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Sulfur, selenium and tellurium pseudopeptides: Synthesis and biological evaluation

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ABSTRACT

A new series of sulfur, selenium and tellurium peptidomimetic compounds was prepared employing the Passerini and Ugi isocyanide based multicomponent reactions (IMCRs). These reactions were clearly superior to conventional methods traditionally used for organoselenium and organotellurium synthesis, such as classical nucleophilic substitution and coupling methods. From the biological point of view, these compounds are of considerable interest because of suspected anticancer and antimicrobial activities. While the sulfur and selenium containing compounds generally did not show either anticancer or antimicrobial activities, their tellurium based counterparts frequently exhibited antimicrobial activity and were also cytotoxic. Some of the compounds synthesized even showed selective activity against certain cancer cells in cell culture. These compounds induced a cell cycle delay in the G0/G1 phase. At closer inspection, the ER and the actin cytoskeleton appeared to be the primary cellular targets of these tellurium compounds, in line with some of our previous studies. As most of these peptidomimetic compounds also comply with Lipinski's Rule of Five, they promise good bioavailability, which needs to be studied as part of future investigations.

1. Introduction

The efficient synthesis of drug-like molecules is a major theme in the field of pharmaceutical chemistry. In this context, multicomponent reactions (MCRs) offer an interesting approach to achieve structural diversity and molecular complexity through comparably straight-forward chemical transformations. Indeed, the easy access to a wide variety of starting materials, comparably mild reaction conditions and the wide spectrum of attainable products place such reactions at the heart of many combinatorial approaches and also form the bedrock for the generation of small product libraries.[1,2]

In pharmaceutical chemistry, isocyanide-based MCRs (IMCRs), such as the Ugi and Passerini reactions, are of particular interest as they allow the synthesis of peptidomimetic compounds, that is, compounds which 'resemble' natural, peptide-like substances. Many peptides possess signaling and regulatory functions, and therefore the potential applications of such peptide-like structures are legion and become immediately apparent.[3] Furthermore, many of these compounds exhibit good solubility, permeability—and hence bioavailability—and are also rather stable chemically and metabolically. In fact, among the various MCRs, the Ugi and Passerini reactions can be considered a breakthrough as far as diversity of products, chemo-, regio- and stereoselectivity and ease of operation are concerned.[4–7]

At the same time, chalcogen containing peptoids often also exhibit pronounced—yet also selective—biological activities,[8–10] especially in the context of redox-modulation. Although, several preparative methods are nowadays available for the preparation of simple chalcogen-containing molecules, there have been only a few reports on the synthesis of more sophisticated compounds, such as larger, multifunctional selenium- or tellurium compounds or biologically 'more amenable' structures, such as redox-active seleno- and telluropeptoids. This may be due to the fact that organoselenium and organotellurium chemistry are not always straight forward. Furthermore, decomposition of the products and difficulties to generate compounds with sufficient purity are often observed.

Recently, we have therefore applied the potential provided by IMCRs to the synthesis of biologically useful chalcogen-compounds, reporting the synthesis of selenium- and tellurium-containing

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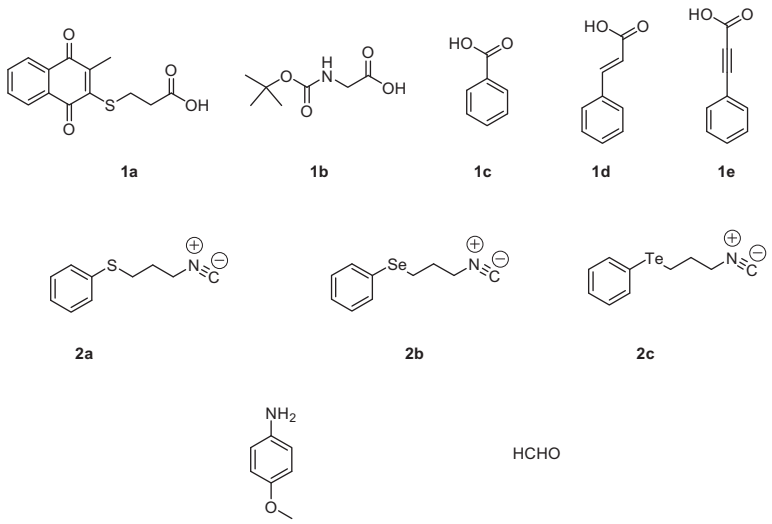

Figure 1. Acid, aldehyde and isonitrile building blocks used in the Passerini and Ugi multicomponent reactions.

peptidomimetic compounds using IMCRs.[11,12] Although the method appears to work well, the scope of the method is rather limited by the accessibility to the isonitrile building block. Chalcogens (sulfur, selenium and tellurium) bearing isonitriles have therefore been synthesized accordingly to overcome this sophisticated limitation.[13] Based on these results, we are now interested to further explore the reactivity of these isonitriles and to generate a library of diverse and highly functionalized organochalcogens of expected biological activity.

2. Results and discussion

Our present study aimed at the synthesis of novel peptidomimetic redox modulating substances with potential biological activity, which was subsequently investigated in different biological assays. As before, the IMCR-based approach turned out to be rather successful for the synthesis of a range of chemically diverse sulfur-, selenium- and tellurium-containing peptidomimetic compounds (**3**–**20**). These compounds were tested in various cancer cell lines and, in the case of the tellurium compounds, showed considerable activity in many of these cell cultures, possibly by selectively damaging the ER and actin cytoskeleton, delaying the cell cycle and inducing apoptosis. Some of these peptidomimetic compounds even exhibited antimicrobial activity which needs to be investigated further. These results will now be presented and discussed in more detail.

2.1. Chemistry

As far as the chemical synthesis is concerned, readily available building blocks, including isonitriles were used in the Passerini and Ugi MCRs for the synthesis of 18 peptidomimetic compounds (**3**–**20**) (Fig. 2). The individual building blocks used are shown in Figure 1, whereby the isonitriles were prepared in a good yield (up to 82%) by dehydration of their corresponding formamides using phosphoryl chloride ($POCl_3$).

In order to optimize the reaction conditions and the yield, the reaction was performed in different solvents. In the case of the sulfur- and the selenium-containing isonitriles, a significant enhancement of the reaction rate and yields (75–94%) was achieved when using water as solvent instead of the classical organic solvents. This could be attributed to various factors, including the hydrophobic effect and enhanced hydrogen bonding in the transition state.[14–16] Additionally, the use of water as solvent permitted the reaction to be conducted rapidly, and as the products are often insoluble in water, it also facilitated their isolation as precipitates. In contrast, low yields and the formation of unfavourable by-products were observed in case of the tellurium-containing isonitrile. Methanol, ethanol and trifluoroethanol, described in the literature as good solvents for MCRs, were used instead; nonetheless, the yield remained low. The best result in case of tellurium isonitrile was achieved using chloroform as a solvent which in turn led to an improvement of yield and a minimization of side products (yields were in the range of 64–96%).

Besides the mild reaction conditions employed and the good yields obtained, this synthetic strategy has additional advantages over conventional methods. Firstly, it is now possible to insert phenyl chalcogenides (especially phenyl telluride) into molecules via IMCRs. Secondly, by keeping the isonitriles fixed whilst varying the other synthetically accessible building blocks as part of a 'mix and match' approach, it has been possible to assemble a diverse arsenal of chalcogen-based compounds, that is, a small library of potential redox modulators which was subsequently tested in biologically relevant in vitro assays.

2.2. Biological evaluation

2.2.1. Preliminary screen for biological activity

As part of this biological evaluation, a preliminary in vitro antitumor screening was performed under the Developmental Therapeutics Program (DTP) offered by National Cancer Institute (NCI) of the National Institute of Health (NIH) in Bethesda, Maryland, United States. Here, compounds[1] **5**, **6**, **9** and **13** were exposed to 58 different cancer cell lines including leukemia cells (CCRF-CEM, HL-60 (TB), K-562, MOLT-4 and SR), non-small cell lung cancer cells (A549/ATCC, EKVX, HOP-62, HOP-92, NCI-H226, NCI-23, NCI-H322M, NCI-H460 and NCI-H522), colon cancer cells (COLO 205, HCC-2998, HCT-116, HCT-15, HT29, KM12, SW-620), melanoma cells (LOX IMVI, MALME-3M, M14, MDA-MB-435, SK-MEL-2, SK-ME-28, SK-MEL-5, UACC-257 and UACC-62), ovarian cancer cells (IGROV1, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, NCI/ADR-RES and SK-OV-3), renal cancer cells (786-0, A498, ACHN, CAKI-1, RXF 393, SN 12C, TK-10 and UO-31), prostate cancer cells (PC-3 and DU-145)

[1] The compounds were chosen according to the National Cancer Institute (NCI) guidelines.

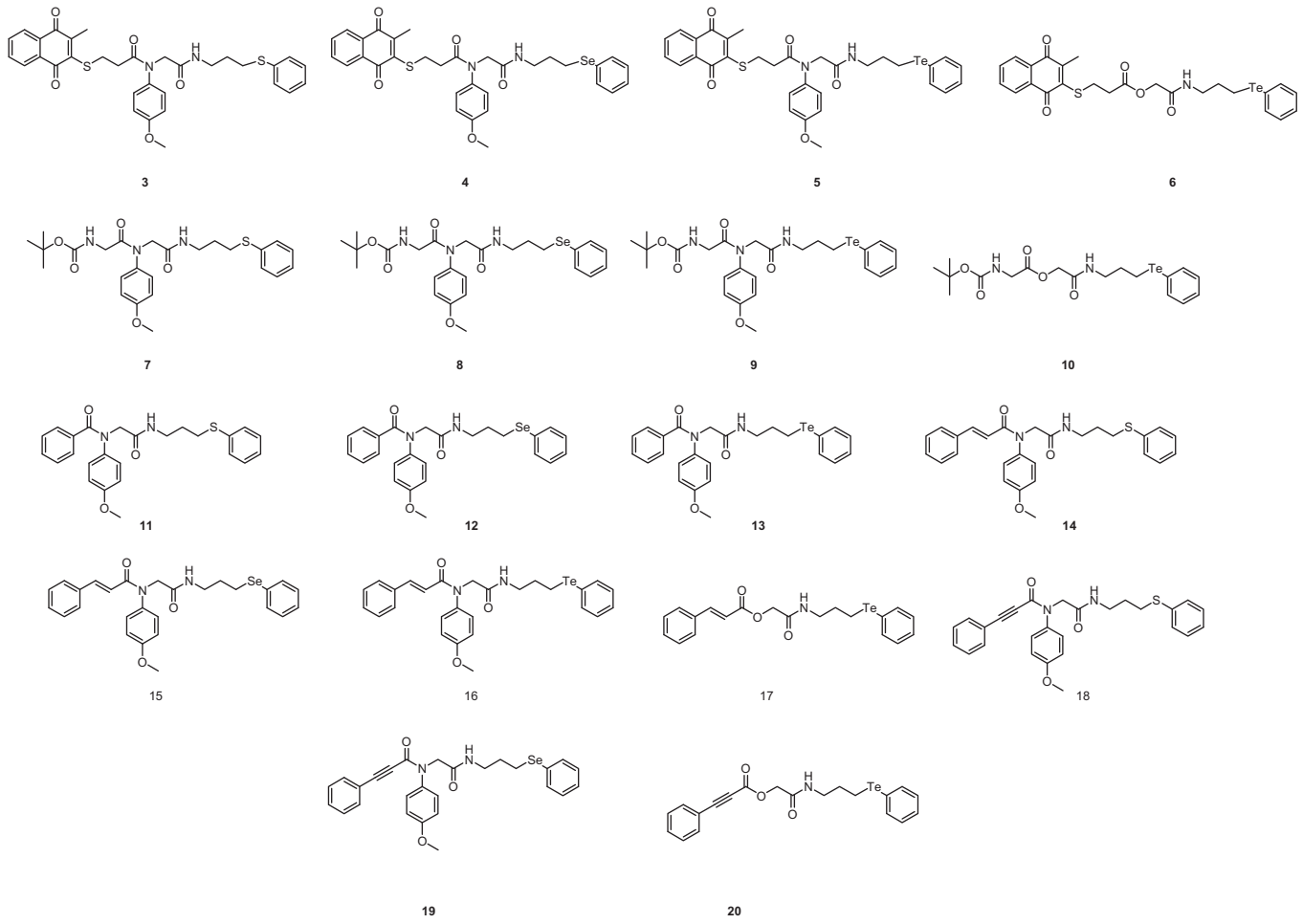

Figure 2. Influence of multifunctional redox compounds on the viability of human cancer and primary cell lines.

and breast cancer cells (MCF7, MDA-MB-231/ATCC, HS 578T, BT-549 and T-47D). As part of this routine screening programme, five different doses were used and cells were incubated with compounds for 48 h. The LC_{50} values obtained for the compounds tested generally were listed in Supporting information and were below 10 mM, pointing towards a general and quite significant biological activity (see below).

Furthermore, a COMPARE analysis was performed for each compound, which revealed that there are some general similarities in the mode of action with known anti-cancer compounds such as cisplatin, methyl mitomycin *C* and anthracycline-based redox agents (e.g., menogaril, deoxydoxorubicin, and MX2 HCl) which are used to treat various types of cancers (see Supporting information).

2.2.2. Cytotoxicity in selected cancer cells and human fibroblast control cells

Based on the above results, the cytotoxic activities were evaluated further and in considerable more detail against human breast adenocarcinoma (MCF-7) and human epidermoid carcinoma (A-431) cancer cell lines, whilst primary human fibroblasts (HF) were used as 'healthy cell' controls. These cell lines were chosen, as they allow a basic evaluation of the activity of the compounds and, in conjunction with PtK2 cells from Potorous tridactylis (see below) also an investigation into the possible modes of action. In the first step, cytotoxicity of the compounds was determined using an MTT assay and 24 h of incubation. The IC_{50} values were estimated from the respective dose response curves and are summarized in Table 1.

Based on these results, the compounds under investigation could be clustered into three different groups: (1) sulfur- and selenium-based compound; (2) tellurium-based compounds and (3) quinone-based compounds. Compounds falling in the first group, that is, containing sulfur (**7**, **11**, **14** and **18**) or selenium (**8**, **12**, **15** and **19**), were unable to reduce the survival of cancer cells and primary cells.

In contrast, tellurium-containing compounds (**9**, **10**, **13**, **16**, **17** and **20**) exhibited a significant cytotoxic effect, whereby the tellurium-containing Passerini products were more toxic than their corresponding Ugi analogues. This may attributed to the particular nature of the Passerini products, that is, structures based on an α-acyloxy amide. Indeed, these compounds contain a readily cleavable ester bond which could be broken enzymatically, hence resulting in potentially more active metabolites. A comparable cytotoxic behaviour was observed in case of the quinone-containing compounds, and regardless if these compounds incorporate sulfur, selenium and/or tellurium atoms.

When comparing the activity of compounds against cancer cells and HF 'normal' cell controls, certain selectivity could be noticed. Compounds **3** and **4** (containing a quinone-redox centre) **6** (containing a quinone-redox centre and tellurium) and **9**, **10**, **17** and **20** (containing tellurium) showed apparent lower toxicities when incubated with HF, pointing to a selective cytotoxic activity against the cancer cells used in this study. The selectivity was more pronounced in case of MCF-7 cells when compared to A-431 cells. Compound **10**, for instance, exhibited considerable selectivity against MCF-7 cells compared to A-431 cells and HF control cells.

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Table 1
Influence of multifunctional redox compounds on the viability of human cancer and primary cells and the partition coefficient values ($\log P_{ow}$) in the octanol–water system

Compound	IC_{50} (μM)			$\log P_{ow}$ [b]	Compound	IC_{50} (μM)			$\log P_{ow}$ [b]
	MCF-7	A-431	HF			MCF-7	A-431	HF	
3	16	17	26	4.20	**12**	[a]	[a]	[a]	3.10
4	8	17	24	4.23	**13**	27	5	7.5	1.72
5	7	1	4	1.97	**14**	[a]	[a]	[a]	4.00
6	12	1	13	3.80	**15**	[a]	[a]	[a]	3.70
7	[a]	[a]	[a]	3.12	**16**	2	6	5	1.31
8	[a]	[a]	[a]	3.30	**17**	1	6	11	3.01
9	5	5	25	4.20	**18**	[a]	[a]	[a]	3.40
10	0.3	10	19	3.83	**19**	[a]	[a]	[a]	2.60
11	[a]	[a]	[a]	3.10	**20**	1	7	7	0.90

[a] The measured IC_{50} was $\geqslant$100 μM.
[b] The lipophilicity was determined by mean of HPLC.

Whilst these findings seem to confirm a considerable, yet also selective activity of some of these compounds, it should be noted that only a limited number of cell types have been tested and a generalization regarding selectivity is not yet feasible. As an alternative explanation, MCF-7 cells may be particularly prone to cell death whilst HF cells are simply normally more resilient.

2.2.3. Mechanistic studies

In order to shed some light on the processes underlying efficiency—and possibly also selectivity—a more detailed mechanistic study has been performed employing a combination of intracellular analytical techniques (so-called 'intracellular diagnostics'). Compared to extensive screening in different cancer and normal cell lines (which has in part already been performed at the NCI, see above), this approach has two advantages. Firstly, it identifies some of the intracellular events responsible for activity, and secondly, it may well provide some information if and why certain compounds are acting selectively.

In the first step, an analysis of the cell cycle was performed in order to indentify irregularities and possible causes of apoptosis (Fig. 3). Indeed, flow cytometric analysis of MCF-7 cells treated with **3**, **10** and **13** at their respective IC_{50}s for 24 h revealed a significant delay in cell cycle progression. Figure 3 reflects a clear increase in the G0/G1 phase and reduction in G2/M phase compared to the methanol control. For example, cell cycle analysis of MCF-7 cells treated with **10**, **3** and **13** showed that 92%, 89% and 85% cells were in G0/G1 phase, respectively, compared to 68% in the methanol treated cells (Table 2). Whilst the precise relation between cell cycle arrest and apoptosis is still not fully understood, it is possible that cell cycle delay may induce an apoptotic response.

Interestingly, an arrest in G0/G1 phase is rather unusual for such compounds, as our previous studies have frequently pointed towards an arrest in the G2/M phase. These issues and their potential implications for the activity and possible uses of the tellurium compounds need to be addressed in more detail as part of subsequent studies.

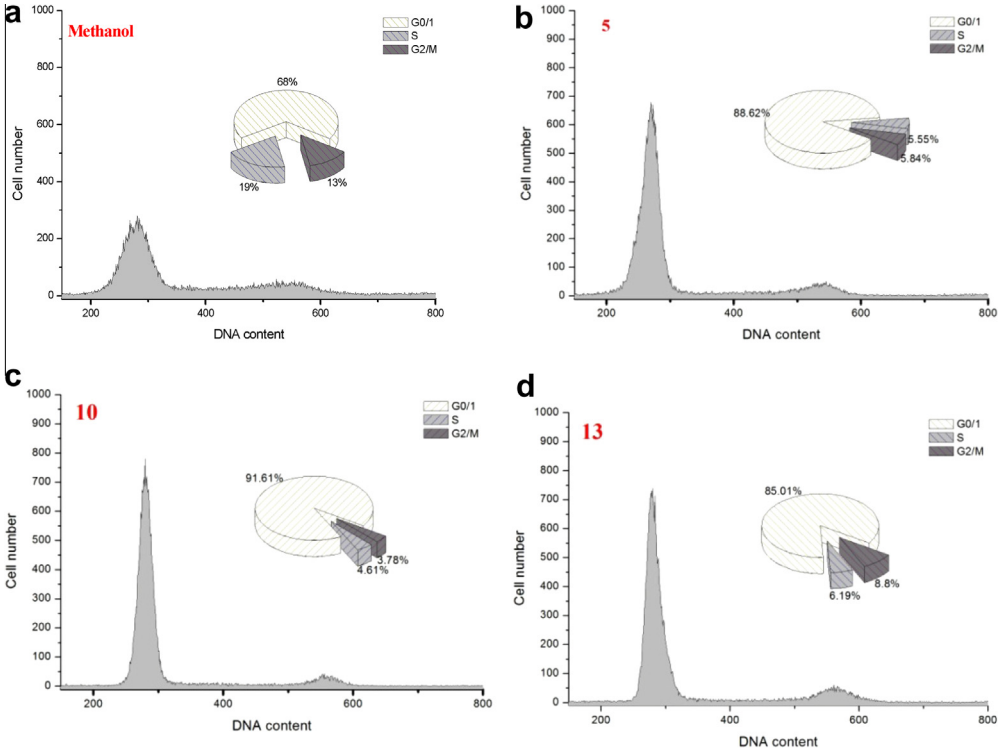

Figure 3. MCF-7 cells were treated with (panel b), **10** (panel c), and **13** (panel d) at their respective IC_{50}s for 24 h. The diagrams show the distribution of the cells according to their DNA content. The inserts give the percentages of cells in different cell cycle phases.

Table 2
Cell cycle distribution of MCF-7 cells treated with different compounds and methanol as negative control

	Cell cycle phase in%		
	G0/G1	S	G2/M
Methanol	68	19	1.6
5	89	5.5	5.8
10	92	6.2	8.8
13	85	6.2	8.8

We have already shown that the quinone-containing chalcogen compounds (**3**, **4**, **5** and **6**) exert their main cytotoxic effects at least in part via modulation of the intracellular redox state.[13] Consequently, these compounds were able to inhibit cancer cell proliferation by induction of Oxidative Stress, cell cycle delay, alteration of the cytoskeleton and apoptosis. Furthermore, we have also shown that the tellurium-containing compounds **6** and **5** are generally able to enhance the oxidation of thiols in the presence of H_2O_2, using a simple in vitro thiophenol oxidation assay.[11,12]

In continuation of our previous work, the ROS levels in (the more robust) A-431 cells were monitored using the standard fluorescent 2′,7′-dichlorodihydrofluorescin diacetate (DCHFA) assay, which can be considered as a general ROS indicator, as DCHFA reacts readily with most common ROS, including H_2O_2, lipid hydroperoxides and $ONOO^-$.[17] As suspected, in the concentration range used, sulfur- and selenium-based compounds were unable to disturb the ROS levels in A-431 melanoma cells over a 1 h treatment period. In contrast, compounds containing tellurium decreased ROS levels in A-431 cells in a concentration-dependent manner (see Supporting information).

In order to evaluate if this decrease in ROS is a cause or consequence of other major cellular events, including the arrest in G0/G1 phase—and if specific cellular components may be affected—immunofluorescence investigations on the cell adherence and associated ER and actin cytoskeletal network were performed, using potorous PtK2 cells as a suitable model (PtK2 cells are an excellent model to study most aspects related to mitosis) (Fig. 4). Interestingly, treatment of PtK2 cells with compound **10** resulted in major morphological changes. Upon treatment, the PtK2 cells begun to lose their typical shape and became round.

At the same time, a distinct loss of cell adhesion, partial lysis of the ER and disappearance of the actin stress fibers were also observed (Fig. 4).

It is possible that these modifications have a major impact on the cell and its ability to function properly and ultimately to divide. Whilst speculative at time, damage to the ER and cytoskeleton are possible targets of the tellurium agents and/or the ROS disturbance as a result of their presence. These issues require more detailed cell biological investigations in the future.

So far, we have considered the impact of the peptidomimetic compounds on cultured human cells. There is, of course, also the chance that such agents may be toxic against lower organisms, such as fungi and bacteria. The antimicrobial activity of the

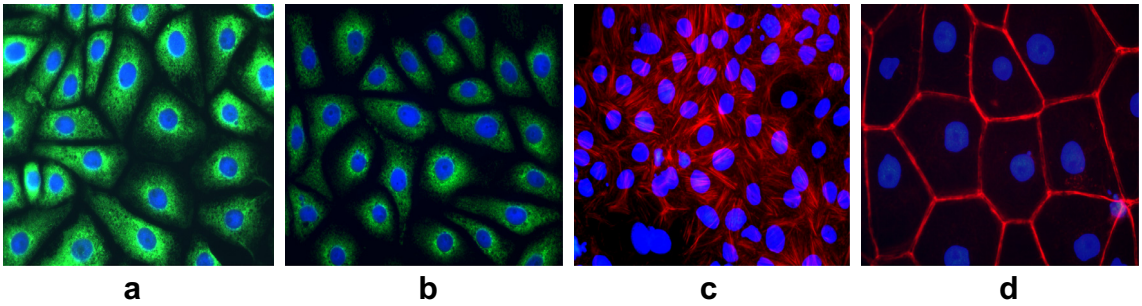

Figure 4. Immunofluorescence investigations of the ER (panel a and b) and the actin cytoskeleton (panel c and d) of PtK2 cells in the absence (panel a and c) and presence of compound **10** (panel b and d). PtK2 cells were incubated with **10** in comparison with control cells. Treated cells show that the ER is affected in general (panel b) and actin stress fibres are barely detectable (panel d). Adhesion seems to be reduced and cells become rounded and detached from each other.

Table 3
Diameters (in mm) of inhibition zones of agar diffusion assays against a variety of fungi and bacteria (growth was quantified after 1 and 2 days)[a]

Compound	*S. cerevisiae*	*C. albicans*	*As. niger*	*M. phlei*	*M. luteus*	*S. aureus*	*K. pneumonia*	*P. aeruginosa*	*E. coli tolC*
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	7	0	0	0	0
5	0	0	0	15	8	11	12	0	12
6	0	0	19	16	11	13	13	12	11
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	12	7	10	12	10	12
10	0	0	0	14	7	16	15	0	12
11	0	0	0	0	0	0	0	0	0
12	0	0	18	0	0	0	0	0	0
13	0	0	10	17	13	12	12	9	11
14	0	0	0	15	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	12	8	11	13	9	11
17	0	0	0	0	7	0	0	0	0
18	0	0	0	0	0	0	0	0	0
19	0	0	19	0	0	0	0	0	0
20	0	0	0	15	9	0	0	0	0

[a] Diameters (mm) of zones of inhibition (agar diffusion assay) are provided. In each case, 6-mm disks with 20 μg of the test compounds were incubated.

compounds was therefore evaluated against three fungal strains, namely *Saccharomyces cerevisiae*, *Candida albicans* and *Aspergillus niger*, and against six bacterial strains, including *Mycobacterium phlei*, *Micrococcus luteus*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Escherichia coli tolC*. A standard agar diffusion assay was used and the diameters (mm) of inhibition zones are summarized in Table 3.

Overall, none of the compounds investigated were particularly toxic against *S. cerevisiae* and *C. albicans*. Indeed, most of sulfur and selenium compounds at the concentrations used showed little toxicity against any of the organisms. In contrast, compounds **6**, **19**, **12** and **13** showed moderate to high toxicity against *A. niger* (with inhibition zone diameters of 19, 19, 18 and 10 mm, respectively). Nonetheless, a convincing antifungal activity could not be confirmed.

In the case of bacteria, most of the compounds showed low to moderate toxicity against *Mycobacterium phlei* and *Micrococcus luteus*. Tellurium compounds were rather toxic against *Staphylococcus aureus and Klebsiella pneumonia*, while most of the quinone-containing compounds were not. The same behavior was noticed in case of *Pseudomonas aeruginosa* and *Escherichia coli tol C.*, yet these two strains generally were less affected.

Whilst the spectrum of activities associated with the peptidomimetic compounds against cancer cells, fungi and bacteria therefore bodes rather well, especially for the tellurium compounds, it should also be noted that these compounds follow the traditional Lipinski's Rule of Five. Here, most of the compounds studied have less than 10 hydrogen bond acceptors and only one or two hydrogen bond donors. The molecular mass in most cases is below 500 Dalton (or slightly above). The experimental partition coefficient values ($\log P_{ow}$) of the compounds in the octanol–water system were measured by the high performance liquid chromatography (HPLC) technique and are found to be below 5 (see Supporting information).

3. Conclusion

A novel library of sulfur-, selenium- and tellurium-based pseudopeptides has been prepared using the isocyanide-based Passerini and Ugi multicomponent reactions and a variety of selected building blocks. The resulting products were obtained in moderate to good yields, turning this approach to a promising synthetic avenue able to provide a rather straight-forward access to many biologically interesting chalcogen-based redox modulating agents. Notably, this strategy is superior to the classical methods as it only requires mild conditions, can be performed easily and relies on a plethora of readily available starting materials. The compounds synthesized were tested for their cytotoxic and antimicrobial activities. Whilst the sulfur and selenium containing compounds generally did not show any major activity, virtually all of the tellurium-containing compounds exhibited both cytotoxic and antimicrobial activities. Interestingly, some of the compounds investigated even showed lower toxicity in HF primary cells compared to cancer cells, appointing towards a possible selective cytotoxic effect. Whilst it is too early to speculate on selectivity and underlying mechanisms, it seems that the tellurium agents are interrupting the ER structure and also actin cytoskeleton, hence causing G0/G1 cell cycle arrest and subsequently inducing apoptosis. The intracellular ROS levels also seemed to be disturbed upon treatment, yet it is still unclear if this is a cause or a rather a consequence of the ER and cytoskeleton damage and cell cycle arrest observed. Additional cell biological studies are clearly required to investigate the exact impact of such peptidomimetic compounds on human and microbial cells and to study the targets and underlying mechanisms involved. The ability to create small libraries of such peptidomimetic compounds with comparable ease will clearly benefit such pharmaceutical and cell biological investigations.

4. Experimental

4.1. Chemistry

4.1.1. Material and methods

All chemical reagents for the synthesis of compounds were purchased from Sigma–Aldrich or Fluka (Germany) and used without further purification unless stated otherwise. Reactions under inert atmosphere were carried out under argon (4.6) using standard oxygen and water free conditions. Silica gel 60 (Machereye-Nagel, 50–200 mm) was used for column chromatography. Unless noted otherwise, the dimensions of columns used were 2.5 cm (diameter) and 25–30 cm (height of silica gel). TLC plates (silica gel 60 F_{254}, 0.20 mm) were purchased from Merck (Germany). NMR spectroscopy: ^{1}H NMR spectra were recorded at 500 MHz, ^{13}C NMR spectra at 125 MHz on a Bruker DRX 500 or Avance 500 spectrometer. Chemical shifts are reported in δ (ppm), expressed relative to the solvent signal at 7.26 ppm ($CDCl_3$, ^{1}H NMR) and at 77.16 ppm ($CDCl_3$, ^{13}C NMR), as well as 3.31 ppm (^{1}H NMR, CD_3OD) and 49.00 ppm (^{13}C NMR, CD_3OD). Coupling constants (J) are given in Hz. LC–MS/MS analysis: Analyses were performed using a TSQ Quantum mass spectrometer equipped withan ESI source and a triple quadrupole mass detector (Thermo Finnigan). HRMS: High-resolution mass spectrometry was performed on an Accela UPLC-system (Thermo-Fisher) coupled to a linear trap-FT-Orbitrap combination (LTQ-Orbitrap), operating in positive ionization mode. These spectra indicated a $\geqslant$95% purity of the prepared compounds.

4.1.2. General procedure for the preparation of chalcogen containing isocyanide building blocks (2a–2c)

A mixture of 1 g of appropriate amine, 1.0–1.2 equiv of formic acid and 1.5–2.0 equiv of acetic anhydride were mixed and heated for 2 h. The progress of the reaction was monitored by TLC, and after the starting material had disappeared, the solvent was evaporated from the reaction mixture to yield the crude *N*-formyl compound, essentially as an oily product which could be purified by chromatography on silica gel, usually with dichloromethane/methanol (10:1).

The *N*-formyl compound (6.05 g, 50.0 mmol) and diisopropylamine (DIPA) (19 ml, 0.14 mol) were dissolved in CH_2Cl_2 (50 ml) and cooled to 0 °C. $POCl_3$ (5.0 ml, 55 mmol) was added slowly and stirring was continued at 0 °C for another 30 min. Sodium carbonate (10 g) in H_2O (50 ml) was added at room temperature in a rate so that the temperature was maintained between 25 and 35 °C. The mixture was stirred for 90 min at room temperature. H_2O and CH_2Cl_2 (25 ml each) were added. The organic layer was separated, washed with H_2O (3 × 25 ml), dried over $MgSO_4$ and purified by chromatography on silica gel, with petrol ether/ethyl acetate (8:1) as eluent.

4.1.3. General procedure (A) for the preparation of α-acyloxy amide via the three-component Passerini reaction

As a general procedure, a mixture of aldehyde (1 mmol), carboxylic acid (1.2 mmol) and isocyanide (1.5 mmol) in 5 ml solvent (H_2O was used in most cases) was stirred at room temperature overnight. Upon completion (monitored by TLC), 10 ml CH_2Cl_2 were added to dissolve the sticky product.

The aqueous layer was extracted three times with CH_2Cl_2, the organic layers were combined, dried over Na_2SO_4 and concentrated to yield a sticky product which was purified by chromatography on silica gel, with petrol ether/ethyl acetate (4:1) as eluent.

4.1.4. General procedure (B) for the preparation of α-aminoacyl amide via the four-component Ugi reaction

As a general procedure, a mixture of aldehyde (1 mmol), amine (1 mmol), carboxylic acid (1.2 mmol), and isocyanide (1.5 mmol) in 5 ml solvent (H_2O was used in most cases) was stirred at room temperature overnight. Upon completion (monitored by TLC), 10 ml CH_2Cl_2 were added to dissolve the sticky product. The water layer was extracted three times with CH_2Cl_2, the organic layers were combined, dried over Na_2SO_4 and concentrated to yield a sticky product which was purified by chromatography on silica gel, with petrol ether/ethyl acetate (5:2). The synthesis of individual products, including building blocks, yield and analytical data are provided as a part of the individual experimental sections.

Compounds **2a**–**c**, **3**, **4**, **5**, and **6** were synthesized according to literature.[13]

4.1.5. *tert*-Butyl ((2-(*N*-(4-methoxyphenyl)-*N*-amino)-*N*-(3-(phenylthio)propyl)acetamide)carbonyl)methylcarbamate (7)

Following general procedure B, the crude residue was purified by column chromatography on silica gel, eluting with petrol ether/ethyl acetate (5:1), afforded desired product **7** as colourless oil (yield = 77%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.31–7.24 (m, 6H), 7.17–7.14 (m, 1H), 6.99 (br s, 1H), 6.88–6.86 (d, J = 8.90 Hz, 2H), 5.48 (s, 1H), 4.26 (s, 2H), 3.78 (s, 3H), 3.66–3.64 (d, J = 5.07 Hz, 2H), 3.37-3.33 (q, J = 6.82, 13.00, 19.77 Hz, 2H), 2.92–2.89 (t, J = 7.24, 14.49 Hz, 2H), 1.86–1.81 (p, J = 7.24, 13.96, 21.11, 28.00 Hz, 2H), 1.49 (s, 9H) ppm; ^{13}C NMR (125.79 Hz, $CDCl_3$) δ 170.13, 168.18, 159.39, 155.84, 136.00, 133.46, 128.82, 128.69, 128.54, 125.71, 114.94, 79.44, 55.25, 53.71, 42.80, 38.22, 30.70, 28.61, 28.08 ppm; LC–MS (ESI): m/z calcd 487.21, R_t = 1.61 min, m/z found 488.2 $[M+1]^+$; HRMS: [M+Na] calcd 510.2039, found 510.2033 [M+Na]. Isotopic pattern of S: m/z (relative abundance %) 488.2214 (100), 489.2247 (25), 490.2172 (5), 510.2033 (100), 511.2067 (25), 512.1991 (5).

4.1.6. *tert*-Butyl ((2-(*N*-(4-methoxyphenyl)-*N*-amino)-*N*-(3-(phenylselanyl)propyl)acetamide)carbonyl)methylcarbamate (8)

Following general procedure B, the crude residue was purified by column chromatography on silica gel, eluting with petrol ether/ethyl acetate (5:1), afforded desired product **8** as colourless oil (yield = 86%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49–7.46 (m, 2H), 7.27–7.21 (m, 5H), 6.89–6.87 (d, J = 8.92 Hz, 2H), 6.77 (br s, 1H), 5.40 (br s, 1H), 4.24 (s, 2H), 3.79 (s, 3H), 3.66–3.64 (d, J = 5 Hz, 2H), 3.37–3.33 (q, J = 6.71, 12.71, 19.56 Hz, 2H), 2.89–2.87 (t, J = 7.37, 14.35 Hz, 2H), 1.93–1.87 (p, J = 7.00, 13.73, 21.00, 27.95 Hz, 2H), 1.41 (s, 9H) ppm; ^{13}C NMR (125.79 Hz, $CDCl_3$) δ 170.18, 168.20, 159.52, 155.88, 133.52, 132.39, 129.89, 128.95, 128.61, 126.73, 115.07, 79.59, 55.37, 53.91, 42.90, 39.21, 29.69, 28.18, 24.68 ppm; LC–MS (ESI): m/z calcd 535.16, R_t = 1.73 min, m/z found 558.10 $[M+Na]^+$; HRMS: [M+H] calcd 536.1585, [M+H] found 536.1658, [M+Na] calcd 558.1478 [M+Na] found 559.1511. Isotopic pattern of Se: m/z (relative abundance %) 536.1658 (100), 537.1692 (26), 538.1660 (15), 539.1694 (5).

4.1.7. *tert*-Butyl ((2-(*N*-(4-methoxyphenyl)-*N*-amino)-*N*-(3-(phenyltellanyl)propyl)acetamide)carbonyl)methylcarbamate (9)

Following general procedure B, the crude residue was purified by column chromatography on silica gel, eluting with petrol ether/ethyl acetate (5:1), afforded desired product **9** as colourless oil (yield = 70%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.66–7.64 (m, 2H), 7.19 (s, 1H), 7.14–7.10 (m, 4H), 6.82–6.79 (d, J = 8.9 Hz, 2H), 6.32 (br s, 1H), 5.20 (br s, 1H), 4.13 (s, 2H), 3.73 (s, 3H), 3.58–3.57 (d, J = 5.28 Hz, 2H), 3.28–3.24 (q, J = 6.55, 13.41, 19.32 Hz, 2H), 2.80–2.77 (t, J = 7.66, 15.15 Hz, 2H), 1.97–1.91 (p, J = 6.95, 14.24, 21.66, 28.65 Hz, 2H), 1.43 (s, 9H) ppm; ^{13}C NMR (125.79 Hz, $CDCl_3$) δ 170.25, 168.30, 159.72, 155.92, 138.45, 133.58, 129.23, 128.68, 127.70, 115.26, 111.46, 79.80, 55.25, 54.22, 43.05, 41.22, 31.52, 28.30, 4.75 ppm; LC–MS (ESI): m/z calcd 585.15, R_t = 1.54 min, m/z found 608.15 $[M+Na]^+$; HRMS: [M+H] calcd 586.1482, [M+H] found 586.1555, [M+H+O] calcd 602.1431 [M+H] found 602.1504, [M+Na] calcd 608.1380 [M+Na] found 608.1656. Isotopic pattern of Te: m/z (relative abundance %) 586.1555 (100), 587.1589 (29), 588.1622 (5), 602.1504 (100), 603.1538 (26), 604.1571 (5), 608.1656 (1).

4.1.8. *tert*-Butyl (((3-(phenyltellanyl)propylcarbamoyl)methoxy)carbonyl)methylcarbamate (10)

Following general procedure A, the crude residue was purified by column chromatography on silica gel, eluting with petrol ether/ethyl acetate (5:1), afforded desired product **10** as colourless oil (yield = 83%). ^{1}H NMR (500 MHz $CDCl_3$) δ 7.66–7.63 (m, 2H), 7.23–7.20 (m, 1H), 7.15–7.11 (m, 2H), 6.70 (br s, 1H), 5.05 (br s, 1H), 4.56 (s, 2H), 3.83–3.82 (d, 2H), 3.30–3.26 (q, J = 6.68, 12.91, 19.81 Hz, 2H), 2.81–2.78 (t, J = 7.50, 15.11 Hz, 2H), 1.99–1.93 (p, J = 7.26, 14.53, 21.55, 29.05 Hz, 2H), 1.37 (s, 9H) ppm; ^{13}C NMR (125.79 Hz, $CDCl_3$) δ 168.99, 166.87, 156.48, 138.42, 129.21, 127.68, 111.50, 80.79, 63.13, 42.88, 40.96, 31.44, 28.30, 4.70 ppm; LC–MS (ESI): m/z calcd 480.09, R_t = 7.21 min, m/z found 497.59 [M+H+O], 497.44 $[M+H+O]^+$; HRMS: [M+H+O] calcd 497.0849, [M+H+O] found 497.0926, [M+Na] calcd 503.0904, [M+Na] found 503.1078. Isotopic pattern of Te: m/z (relative abundance %) 495.0908 (93), 497.0926 (100), 498.0959 (22), 499.0993 (7), 503.1078 (2).

4.1.9. *N*-((3-(Phenylthio)propylcarbamoyl)methyl)-*N*-(4-methoxyphenyl)benzamide (11)

Following general procedure B, the crude residue was purified by column chromatography on silica gel, eluting with petrol ether/ethyl acetate (5:1), afforded desired product **11** as colourless oil (yield = 75%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.25–7.22 (m, 4H), 7.19–7.17 (m, 2H), 7.11–7.07 (m, 3H), 6.94–6.91 (m, 2H), 6.64–6.61 (m, 3H), 4.38 (s, 2H), 3.63 (s, 3H), 3.36–3.32 (q, J = 6.48, 12.34, 19.75 Hz, 2H), 2.87–2.84 (t, J = 7.35, 18.85 Hz, 2H), 1.82–1.76 (p, J = 6.98, 13.85, 20.83, 27.92 Hz, 2H) ppm; ^{13}C NMR (125.79 Hz, $CDCl_3$) δ 171.49, 168.18, 158.52, 136.70, 136.30, 135.09, 130.26, 129.52, 129.38, 129.15, 129.06, 128.52, 128.04, 126.28, 114.70, 55.57, 38.62, 31.32), 29.06 ppm; LC–MS (ESI): m/z calcd 434.17, R_t = 15.69 min, m/z found 435.2 $[M+H]^+$; HRMS: [M+H] calcd 435.1764, [M+H] found 435.1737, [M+Na] calcd 457.1562 [M+Na] found 457.1556. Isotopic pattern of S: m/z (relative abundance %) 435.1737 (100), 436.1770 (29), 437.1695 (5), 457.1556 (100), 458.1590 (25), 459.1514 (5).

4.1.10. *N*-((3-(Phenylselanyl)propylcarbamoyl)methyl)-*N*-(4-methoxyphenyl)benzamide (12)

Following general procedure B, the crude residue was purified by column chromatography on silica gel, eluting with petrol ether/ethyl acetate (5:1), afforded desired product as colourless oil (yield = 79%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40–7.38 (m, 2H), 7.24–7.22 (m, 2H), 7.16–7.14 (m, 4H), 7.11–7.07 (m, 2H), 6.94–6.91 (m, 2H), 6.64–6.61 (m, 2H), 4.36 (s, 2H), 3.63 (s, 3H), 3.33–3.29 (q, J = 6.57, 13.14, 19.41 Hz, 2H), 2.83–2.81 (t, J = 7.06, 14.52 Hz, 2H), 1.86–1.81 (p, J = 6.98, 13.96, 21.31, 28.29 Hz, 2H) ppm; ^{13}C NMR (125.79 Hz, $CDCl_3$) δ 171.22, 168.88), 158.26, 136.44, 134.84, 132.60, 130.00, 129.86, 129.06, 128.79, 128.27, 127.79, 126.89, 114.44, 55.31, 55.28, 39.23, 29.78), 24.78 ppm; HRMS: [M+H] calcd 483.1109, [M+H] found 483.1181, [M+Na] calcd 505.1007 [M+Na] found 505.1001. Isotopic pattern of Se:

m/*z* (relative abundance %) 483.1181 (100), 484.1215 (30), 485.1183 (20), 486.1217 (8), 505.1001 (100), 506.1034 (30), 507.1003 (20), 508.1036 (5).

4.1.11. *N*-((3-(Phenyltellanyl)propylcarbamoyl)methyl)-*N*-(4-methoxyphenyl)benzamide (13)

Following general procedure B, the crude residue was purified by column chromatography on silica gel, eluting with petrol ether/ethyl acetate (5:1), afforded desired product as colourless oil (yield = 69%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78–7.75 (m, 2H), 7.38–7.30 (m, 5H), 7.24–7.22 (m, 3H), 7.06–7.04 (m, 2H), 6.77–6.75 (m, 2H), 4.49 (s, 2H), 3.78 (s, 3H), 3.43–3.38 (p, J = 6.68, 13.09, 19.56, 25.44 Hz, 2H), 2.93–2.90 (t, J = 7.52, 15.16 Hz, 2H), 2.08–2.03 (p, J = 6.84, 14.37, 21.49, 28.74 Hz, 2H) ppm; ^{13}C NMR (125.79 Hz, $CDCl_3$) δ 171.24, 170.80, 168.90, 158.30, 138.48, 136.46, 134.85, 130.04, 129.22, 128.82, 128.27, 127.82, 127.69, 114.47, 55.36, 55.35, 41.11, 31.52, 4.67 ppm; LC–MS (ESI): *m*/*z* calcd 532.10, R_t = 1.54 min, *m*/*z* found 533.0 $[M+H]^+$; HRMS: [M+H+O] calcd 549.1006, [M+H+O] found 549.1028. Isotopic pattern of Te: *m*/*z* (relative abundance %) 549.1028 (100), 550.1061 (30), 551.1095 (6).

4.1.12. (*E*)-*N*-((3-(Phenylthio)propylcarbamoyl)methyl)-*N*-(4-methoxyphenyl)cinnamamide (14)

Following general procedure B, the crude residue was purified by column chromatography on silica gel, eluting with petrol ether/ethyl acetate (5:1), afforded desired product as colourless oil (yield = 87%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.61–7.58 (m, 1H), 7.24–7.04 (m, 11H), 6.88–6.71 (m, 3H), 6.30–6.27 (m, 1H), 4.28 (s, 2H), 3.73 (s, 3H), 3.33–3.29 (q, J = 6.73, 12.95, 19.68 Hz, 2H), 2.87–2.84 (t, J = 7.16, 14.50 Hz, 2H), 1.81–1.75 (p, J = 6.94, 13.99, 21.04, 27.98 Hz, 2H) ppm; ^{13}C NMR (125.79 Hz, $CDCl_3$) δ 169.02, 167.10, 159.11, 142.92, 136.08, 134.87, 134.73, 129.78, 129.13, 128.82, 128.75, 128.64, 127.90, 125.90, 117.71, 114.83, 55.42, 54.71, 38.33, 30.99, 28.82 ppm; LC–MS (ESI): *m*/*z* calcd 460.18, R_t = 14.10 min, *m*/*z* found 461.2 $[M+H]^+$; HRMS: [M+H] calcd 461.1821, [M+H] found 461.1893. Isotopic pattern of Se: *m*/*z* (relative abundance %) 461.1893 (100), 462.1927 (30), 463.1851 (5), 464.1885 (2).

4.1.13. (*E*)-*N*-((3-(Phenylselanyl)propylcarbamoyl)methyl)-*N*-(4-methoxyphenyl)cinnamamide (15)

Following general procedure B, the crude residue was purified by column chromatography on silica gel, eluting with petrol ether/ethyl acetate (5:1), afforded desired product as colourless oil (yield = 92%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.62–7.59 (m, 1H), 7.39–7.37 (m, 2H), 7.24–7.08 (m, 9H), 6.88–6.64 (m, 3H), 6.30–6.27 (m, 1H), 4.27 (s, 2H), 3.75 (s, 3H), 3.31–3.27 (q, J = 7.10, 13.37, 20.20 Hz, 2H), 2.83–2.80 (t, J = 7.25, 14.87 Hz, 2H), 1.86–1.81 (p, J = 6.96, 14.21, 21.31, 28.42 Hz, 2H) ppm; ^{13}C NMR (125.79 Hz, $CDCl_3$) δ 169.03, 167.14, 159.15, 142.96, 134.88, 134.76, 132.56, 129.91, 129.81, 129.02, 128.76, 128.67, 127.93, 126.82, 117.71, 114.83, 55.46, 54.78, 39.22, 29.82, 24.78 ppm; LC–MS (ESI): *m*/*z* calcd 508.13, R_t = 1.71 min, *m*/*z* found 509.10 $[M+H]^+$; HRMS: [M+H] calcd 509.1265, [M+H] found 509.1338. Isotopic pattern of Se: *m*/*z* (relative abundance %) 509.1338 (100), 510.1371 (30), 511.1340 (17), 512.1373 (7), 513.1407 (2).

4.1.14. (*E*)-*N*-((3-(Phenyltellanyl)propylcarbamoyl)methyl)-*N*-(4-methoxyphenyl)cinnamamide (16)

Following general procedure B, the crude residue was purified by column chromatography on silica gel, eluting with petrol ether/ethyl acetate (5:1), afforded desired product as colourless oil (yield = 77%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.64–7.61 (m, 3H), 7.40–7.3 (m, 5H), 7.25–7.2 (m, 3H), 6.71–7.02 (m, 3H), 6.75 (br s, 1H), 6.30–6.27 (m, 1H), 4.26 (s, 2H), 3.76 (s, 3H), 3.28–3.24 (q, J = 6.42, 12.84, 19.69 Hz, 2H), 2.80–2.77 (t, J = 7.59, 15.19 Hz, 2H), 1.96–1.91 (p, J = 6.94, 14.52, 21.47, 28.88 Hz, 2H) ppm; ^{13}C NMR (125.79 Hz, $CDCl_3$) δ 169.30, 167.42, 159.43, 143.25, 138.64, 135.13, 135.03, 130.10, 129.42, 129.02, 128.95, 128.21, 127.87, 117.97, 115.15, 111.66, 55.74, 55.06, 41.33, 31.78, 4.95 ppm; LC–MS (ESI): *m*/*z* calcd 558.12, R_t = 17.19 min, *m*/*z* found 558.80 [M+H], 582.1 [M+Na]; HRMS: [M+H+O] calcd 575.1162, [M+H+O] found 575.1184. Isotopic pattern of Te: *m*/*z* (relative abundance %) 575.1184 (100), 576.1218 (30), 577.1251 (5), 578.1285 (2).

4.1.15. (*E*)-(3-(Phenyltellanyl)propylcarbamoyl)methyl cinnamate (17)

Following general procedure A, the crude residue was purified by column chromatography on silica gel, eluting with petrol ether/ethyl acetate (5:1), afforded desired product as colourless oil (yield = 90%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.73–7.64 (m, 3H), 7.57–7.45 (m, 2H), 7.37–7.33 (m, 2H), 7.22–7.18 (m, 2H), 7.11–7.15 (m, 2H), 6.50–6.35 (m, 1H), 6.12 (br s, 1H), 4.60 (s, 2H), 3.35–3.31 (q, J = 6.63, 13.07, 19.96 Hz, 2H), 2.82–2.79 (t, J = 7.28, 14.82 Hz, 2H), 2.00–1.95 (p, J = 7.09, 14.27, 21.45, 28.81 Hz, 2H) ppm; ^{13}C NMR (125.79 Hz, $CDCl_3$) δ 167.23, 165.42, 146.79, 138.51, 133.96, 130.87, 129.28, 129.02, 128.28, 127.81, 116.38, 111.33, 63.07, 40.80, 31.47, 4.60 ppm; LC–MS (ESI): *m*/*z* calcd 453.06, R_t = 9.01 min, *m*/*z* found 469.85 $[M+O]^+$; HRMS: [M+H+O] calcd 470.0584, [M+H+O] found 470.0606. Isotopic pattern of Te: *m*/*z* (relative abundance %) 470.0606 (100), 471.0639 (23), 472.0673 (4).

4.1.16. *N*-((3-(Phenylthio)propylcarbamoyl)methyl)-*N*-(4-methoxyphenyl)-3-phenylpropiolamide (18)

Following general procedure B, the crude residue was purified by column chromatography on silica gel, eluting with petrol ether/ethyl acetate (5:1), afforded desired product as colourless oil (yield = 86%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.29–7.24 (m, 4H), 7.19–7.16 (m, 5H), 7.10–7.06 (m, 3H), 6.84–6.81 (m, 2H), 6.47 (br s, 1H), 4.27 (s, 2H), 3.74 (s, 3H), 3.35–3.31 (q, J = 6.77, 12.89, 19.49 Hz, 2H), 2.88–2.85 (t, J = 7.09, 14.34 Hz, 2H), 1.83–1.77 (p, J = 7.09, 13.86, 20.94, 27.71 Hz, 2H) ppm; ^{13}C NMR ($CDCl_3$, 125.79 Hz) δ 168.15, 159.44, 155.33, 136.02, 134.81, 132.51, 130.20, 129.29, 128.98, 128.92, 128.35, 126.03, 120.03, 114.39, 92.68, 81.96, 55.53, 53.62, 38.51, 31.08, 28.79 ppm; LC–MS (ESI): *m*/*z* calcd 458.17, R_t = 1.56 min, *m*/*z* found 459.2 $[M+H]^+$; HRMS: [M+H] calcd 459.1664, [M+H] found 481.1734, [M+Na] calcd 481.1562 [M+Na] found 481.1556. Isotopic pattern of S: *m*/*z* (relative abundance %) 459.1734 (100), 460.1770 (30), 461.1695 (4), 481.1556 (100), 482.1590 (30), 483.1514 (5).

4.1.17. *N*-((3-(Phenylselanyl)propylcarbamoyl)methyl)-*N*-(4-methoxyphenyl)-3-phenylpropiolamide (19)

Following general procedure B, the crude residue was purified by column chromatography on silica gel, eluting with petrol ether/ethyl acetate (5:1), afforded desired product as colourless oil (yield = 90%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42–7.39 (m, 2H), 7.27–7.22 (m, 3H), 7.18–7.13 (m, 5H), 7.11–7.07 (m, H), 6.84–6.82 (m, 2H), 6.46 (br s, 1H), 4.26 (s, 2H), 3.74 (s, 3H), 3.32–3.28 (q, J = 6.94, 13.23, 19.84 Hz, 2H), 2.84–2.80 (t, J = 7.07, 14.40 Hz, 2H), 1.87–1.81 (p, J = 6.91, 13.92, 20.72, 27.74 Hz, 2H) ppm; ^{13}C NMR (125.79 Hz, $CDCl_3$) δ 168.08, 159.40, 155.28, 134.79, 132.61, 132.48, 130.18, 129.87, 128.06, 128.98, 128.33, 126.86, 120.01, 114.36, 92.62, 81.97, 55.51, 53.54, 39.34, 29.76, 24.77 ppm; LC–MS (ESI): *m*/*z* calcd 506.11, R_t = 11.45 min, *m*/*z* found 507.54 $[M+H]^+$; HRMS: [M+H] calcd 507.1109, [M+H] found 507.1181. Isotopic pattern of Se: *m*/*z* (relative abundance %) 507.1181 (100), 508.1215 (33), 509.1183 (18), 510.1217 (7), 511.1250 (1).

4.1.18. *N*-((3-(Phenyltellanyl)propylcarbamoyl)methyl)-3-phenylpropiolamide (20)

Following general procedure A, the crude residue was purified by column chromatography on silica gel, eluting with petrol ether/ethyl acetate (5:1), afforded desired product as colourless oil (yield = 86%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70–7.65 (m, 2H), 7.55–7.51 (m, 2H), 7.36–7.31 (m, 3H), 7.22–7.21 (m, 1H), 7.15–7.10 (m, 2H), 6.60 (br s, 1H), 4.65 (s, 1H), 4.60 (s, 2H), 3.64–3.61 (m, 2H), 2.75–2.72 (m, 2H), 1.72–1.67 (m, 2H) ppm; ^{13}C NMR (125.79 Hz, $CDCl_3$) δ 167.40, 166.55, 152.53, 133.43, 133.37, 131.44, 131.39, 128.97, 128.93, 126.85, 119.28, 88.87, 79.81, 63.97, 43.75, 36.63, 32.89 ppm; HRMS: [M+O] calcd 466.0427, [M+O] found 466.0449. Isotopic pattern of Te: *m*/*z* (relative abundance %) 468.0449 (100), 469.0483 (21), 470.0516 (5).

4.2. Biological assays

4.2.1. Cancer cell line screening at the National Cancer Institute

The 58 cancer cell line screen was performed at the National Cancer Institute (NCI) at the NIH (US). These single dose tests (at 10 μM) were performed for cell lines clustered in cells representing leukemia, non-small cell lung cancer, colon cancer, cancer of the central nervous system, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer. All compounds were selected for five dose testing. These cell culture based investigations followed the NCI's standard protocol for cytotoxicity screens, details of which can be obtained directly from the NCI website at http://dtp.nci.nih.gov.

4.2.2. Cytotoxicity assays

All cell lines required for these studies were obtained from the DSMZ (Braunschweig, Germany). Human fibroblasts (HF) isolated from human male foreskin were a generous gift of Dr. Thierauch, Bayer-Schering Pharma AG. All cells were grown at 37 °C and 10% CO_2 in the following media: MCF-7 in DMEM supplemented with 1% L-glutamine and 1% non essential amino acids, A-431 in RPMI 1640 (Gibco), and HF in MEM (Gibco) supplemented with 1% L-glutamine. All media were supplemented with 10% fetal calf serum (Lonza or Gibco).

MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] (Sigma) was used to measure the metabolic activity of cells (alive cells are able to reduce MTT to a violet coloured formazan product which can be quantified spectrophotometrically). Briefly, 120 μl aliquots of a cell suspension (50,000 cells ml^{-1}) in 96-well microplates were incubated at 37 °C and 10% CO_2 and allowed to grow for two days. Then 60 μl of serial dilutions of the compounds were added. After 24 h of incubation at 37 °C and 10% CO_2, 20 μl MTT in phosphate buffered saline (PBS) were added to a final MTT concentration of 0.5 mg ml^{-1}. After 2 h the precipitate of formazan crystals was centrifuged and the supernatant discarded. The precipitate was washed with 100 μl PBS and dissolved in 100 ml isopropanol containing 0.4% hydrochloric acid. The resulting colour was quantified at 590 nm using an ELISA plate reader. All investigations were carried out in two parallel experiments. The IC_{50} values were determined from the dose–response curves as the concentrations of compounds, which resulted in 50% of the absorbance of untreated control cells.

4.2.3. In vitro studies

4.2.3.1. Detection of ROS. The ability of compounds to induce intracellular ROS formation was determined using DCHFA assays. Cells were seeded in 96 wells plates at a density of 10^5 cells per well and treated with different concentrations of the test compounds for 1 h. The cells (180 μl) were then incubated with 20 μl of 10 μM DCHFA probe for 30 min in the dark. The fluorescence was measured immediately in a fluorescence spectrophotometer (DCHFA, $\lambda_{ex}/\lambda_{em}$ = 485 ± 20/528 ± 20 nm). Results were expressed as arbitrary units per 10^5 cells.

4.2.3.2. Cell cycle analysis. Treated MCF-7 cells (10^6) were fixed with cold (−20 °C) methanol (70%) at 4 °C for one day. Cells were washed with PBS and then treated with saponin (0.1% in PBS). Finally, propidium iodide (500 μl, 20 μg ml^{-1}) and RNAse (1 mg ml^{-1}) were added. After 30 min, samples were analyzed by FacScan.

4.2.3.3. Immunofluorescence microscopy. Cells were grown on cover slips in 4-well plates, compounds were added after the cells had become semi-confluent and were incubated for 24 h. Subsequently, cells were fixed with 3.7% paraformaldehyde (followed by Triton-X 100 (0.1%) treatment for 5 min) or ice cold methanol/acetone (50:50) for 10 min and then washed with phosphate-buffered saline (PBS). Primary antibodies were added and incubated for 45 min and washed with PBS. Secondary antibodies were then added to the cells and incubated for further 45 min. After washing with PBS, 4′,6-diamidino-2-phenylindole (DAPI) was added and kept at room temperature for 5 min. Cover slips were mounted in anti-fade mounting medium (Molecular Probes). Images were taken with a CCD camera attached to a fluorescence microscope. The following antibodies were used: anti-GRP94, anti-α-tubulin, anti-mouse Alexa Fluor 488 and anti-rat Alexa Fluor 488. The actin filaments were stained with phalloidin Alexa Fluor 594 for 45 min.

4.2.3.4. Lipophilicity measurements. High performance liquid chromatography (HPLC) was used as a rapid method for the determination of lipophilicity. In order to correlate the measured HPLC data of a compound with its *P* value, a calibration graph of log *P* versus chromatographic data using at least six reference points has to be established. The retention times (t_R), of the compounds were determined using a Hewlett Packard series 1090 HPLC fitted with a diode array detector and UV detection with maximum absorbance at λ = 230 and λ = 245 nm. Chromatographic conditions were as follows: column EC 125/2 mm and precolumn, Nucleosil 120-5-C_{18}; flow rate 0.5 ml/min. Isocratic elution was performed with water/methanol at a volume ratio of (3:1) as the mobile phase. The dead time (t_0) of the system was determined using thiourea (as unretained substance) with a t_0 value of 0.728 min.

The retention factors under isocratic conditions, *k*, were calculated according to the equation $k = (t_R - t_0)/t_0$. The calibration lines for the RP-HPLC retention factors ($Y = A + B * X$, with $Y = \log k$ and $X = \log P_{ow}$) were established by linear regression with the reference data.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.05.019.

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