3(2H)-one-1-oxide (**11**)

Dodecanethiol (1.2 mmol, 0.28 mL) was added to a solution of 2-tert-butyl-5-chloroisothiazol-3(2*H*)-one-1-oxide (1.2 mmol, 0.25 mg) in dry dichloromethane at room temperature. Triethylamine (1.2 mmol, 0.15 μL) was added dropwise, and the mixture was stirred for 3 h at room temperature. Dichloromethane (50.0 mL) was added, and the organic layer was extracted with H_2O (3 × 50 mL) and brine (1 × 50 mL), and dried with Na_2SO_4. The solvent was evaporated under reduced pressure, and the residue was purified by column chromatography using EtOAc/Hex 1:25 (v/v) as eluent. The compound was obtained as a white solid (241 mg, 57%): $R_f = 0.42$ (EtOAc/Hex, 1:4); mp: 62 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 0.87$ (t, $J = 6.5$ Hz, 3H), 1.20–1.50 (m, 18H), 1.62 (s, 9H), 1.65–1.85 (m, 2H), 2.98 (t, $J = 7.3$ Hz, 2H), 6.07 ppm (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 14.3$, 22.8, 28.3, 28.9, 29.1, 29.3, 29.5, 29.6, 29.7, 32.0, 33.6, 58.9, 118.0, 165.5, 167.2 ppm. MS (ESI): m/z 374.1 $[M+H]^+$.

5.12. Methyl 3-[5-(dodecylthio)-1-oxido-3-oxoisothiazol-2(3H)-yl] propanoate (**12**)

The product was obtained using the same procedure as for compound **11** starting from dodecanethiol and methyl 3-(5-chloro-1-oxido-3-oxoisothiazol-2(3*H*)-yl)propanoate (**6**). The compound was purified using column chromatography with EtOAc/Hex (v/v) 1:10 as eluent and was obtained as a white solid (335 mg, 69%): $R_f = 0.39$ (EtOAc/Hex, 1:2); mp: 55.7 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 0.86$ (t, $J = 6.5$ Hz, 3H), 1.20–1.50 (m, 18H), 1.65–1.85 (m, 2H), 2.72 (dt, $J = 4.0$, 7.1 Hz, 2H), 3.01 (t, $J = 7.3$ Hz, 2H), 3.69 (s, 3H), 3.98 (t, $J = 6.8$ Hz, 2H) 6.15 ppm (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 14.23$, 22.8, 28.2, 28.8, 29.1, 29.4, 29.5, 29.6, 29.7, 32.0, 33.8, 33.9,

37.2, 52.1, 115.8, 166.4, 168.3, 171.4 ppm; HRMS: m/z $[M+H]^+$ calcd for $C_{19}H_{34}NO_4S_2$: 404.1924, found: 404.1925.

5.13. Methyl 3-[4-chloro-5-(dodecylthio)-1-oxido-3-oxoisothiazol-2(3H)-yl]propanoate (**13**)

The product was obtained using procedure 2 starting from **12** and was purified using column chromatography with EtOAc/Hex 1:10 (v/v) as eluent. The compound was obtained as a colorless oil (254 mg, 58%) (the starting material **12** was isolated in a yield of 31%): $R_f = 0.49$ (EtOAc/Hex, 1:2); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 0.86$ (t, $J = 6.5$ Hz, 3H), 1.20-1.80 (m, 20H), 1.65–1.85 (m, 2H), 2.65–2.80 (m, 2H), 2.85–3.00 (m, 2H), 3.69 (s, 3H), 3.95–4.10 ppm (m, 2H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 14.4$, 28.8, 29.0, 29.2, 29.3, 29.6, 29.7, 29.8, 30.9, 32.1, 33.2, 38.4, 39.9, 40.7, 52.4, 95.8, 167.8, 169.5, 171.2 ppm; HRMS: m/z $[M+H]^+$ calcd for $C_{19}H_{33}^{35}ClNO_4S_2$: 438.1534, found: 438.1532.

5.14. 5-Chloro-2-ethyl-4-methylisothiazol-3(2H)-one (**19**)

The product was obtained using procedure 3 starting from 3,3′-dithiobis(N-ethyl-2-methylpropanamide) and was purified using column chromatography with EtOAc/Hex 1:6 (v/v) as eluent. The compound was obtained as a yellow liquid (64 mg, 38%): $R_f = 0.34$ (EtOAc/Hex, 1:2); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.26$ (t, $J = 7.2$, 3H), 1.97 (s, 3H), 3.78 ppm (q, $J = 7.3$, 2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ 10.9, 14.8, 39.0, 122.0, 138.4, 166.5 ppm; HPLC: purity 98%, RT = 9.2, mobile phase: H_2O:CH_3CN:TFA 60:40:0.1; HRMS: m/z $[M+H]^+$ calcd for $C_6H_9^{35}ClNOS$: 178.0088, found: 178.0088.

5.15. Methyl 3-(5-chloro-4-methyl-3-oxoisothiazol-2(3H)-yl)propanoate (**20**)

The product was obtained using procedure 3 starting from 3,3′-dithiobis[N-(methyl 2-aminopropionate)-2-methylpropanamide] and purified using column chromatography with EtOAc/Hex 1:4 (v/v) as eluent. The compound was obtained as a yellow gum (122 mg, 52%): $R_f = 0.44$ (EtOAc/Hex, 1:1); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.98$ (s, 3H), 2.71 (t, $J = 6.2$ 2H), 3.70 (s, 3H), 4.02 ppm (t, $J = 6.3$ 2H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 10.9$, 33.8, 39.8, 52.2, 121.5, 139.6, 166.9, 171.7; HPLC: purity 99%, RT = 11.4 min, mobile phase: H_2O:CH_3CN:TFA 60:40:0.1; HRMS: m/z $[M+H]^+$ calcd for C8H1135ClNO3S: 236.0142, found: 236.0141.

5.16. 5-Chloro-2-ethyl-4-methylisothiazol-3(2H)-one-1-oxide (**21**)

The product was obtained using procedure 1 starting from **19** and purified using column chromatography with EtOAc/Hex 1:2 (v/v) as eluent. The compound was obtained as a colorless liquid (159 mg, 82%): $R_f = 0.72$ (EtOAc/Hex, 1:1); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.32$ (t, $J = 7.3$, 3H), 2.05 (s, 3H), 3.69–3.87 ppm (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 10.9$, 14.8, 39.0, 122.0, 138.4, 166.5 ppm; HPLC: 98%, RT = 11.8 min, mobile phase: H_2O:CH_3CN:TFA 60:40:0.1; HRMS: m/z $[M+H]^+$ calcd for $C_6H_9^{35}ClNO_2S$: 194.0037, found: 194.0032.

5.17. Methyl 3-(5-chloro-4-methyl-1-oxido-3-oxoisothiazol-2(3H)-yl)propanoate (**22**)

The product was obtained using procedure 1 starting from **20** and purified using column chromatography with EtOAc/Hex 1:4 (v/v) as eluent. The compound was obtained as a colorless oil (181 mg, 72%): $R_f = 0.56$ (EtOAc/Hex, 1:1); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 2.04$ (s, 3H), 2.69–2.77 (m, 2H), 3.69 (s, 3H), 4.00 ppm (t, $J = 6.7$, 2H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 10.8$, 33.6, 37.4, 52.2, 133.6, 149.8, 165.6, 171.2 ppm; HPLC: purity 97%, RT = 11.2, mobile phase H_2O:CH_3CN:TFA 60:40:0.1; HRMS: m/z $[M+H]^+$ calcd for $C_8H_{11}^{35}ClNO_4S$: 252.0092, found 252.0091.

5.18. Determination of the propane-1-thiol concentration

The concentration of free thiol was quantified using 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB). A DTNB calibration curve was determined by measurement of the UV extinction at 401 nm for DTNB concentration between 1 mM and 10 μM to which an excess (10 mM) propane-1-thiol was added. Subsequently, 100 μL of a 1 mM propane-1-thiol solution was mixed with 100 μL 20 mM DTNB solution. The UV absorbance was measured and the –SH content was calculated from the calibration curve. No significant differences were observed between the calculated (0.50 mM) and measured (0.48 mM) concentrations.

5.19. Reaction of isothiazolone with propane-1-thiol

The isothiazolone(-1-oxide) (50 μmol) was dissolved in CH_3CN (2 mL). A 0.1 M solution of propane-1-thiol in CH_3CN (50 μmol, 500 μL) was added, and the progress of the reaction was followed by HPLC analysis. The mobile phase contained H_2O:CH_3CN:TFA 50:50:0.1 and the compounds were identified on the basis of their retention times.

5.20. Reaction of isothiazolone with sodium propane-1-thiolate

A solution of 0.1 M sodium propane-1-thiolate in water (30 mL) was prepared from propane-1-thiol (272 μL, 3 mmol) and NaOH (108 mg, 2.7 mmol). The isothiazolone(-1-oxide) (50 μmol) was dissolved in CH_3CN (2 mL). A 0.1 M solution of sodium propane-1-thiolate (0.1, 0.5 or 1.0 equiv) was added, and the progress of the reaction was monitored by HPLC. The mobile phase contained H_2O:CH_3CN:TFA 50:50:0.1 and the compounds were identified on the basis of their retention times.

5.21. *Log* P *measurement*

The compound (4 mmol) was dissolved in 1-octanol (1 mL). Water (1 mL) was added and the mixture was vigorously shaken with a vortex mixer (3 × 5 min) and centrifuged (2000*g*, 10 s). The two layers were separated and analyzed by HPLC The mobile phase contained H_2O:CH_3CN:TFA 50:50:0.1. The log P was calculated by comparison of the peak area of the analyte in both layers.

5.22. Enzyme inhibition studies

A fluorescent histone acetyltransferase assay described by Trievel et al. was used for enzyme inhibition studies [27]. Enzyme inhibition was measured by determination of the residual enzyme activity after 15 min incubation with the inhibitor. The enzyme activity was measured by detection of CoA–SH by the fluorescent dye 7-(diethylamino)-3-(40-maleimidylphenyl)-4-methylcoumarin (CPM). The CoA–SH concentrations measured with no inhibitor present were around 50 μM. The inhibitor concentrations were maximal 10 μM, so that inhibitory effects of more than 20% at 10 μM inhibitor concentration cannot be explained by direct reaction of the inhibitors with CoA–SH. Compounds that showed more than 50% inhibition at 10 μM ($n = 3$) were subjected to IC_{50} determination ($n = 3$). The human recombinant histone acetyltransferase PCAF (p300/CREB-bindingprotein Associated Factor) was obtained from Biomol International. The histone H3 peptide (Ac-QTARKSTGGKAPRKQLATKNH2) was purchased from Pepscan (Lelystad, NL).

5.23. Cell growth inhibition

All cell culture reagents were purchased from Invitrogen. The human cancer cells HEP G2 (liver) were maintained in Dulbecco's modified Eagle's Medium (DMEM) with 10% heat-inactivated fetal calf serum (FBS), 50 IU/mL penicillin, and 50 mg/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator. Cell proliferation was measured by a Crystal Violet assay. The 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. The medium was then aspirated, and the cells were fixed with 50 μL 1% crystal violet in 70% ethanol for 30 min. 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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Appendix. Supplementary data

The synthesis and the characterizations of the precursors of the final products numbered **15**–**18** can be found in the supporting information. HPLC chromatograms of the reaction of compounds **3** and **6** with thiols or thiolates and also the ^{1}H and ^{13}C NMR spectra for reaction of **6** with propane-1-thiolate can be found in the supporting information. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejmech.2009.07.025.

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