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Inactivation of the Human Papillomavirus-16 E6 Oncoprotein by Organic Disulfides

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Abstract—We are investigating compounds that could be useful in the treatment of neoplastic lesions of the cervix by acting on the oncoprotein E6 of human papillomavirus-16. The E6 protein contains two potential zinc-binding domains that are required for most of its functions. We have published tests that measure (i) the release of zinc ions after chemical alteration of the cysteine groups of these zinc-binding domains (TSQ assay), (ii) the interaction of E6 with the cellular proteins E6AP and E6BP (BIACORE assay), and (iii) the viability of tumor cell lines that require the continuous expression of HPV oncoproteins (WST1 assay). Based on these tests, we identified 4,4′-dithiodimorpholine as a potential lead compound. In this study we examined whether the dithiobisamine moiety of 4,4′-dithiodimorpholine may be an important molecular prerequisite for further drug development in this system. We have evaluated 59 new substances including organic disulfides and those containing the dithiobisamine moiety, as well as structural analogues. The compounds with significant reactivity in all three assays were observed only for dithiobisamine derivatives with saturated cyclic amines and aryl substituted piperazines. The identity of these substances suggests that the N–S–S–N moiety is necessary but not sufficient for reactivity in our assays, and that dithiobisamine based substances are useful as lead compounds that target the cysteine groups of HPV-16 E6 zinc fingers.

Introduction

Human papillomaviruses (HPVs) cause benign and malignant neoplasia of mucosal and cutaneous epithelia.[1,2] More than 80 HPV types are known, and they fall into various distantly related groups. Within each group, members are closely related to one another, infect similar target cells, and give rise to similar pathology.[3] Most research has been done on HPV-16 and HPV-18, the most prevalent viruses found in cervical cancer and its precursor lesions, and on HPV-6 and HPV-11, which cause genital warts. HPV genomes contain eight genes, among them the oncogenes E6 and E7. The oncoproteins E6 and E7 of different HPV types are homologous to one another, respectively, and have similar, but not completely identical functions. In the case of HPV-16, E6 and E7 are expressed in benign lesions, which maintain a productive viral infection, as well as in malignant lesions, which most often contain chromosomally integrated viral genomes.[4]

The function of these two oncoproteins is required for focus formation in cell culture,[5] establishment of benign neoplasia,[6] progression toward malignancy,[7] and maintenance of the malignant phenotype.[8,9] The E6 oncoprotein alone is sufficient to induce tumors in transgenic mice.[10] E6 proteins exert their function by interacting on the molecular level with a large number of cellular proteins, although they have only a small size, approximately 150 amino acids. Presently, protein–protein interactions have been reported between the E6 protein and p53, E6AP, E6BP, paxillin, CBP, AP-1, hDLG, IRF-3, Myc, hMCM7, Bak, and E6TP-1;[6,11–22] for reviews.[23,24] A major part of the function of the E7 protein apparently stems from its ability to interact with the cell cycle repressor Rb.[25]

The comparison of the E6 oncoproteins of different HPV types has identified numerous conserved amino acid residues. The most conspicuous motifs are two Cys-X2-Cys-X29-Cys-X2-Cys sequences (where X represents any amino acid and the number represents

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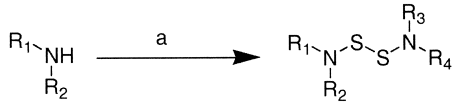
the number of residues), which are responsible for the ability of E6 proteins to bind zinc,[26] and are likely to establish zinc fingers with structures specific for these HPV proteins.[27] The E7 oncoprotein has a singular presumed zinc finger structure. Mutations of conserved amino acids, in particular mutation of the cysteine residues of the Cys-X2-Cys-X29-Cys-X2-Cys motifs, have most often pleiotropic functional consequences, suggesting that mutations of these amino acid residues lead to changes of the E6 structure that are incompatible with many or all of the above mentioned protein–protein interactions.[6,23]

These cysteine residues of E6 present themselves as targets for drugs, since their alteration by biochemical reactions should negatively affect structure and function in a manner similar to mutations. Papillomavirus proteins are likely to be much better targets for antiviral and anticancer drugs than the proteins of RNA viruses, since the genomes of papillomaviruses do not evolve as rapidly as RNA genomes, and remain completely unchanged over time spans as long as decades.[3] Therefore it is likely that anti-HPV drugs will be more consistently useful than drugs against RNA viruses.

We have recently described novel screens for anti-HPV-E6 drugs.[28] Our experiments were based on two assumptions. Firstly, we assumed that compounds that chemically alter any of the eight cysteine groups, either by oxidation or by formation of adducts of the thiol groups, would render E6 incapable of binding zinc and of maintaining its tertiary structure. Secondly, we established a hierarchy of tests that measured (1) the release of zinc ions from E6, (2) the ability of E6 to bind the cellular proteins E6AP and E6BP, and (3) the proliferation of cell lines whose survival depends on continuous expression of E6 and E7 from endogenous HPV genomes. In these tests we examined 36 compounds that might react with thiol groups, and we identified one particular substance, 4,4′-dithiodimorpholine (**C16**), as a powerful and specific inhibitor of E6 function. Based on these findings, we examined 59 new compounds in this study. Most of these substances were designed and synthesized based on the assumption that the dithiobisamine moiety of 4,4′-dithiodimorpholine is an important prerequisite for its reactivity, as it is generally known that the bond-disassociation energy of disulfide in the dithiobisamine is weaker than in the dithioether.[29] As a result, the formation of disulfide with the cysteine group is probably the driving force for such an exchange to take place. We further speculated that modification of the side chains around the N–S–S–N moiety might alter its reactivity and ability to access the E6 protein. Yet other substances were designed to test whether structurally analogous substances without the dithiobisamine moiety might fail to show reactivity with the E6 protein.



Scheme 1. (a) S_2Cl_2/−78 °C/ petroleum ether.

Results

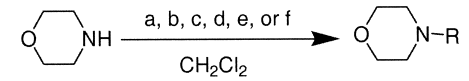
Design of organic disulfides with the potential to react with zinc coordinating cysteine thiol groups of HPV-16 E6

In our previous screen of disulfides, azoics and nitroso aromatics we identified only one compound, the dithiobisamine dithiodimorpholine (**C16**), with activity in three established assays and therefore as a potential lead compound.[28] We hypothesized that related dithiobisamine derivatives might have a similar or better reactivity with the E6 protein. To investigate this, we synthesized dithiobisamines with the general formula: R^1/R^2–N–S–S–N–R^3/R^4 and other disulfides derived from cyclic amines, acyclic amines, aryl substituted piperazine, aromatic amines, and additional analogues.

The synthesis of bisthioamines was carried out according to the condition reported by Katritzky et al.,[30] with some slight modifications. The secondary amine reacted readily with disulfur dichloride in petroleum ether to give the bisthioamines in 10–80% yield after purification (Scheme 1). In our hands, we found that a low temperature (−78 °C) and a very dilute reaction solution suppress the formation of side products very efficiently. Sometimes, co-solvent such as THF was added to increase the solubility of the amine in the reaction mixture. Purification and characterization by NMR and MS of the bisthioamines were carried out.

We have also designed and synthesized a series of structural analogues of bisthioamines **M50**–**M55** containing the morpholine moiety of **C16** but without the disulfide or the diamine. They were prepared by reacting the morpholine with phenyl isothiocyanate, 4-nitrophenyl chloroformate, naphthalene-1-sulfonyl chloride, benzoyl chloride, 1,1′-thiocarbonyl diimidazole and sulfuryl chloride respectively (Scheme 2). In the case of **M51**, 0.5 equivalent of 4-nitrophenyl chloroformate was used as our initial intention was to make a dimorpholine urea. However, **M51** was obtained because the carbamate was too stable to be displaced by another molecule of morpholine. In the synthesis of **M55**, the more reactive sulfuryl chloride was preferred over 1,1-sulfonyl diimidazole as it gave better yield.

Table 1 summarizes all substances examined in this study and introduces a shorthand nomenclature for this paper. It shows disulfides derived from (A) cyclic amines (**C16** and **M1**–**M11**) and aryl and arylalkyl substituted piperazines (**M12**–**M26**), (B) acyclic amines



Morpholine

M50, R = $C_6H_5NHC(S)$-,
M51, R = 4-$NO_2C_6H_4OC(O)$-,
M52, R = 1-$C_{10}H_7SO_2$-,
M53, R = $C_6H_5C(O)$-,
M54, R = 4-$OC_4H_8NC(S)$-.
M55, R = 4-$OC_4H_8NSO_2$-

Scheme 2. (a) C_6H_5NCS; (b) 4-$NO_2C_6H_4OC(O)Cl$; (c) 1-$C_{10}H_7SO_2Cl$; (d) C_6H_5COCl; (e) $C(S)Im_2$; (f) SO_2Cl_2.

(**M27**, **M29**–**M32**) and formamide (**M28**), and (C) formamidine (**M33**), imine and aromatic heterocycles (**M34**–**M40**), benzyl amines (**M41**–**M43**), and anilines (**M44**–**M46**). Table 1 also shows structurally analogous substances (C) (**M47**–**M59**), including thiuram disulfides (**M48**–**M49**) and compounds containing the morpholino moiety of **C16** but without the disulfide (**M50**–**M55**), piperidinomethane (**M56**) as well as other disulfides without the bisamine moiety (**M57**–**M59**).

Zinc ejection after in vitro treatment of HPV-16 E6 protein with organic disulfides

The TSQ assay measures the release of zinc after topological or chemical change of the E6 protein. Our assay conditions require the absence of glutathione, whose reducing power can antagonize the reactivity of the compound being tested.[28] Zinc release is expressed as a percentage of the total zinc available in the E6 protein that is determined by oxidizing all thiol groups with H_2O_2, a strong oxidizing agent. Table 1 shows the outcome of this experiment. Only two compounds, the dithiodiphthalimide **M47** and the thiuram disulfide **M49**, were as active as H_2O_2 in releasing zinc, and another nine substances in addition to the previously characterized 4,4′-dithiodimorpholine (**C16**) released between 50 and 100% of the zinc compared with the H_2O_2 value and were classified as compounds of high TSQ-activity. Ten substances released between 30 and 50% of the zinc and were classified as compounds with moderate TSQ-activity, 21 substances released between 10 and 30% of the zinc and were defined as compounds with little TSQ-activity, while 17 substances released less than 10% and can be considered inactive, as these values did not differ much from the TSQ fluorescence obtained with the solvent DMSO alone (6.1%).

Most of the compounds tested were colorless but we noted that some compounds with yellow color at 1 mM stock solutions such as **M18**, **M19** and **M44**–**M46** had TSQ values of 0%. When these compounds were examined for TSQ-activity in the presence of 1 mM $ZnCl_2$, reduced fluorescence was observed when compared to DMSO only (data not shown), suggesting that these compounds interfered with the TSQ-fluorescence.

Disulfides derived from cyclic amines (**C16** and **M1**–**M11**) were highly (**C16**, **M6** and **M8**–**M10**) or moderately active (**M1**–**M4** and **M7**) in the TSQ assay. Disulfides derived from aryl and arylalkyl substituted piperazines (**M12**–**M26**) were mostly inactive or had little activity, except for three compounds, **M12** and **M13** with high activity and **M14** with moderate activities. Among them, the halogen-substituted aryl and arylalkyl piperazine compounds (**M23**–**M26**) had particularly low TSQ values (less than 12%).

Within the group of acyclic amines and formamide (**M27**–**M32**) only two disulfides (**M27**–**M28**) showed high TSQ-activities whereas all the other compounds in this group had little activity. Similarly, 10 out of 14 disulfides derived from formamidine, imine, aromatic amines and heterocycles (**M33**–**M46**) had no or little activity, while **M33**, **M38**, **M39** and **M42** had moderate activity with 30–50% zinc release. Among these three, only **M42** had the N–S–S–N moiety. Within the heterogeneous group of structural analogues (**M47**–**M59**) we found that the dithiodiphthalimide **M47** and the thiuram disulfides (**M48**–**M49**) had the highest values in the TSQ assay, whereas all the other structural analogues were inactive (**M50**–**M59**).

Effects of compounds on the protein interaction between HPV-16 E6 and E6AP and E6BP

BIACORE technology allows real time analysis of protein–protein interactions without the need for labeling the reaction partners. It is based on the detection of small changes in the refractive index on the surface of a thin gold film coated with a dextran matrix on which one of the reactant proteins is covalently linked.[31] Here, we monitored the interaction of E6 with E6AP and with E6BP by passing GST-E6 incubated with the chemical compounds over immobilized E6AP or E6BP. The tests are based on the assumption that zinc-releasing compounds would lead to a change in the tertiary structure of E6 protein, which would result in the inability of E6 to form the heterologous complexes with E6AP and E6BP. In contrast to the TSQ assays, these reactions take place in the presence of glutathione and resemble the in vivo situation, where glutathione may undergo disulfide exchange reactions and compete as a substrate with the E6 protein.[28]

Of the 60 compounds examined for their ability to inhibit E6 binding to E6BP or E6AP, 23 compounds were observed to reduce the E6-E6BP and E6-E6AP interaction by at least 50% (Figure 1, Table 1). The majority of these compounds belonged to the group of cyclic amines (11 out of 12 compounds, **C16** and **M1**–**M10**) that also scored positive (>30% zinc release) in the TSQ assay, as well as from the aryl and arylalkyl piperazines (6 out of 10, **M12**, **M13**, **M15**–**M17** and **M19**). Consistent with the outcome of the TSQ assay, most of the compounds from groups B and C were not active in the BIACORE assay except the acyclic amines **M27** and **M30**, formamidine **M28**, ethyl *N*-benzylglycine ester disulfide **M41**, dithiodiphthalimide **M47** and thiuram disulfide **M48**. However, the other thiuram disulfide **M49** was not active in the BIACORE assays, even though it had highest activity in the TSQ assay.

Effects of the compounds on cell viability

In addition to studying the effects of these 60 compounds in in vitro assays, we also examined their effects on cell viability (WST1 assay). Previously, we observed that **C16** was able to induce cell death (apoptosis) specifically in HPV-infected cell lines apparently due to the increased stability of p53 after inactivation of the E6 protein.[28] Similarly, here we compared the effects of our new compounds on cell viability of HPV-positive cells (SiHa, HeLa, and CaSki) and HPV-negative cells (HaCat, MCF7 and HepG2). At a concentration of 50 μM, 17 of the compounds (**M5**, **M6**, **M8**, **M9**, **M20**, **M24**, **M27**, **M28**, **M34**, **M38**, **M41**, **M42**, **M44**, **M45** and **M47**–**M49**) were found to be unspecifically cytotoxic

Table 1. Evaluation of disulfides and other compounds by in vitro and cell-based assays

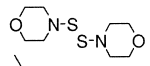
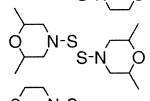
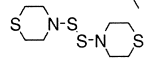
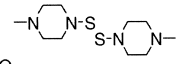
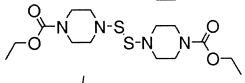
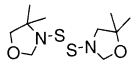
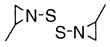
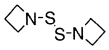
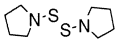
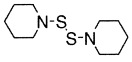
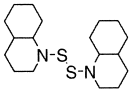
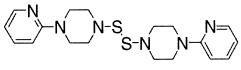
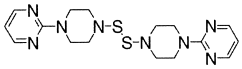
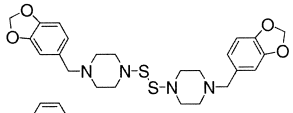
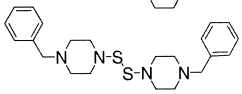
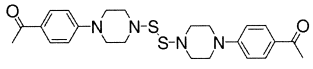
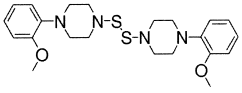
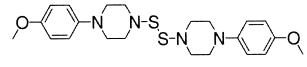
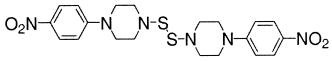
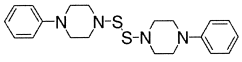
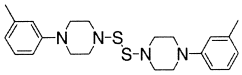
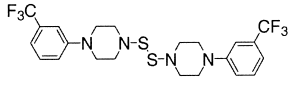
Compounds[a]		TSQ[b]	BIA[c]	WST[d]	WST-Spec[e]	Group[f]
A: Disulfides derived from cyclic amines						
C16		63	+	+	+	1
M1		40	+	+	+	1
M2		42	+	+	+	1
M3		45	+	+	+	1
M4		37	+	+	+	1
M5		28	+	+	–	4
M6		71	+	+	–	3
M7		31	+	–	–	3
M8		55	+	+	–	3
M9		51	+	+	–	3
M10		54	+	+	+	1
M11		19	–	–	–	5
M12		51	+	+	+	1
M13		67	+	+	+	1
M14		41	–	+	+	2
M15		24	+	+	+	2
M16		24	+	+	+	2
M17		12	+	+	+	2
M18		0[g]	–	+	+	4
M19		0[g]	+	–	–	4
M20		24	–	+	–	5
M21		19	–	–	–	5
M22		22	–	–	–	5

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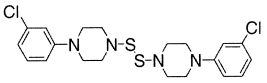
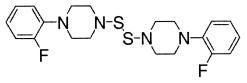
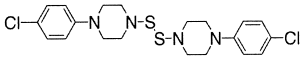
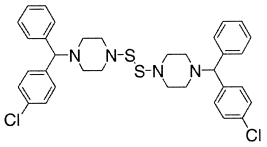
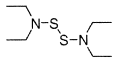
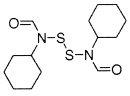
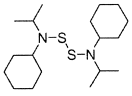
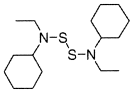
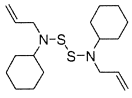
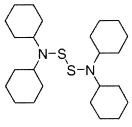
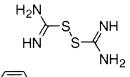
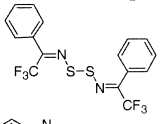
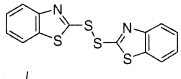
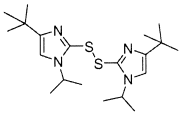
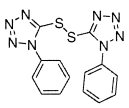
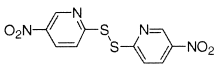
Table 1 (*continued*)

Compounds[a]		TSQ[b]	BIA[c]	WST[d]	WST-Spec[e]	Group[f]
M23		7	–	–	–	5
M24		12	–	+	–	5
M25		11	–	–	–	5
M26		9	–	–	–	5
B: Disulfides derived from acyclic amines and formamide						
M27		57	+	+	–	3
M28		55	+	+	–	3
M29		12	–	–	–	5
M30		11	+	–	–	4
M31		28	–	–	–	5
M32		15	–	–	–	5
C: Disulfides derived from aromatic amines, heterocycles and other analogues						
M33		33	–	–	–	4
M34		8	–	+	–	5
M35		3	–	–	–	5
M36		18	–	–	–	5
M37		0	–	–	–	5
M38		48	–	+	–	4

(*continued on next page*)

Table 1 (*continued*)

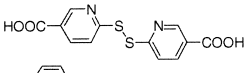
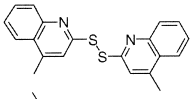
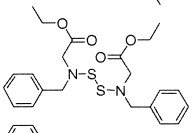
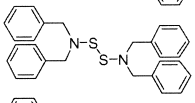
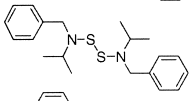
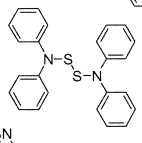
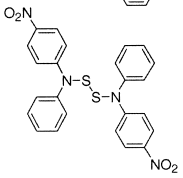
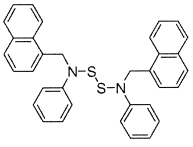
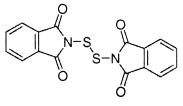
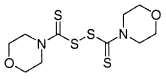
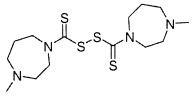
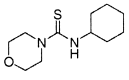
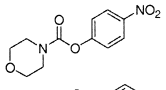
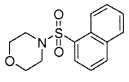
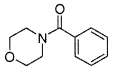
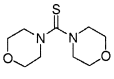
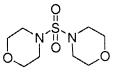
Compounds[a]		TSQ[b]	BIA[c]	WST[d]	WST-Spec[e]	Group[f]
M39		36	–	–	–	4
M40		18	–	–	–	5
M41		22	+	+	–	4
M42		38	–	+	–	4
M43		11	–	–	–	5
M44		0[g]	–	+	–	5
M45		0[g]	–	+	–	5
M46		0[g]	–	–	–	5
M47		103	+	+	–	3
M48		81	+	+ +	–	3
M49		106	–	+	–	4
M50		10	–	–	–	5
M51		6	–	–	–	5
M52		10	–	–	–	5
M53		7	–	–	–	5
M54		8	–	–	–	5
M55		9	–	–	–	5

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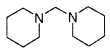
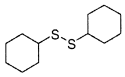
Table 1 (*continued*)

Compounds[a]		TSQ[b]	BIA[c]	WST[d]	WST-Spec[e]	Group[f]
M56		3	–	ND	ND	5
M57		1	–	–	–	5
M58		2	–	–	–	5
M59		13	–	–	–	5

[a]All the compounds are denoted by the letter M and a corresponding number, except dithiodimorpholine (**C16**).
[b]The value represents the % of zinc released compared to release by 0.3% H_2O_2. The concentration of compound used was 13 μM. The values represent 2–5 experiments done in duplicate with standard deviations of < 5%.
[c]This assay measures the compound's ability to interfere in the binding of E6 with the cellular coactivators E6BP and E6AP. A positive score indicates those compounds having values less than 50% of the binding observed in the presence of DMSO. See Figure 1 for values obtained.
[d]A positive score indicates that the compound is cytotoxic at 50 μM for at least one of the following cell lines: HeLa, SiHa, CaSki, HaCat, MCF7 and HepG2.
[e]A positive score indicates that the compound at 50 μM is cytotoxic for HPV-infected cells, HeLa, SiHa and CaSki, compared to the HPV-negative cell lines, HaCat, MCF7 and HepG2. See Figure 2 for details.
[f]The compounds have been categorized into five different groups: Group 1 includes those compounds with TSQ values greater than 30% that were positive in the BIACORE assay and specific in the WST1 assay. Group 2 consists of the compounds that were specific in the WST1 assay and either positive in the BIACORE assay or with TSQ values greater than 30%. Group 3 contains compounds with TSQ values greater than 30% and positive in the BIACORE assay, but were non-specifically cytotoxic. Group 4 is for those compounds that demonstrated activity in only one of the assays. Compounds in Group 5 were inactive in all three assay systems.
[g]These compounds were observed to interfere with TSQ activity in the presence of 1 mM $ZnCl_2$.

against the cells examined (Table 1 and Figure 2). 12 compounds in addition to C16 specifically affected cell viability in HPV-infected cells (Table 1, Figure 2). These 12 HPV-specific compounds consisted only of cyclic amines (**M1**–**M4** and **M10**), and aryl and arylalkyl substituted piperazines (**M12**–**M18**). These compounds were also active in the TSQ and/or BIACORE assays, except **M18**. No cytotoxicity was observed with the remaining classes of compounds. Surprisingly, we noted that many of the compounds that specifically inhibited the growth of HPV containing cell lines had an opposite stimulating effect on the growth of the control HepG2 cells (Fig. 2). The 30 compounds affecting specifically or unspecifically the cell viability were further examined at lower concentrations. Most compounds were found to be inactive at 1–10 μM, except the thiuram disulfide **M48**, which was still unspecifically toxic at 1 μM and inactive at 0.1 μM (data not shown).

Grouping of compounds based on their reactivity in the TSQ, BIACORE and WST1 assays

Based on the results from the three different assays, we have categorized the compounds tested into five groups (Table 1). Group 1 includes compounds that were specific in the WST1 assay (WST1-spec) and positive in the BIACORE assay with TSQ values greater than 30% (**C16**, **M1**–**M4**, **M10**, **M12** and **M13**). These groups represent the most promising compounds for further evaluation.

Group 2 consists of the compounds that were specific in the WST1 assay and were positive in either the BIACORE assay (**M15**–**M17**) or with a TSQ value greater than 30% (**M14**). Group 3 contains compounds (**M6**, **M7**, **M8**, **M9**, **M27**, **M28**, **M47** and **M48**) that were positive in BIACORE assay with TSQ values greater than 30%, but were not specific in the WST1 assay.

Group 4 is for those compounds that demonstrated activity in only one of the assays. These include those compounds with TSQ values greater than 30% (**M33**, **M38**, **M39**, **M42** and **M49**), positive in the BIACORE assay (**M5**, **M19**, **M30**, **M41**) or specific in the WST1 assay (**M18**). Group 5 includes the compounds that were inactive in all three assay systems.

Discussion

In an attempt to identify therapeutically useful new compounds for the treatment of human papillomavirus associated cervical cancer we previously established a hierarchy of assay systems that target the HPV E6 oncoprotein.[28] The initial screen of 36 substances included a large group of disulfide based organic compounds. These substances were chosen under the assumption that disulfide exchange reactions would either lead to adducts between the organic compound and the cysteine residues in E6, or to oxidation of two or more cysteine residues and disulfide bond formation within E6.[32] In both cases, the metal chelating property of the cysteine residues would be disrupted, the zinc ion ejected, and the integrity of the zinc finger disturbed. Among the disulfides that we examined, 4,4′-dithiodimorpholine was the only substance that scored positive in all three

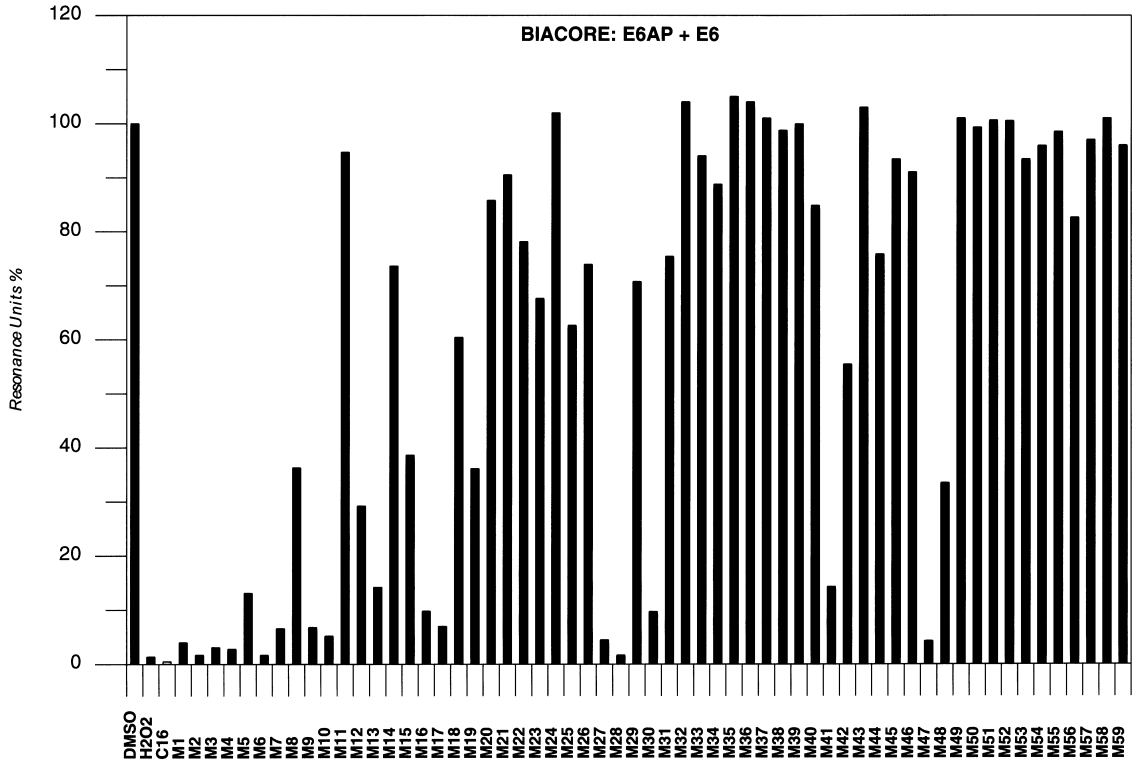


Figure 1. Interaction of HPV-16 E6 with E6AP measured with BIACORE technology. The interactions of E6 with E6AP were monitored using a BIACORE 2000. GST-E6AP was immobilized on a CM-5 sensor chip. HPV16 GST-E6 was incubated with 400 μM compound for 2 h and subsequently passed over the immobilized ligand in sequential flow mode. The amount of E6 bound to E6BP or E6AP was measured by the increase in resonance units after E6 was passed over the ligand. The values represent the % binding compared to the value obtained in the presence of DMSO only after subtracting the value obtained with GST alone (which represents non-specific binding). Similar results were obtained when E6BP was examined (data not shown).

tests. Furthermore, dithiodimorpholine specifically induced p53 protein levels, associated with the induction of apoptosis, and thereby inhibited specifically the growth of HPV-containing cell lines.[28] All these data are in agreement with established functions of E6.

Together with its ability (chemical reactivity) or access (bulkiness) to react with the E6-zinc-binding domain in vitro one additional crucial requirement in our initial screen, that led to the identification of dithiodimorpholine, was the resistance of compounds in vitro (BIACORE assay) and in cell culture (WST1 assay) towards GSH mediated inactivation.[28] Based on this observation, we hypothesized that the dithiobisamine moiety of this substance may render this disulfide bond specifically reactive both in the TSQ and BIACORE assays and in cultured cells and that related dithiobisamine derivatives may have a similar or better reactivity with the E6 protein.

We have now examined 59 structurally related compounds, most of them which were newly synthesized, in order to define the structural requirements necessary for their inhibition of HPV E6 activity. Based on their reactivity profile, we identified 21 compounds with activity $>30\%$ zinc release in the TSQ assay, of which 15 were also active in the BIACORE assay and 7 of these compounds in addition to dithiodimorpholine (**C16**) also specifically inhibited the growth of HPV containing cell lines (Table 1, Group 1 compounds). Those compounds that scored positive in all three assays were derivatives of monocyclic amines with the dithiobisamine moiety (**C16**, **M1**–**M4** and **M10**) or were pyridyl and pyrimidinyl piperazines with the dithiobisamine moiety (**M12** and **M13**). Dithiobisamines with acyclic amines, aromatic amines or other structural analogues were partially active in some of the assays, especially in releasing zinc from E6 (TSQ), but lacked in general activity in the BIACORE assay as well as the ability to specifically inhibit the growth of HPV containing cell lines. Many of these compounds were inactive in the BIACORE as well as in cell culture, which might indicate their inactivation under GSH conditions.[28] This possibility is excluded for compounds that were active in the BIACORE assay and unspecifically toxic in cell culture (**M5**, **M6**, **M8**, **M9**, **M27**, **M28**, **M41**, **M47** and **M48**) or only unspecifically toxic in cell culture (**M20**, **M24**, **M34**, **M38**, **M42**, **M44**, **M45** and **M49**). Probably these unspecific toxic compounds affect cellular zinc binding proteins that are crucial for cell survival. There are three compounds (**M7**, **M19** and **M30**) that are only active in the BIACORE, but lack toxicity in cell culture. Potentially the penetration of these compounds into cells is inefficient or another possibility could be the intracellular stability of these compounds being affected by additional factors than GSH.

The mechanism of reaction between the disulfide and the zinc-finger is not fully understood. There are a few possibilities. Firstly, it might be an electrophilic attack on the sulfur atoms of the zinc-coordinated cysteines of

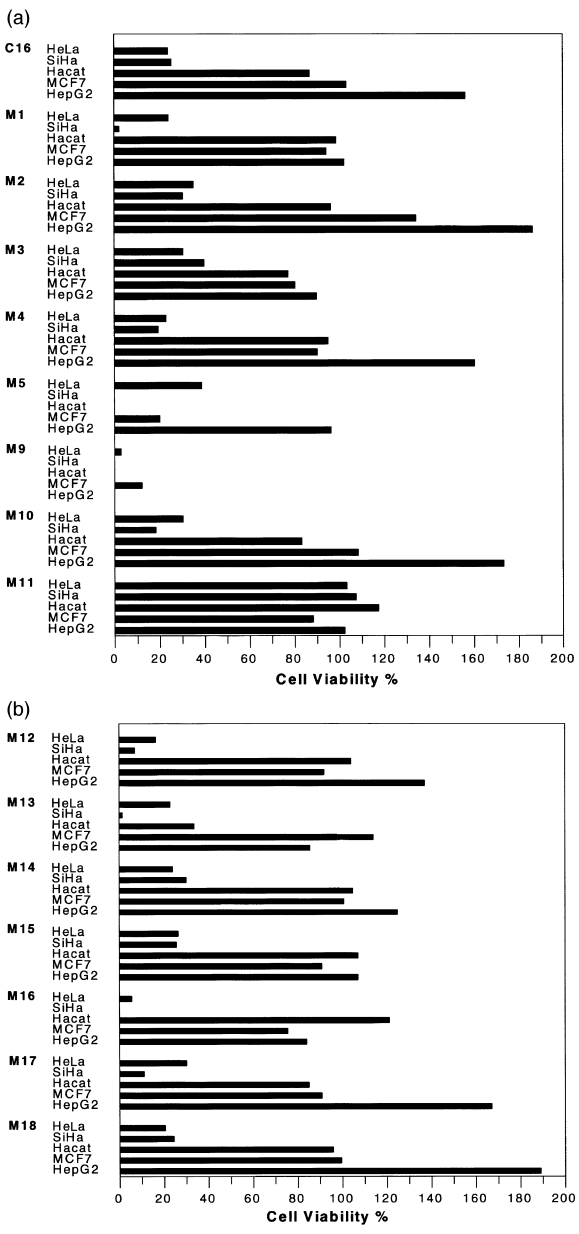


Figure 2. Viability assays of HPV-positive (HeLa, SiHa) and HPV-negative (HaCat, MCF7 and HepG2) cell lines incubated with dithiobisamine compounds. Indicated cell lines were incubated with 50 μM of dithiobisamine compounds containing cyclic amine moieties (a) **C16**, **M1**–**M11** or (b) aryl substituted piperazine moieties **M12**–**M18** for two times in three days as described.[28] Cell viability was determined through absorption readings of WST1, a tetrazolium salt that measured the activity of mitochondrial dehydogenase (WST1 assay). Several of the compounds in (a) **C16**, **M1**–**M4** and **M10** and (b) **M12**–**M18** demonstrated substantial and specific inhibition of cell viability in HPV-positive cervical epithelial tumor cell lines (HeLa and SiHa), but had little or no effect on HPV-negative cell lines, immortalized skin epithelial cells (HaCat) and mammary and liver epithelial cancer cells (MCF7 and HepG2 respectively), except **M13** which did not affect MCF7 and HepG2 only. Other dithiobisamine compounds with cyclic amine moieties such as **M5** and **M9** demonstrated unspecific cytotoxicity towards HPV-positive and HPV-negative cell lines or did not inhibit cell proliferation of any cell line examined as observed with **M11**. CaSki, another HPV-positive cell line, demonstrated similar profiles of cell viability observed with the HeLa and SiHa cells after treatment with compounds (data not shown). All values were normalized to the values obtained in the presence of dimethyl sulfoxide (DMSO) which was set as 100%. Values represent 2–8 independent experiments with at least duplicated measurements, and standard deviations of $< 10\%$.

E6 by the disulfide.[33] Secondly, the disulfide bond of dithiobisamine might cleave to generate an R1R2NS radical which reacts further with the sulfur atoms of the zinc finger resulting in mixed disulfides. Thirdly, it might also be a combination of both the electrophilic and radical reactions. Our finding that compounds **M50**–**M56** were inactive was not at all surprising, since these compounds are not capable of undergoing electrophilic or radical reactions.

Based on our results, we have found that the dithiobisamine moiety N–S–S–N is crucial but not sufficient for activity and specificity. While dithiodimorpholine (**C16**) was active, structural analogues with the morpholino ring such as thiourea (**M50**, **M54**), carbamate (**M51**) sulfonamide (**M52**), amide (**M53**), and sulfone (**M55**) derivatives were inactive. Similarly, while dithiodipiperidine (**M10**) was active, structural analogues that do not contain the disulfide or amine such as piperidinomethane (**M56**), cyclohexyldisulfide (**M57**), *trans*-1,2-dithiane-4,5-diol (**M58**) and 3H-1,2-benzodithiol-3-one, 1,1-dioxide (**M59**) were also found to be inactive. Finally, the most active compounds (Group 1) are derived from a six-membered saturated cyclic amine. While the Group 1 compounds differed in their extent of zinc release (37–67%, Table 1) and inhibition of the complex formation between E6 and E6AP or E6BP (Fig. 1), they were similar in activity at 50 μM with respect to the specific inhibition of HPV-infected cells (Fig. 2).

In addition to dithiobisamines we have also examined thiuram disulfides (**M48** and **M49**) with a cyclic amine ring including the morpholino ring of **C16** in **M48**. These compounds showed highest reactivity in the TSQ assay, in contrast to our previous study with tetraethylthiuram disulfide (Disulfiram), which had only moderate activity in the TSQ assay.[28] Thiuram disulfides containing ring structures such as cyclopentamethylene rings were also reported to be highly reactive with cysteine groups of the human immunodeficiency virus 1 (HIV-1) p7 zinc finger.[34] In this study, the compounds were inactive in in vivo experiments determining the replication of HIV-1. In our studies, **M48** was found to be positive in the BIACORE assay and cytotoxic to all the cell lines tested even at 1 μM, suggesting that the compound is highly resistant to intracellular inactivation (i.e., by GSH) compared to the compounds identified in Group 1. Because thiuram disulfides and the metabolite dithiocarbamates have been implicated in a variety of therapeutic applications including chemoprevention,[32,35–37] inflammation,[38] anticancer enhancers,[39] inhibition of HIV-progression,[40] anti-hepatotoxic activity[41] and inhibition of cisplatinum nephrotoxicity,[42] derivatives of **M48** should be of interest for further evaluation.

Human papillomaviruses are associated with more than 95% of cervical cancers. Cervical cancer is the second-most common cancer among women worldwide and the leading cancer among women in Africa, Asia, and South America with over 300,000 deaths per year. The current treatment for advanced cervical cancer is surgery, followed in some cases by radiation treatment.

Recently, it has been reported that the addition of the drug cisplatin with radiation treatment reduced the risk of death by 30 to 50% in the first few years after diagnosis of cervical cancer.[43–45] While these results are quite dramatic, cisplatin is a non-specific drug with known side effects[42] and induces p53 in both HPV-positive and HPV-negative cells.[46,47] In contrast, dithiodimorpholine (C16) strongly induced p53 only in HPV-positive cells,[28] suggesting that dithiobisamine-based compounds may provide greater specificity than cisplatin in the treatment of cervical cancer. Furthermore, now we define a specific class of dithiobisamine compounds including cyclic amines and aryl substituted piperazines that inactivate the HPV-16 E6 protein and induce cell death specifically in HPV-infected cells. Hopefully, further evaluation of these compounds and their derivatives will allow for a potent therapeutic agent against HPV related diseases.

Experimental

Chemicals and reagents

All reagents were from commercial sources unless otherwise mentioned. The compounds **M6**, **M7**, **M28** and **M34** were obtained from the collection of the National Cancer Institute, Bethesda, Maryland. **C16** was purchased from Tee Hai Chemicals. Compounds **M33**, **M35**–**M38**, **M40**, **M47**, **M48** and **M56**–**M59** were obtained from Aldrich. **M39** and **M49** were from Sigma and Acros Organics, respectively.

Dithiobisamines

The synthesis of all other dithiobisamines not mentioned above was according to Katritzky et al.,[30] with modifications as described. A solution of the secondary amine (0.5 mmol) in petroleum ether (40 mL) was precooled to −78 °C before disulfur dichloride (10 μL, 0.125 mmol) was added. The solution was vigorously stirred for 15 min at −78 °C and another 30 min at room temperature. Water (20 mL) was added and the desired compound was extracted into the organic phase using diethyl ether (3×10 mL). The combined organic layers were dried over magnesium sulfate, filtered and concentrated in vacuo. The compound was purified on preparative TLC plate. High-resolution mass spectra were obtained with a VG7035. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker DMX 400 and 100 MHz respectively. Only data for Group 1 compounds are reported below. The preparative TLC solvent system used for **M1** and **M2** was 12.5% ethyl acetate in hexane, for **M4**, **M10** and **M12** was 25% ethyl acetate in hexane and for **M3** was 9% methanol in chloroform.

Di-2,6-dimethylmorpholin-4-yl disulfide (M1). Yellow solid. Yield: 13.5 mg (37%). **M1** was a mixture of diastereoisomers. ^{1}H and ^{13}C NMR were identical to those reported.[30] HRMS calcd for $C_{12}H_{24}N_2O_2S_2$ (M^+) 292.12793, found 292.12886.

Dithiomorholin-4-yl disulfide (M2). Yellow solid. Yield: 12.3 mg (37%). ^{1}H NMR ($CDCl_3$) δ: 2.73 (br t, J = 4.9 Hz, 8H), 3.10 (br t, J = 4.9 Hz, 8H). ^{13}C NMR ($CDCl_3$) δ: 28.8, 58.2. HRMS calcd for $C_8H_{16}N_2S_4$ (M^+) 268.01962, found 268.01968.

Di-4-methylpiperazinyl disulfide (M3). Yellow solid. Yield: 22.4 mg (68%). ^{1}H NMR ($CDCl_3$) δ: 2.25 (s, 6H), 2.45 (br s, 8H), 2.82–2.84 (m, 8H). ^{13}C NMR ($CDCl_3$) δ: 45.78, 45.82, 55.57. HRMS calcd for $C_{10}H_{22}N_4S_2$ (M^+) 262.12860, found 262.12728.

Di-4-(1-ethoxycarbonyl)piperazinyl disulfide (M4). White solid. Yield: 15.4 mg (33%). ^{1}H NMR ($CDCl_3$) δ: 1.27 (t, J = 7.1 Hz, 6H), 2.78 (br s, 8H), 3.53 (br s, 8H), 4.14 (q, J = 7.1 Hz, 4H). ^{13}C NMR ($CDCl_3$) δ: 14.6, 44.1, 55.5, 61.6, 155.3. HRMS calcd for $C_{14}H_{26}N_4O_4S_2$ (M^+) 378.13956, found 378.13895.

Dipiperidyl disulfide (M10). Yellow solid. Yield: 18.7 mg (65%). The ^{1}H NMR spectrum of **M10** was slightly differed from those reported by Diaz. ^{1}H NMR ($CDCl_3$) δ: 1.36–1.43 (m, 4H), 1.63–1.70 (m, 8H), 2.78 (br t, J = 5.3 Hz, 8H). ^{13}C NMR ($CDCl_3$) δ: 22.8, 27.0, 57.4. HRMS calcd for $C_{10}H_{20}N_2S_2$ (M^+) 232.10680, found 232.10680.

Di-4-(2-pyridyl)piperazinyl disulfide (M12). Yellow syrup. Yield: 27.9 mg (58%). ^{1}H NMR ($CDCl_3$) δ: 2.92–2.96 (m, 8H), 3.60–3.61 (m, 8H), 6.63–6.66 (m, 4H), 7.47–7.51 (m, 2H), 8.18–8.19 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 45.8, 55.4, 107.1, 113.6, 137.5, 147.9, 159.0. HRMS calcd for $C_{18}H_{24}N_6S_2$ (M^+) 388.15039, found 388.15038.

Di-4-(pyrimidin-2-yl)piperazinyl disulfide (M13). White solid. Yield: 25.1 mg (51%). ^{1}H NMR ($CDCl_3$) δ: 2.87 (br t, J = 4.5 Hz, 8H), 3.88 (br t, J = 4.8 Hz, 8H), 6.50 (t, J = 4.7 Hz, 2H), 8.30 (d, J = 4.7 Hz, 4H). ^{13}C NMR ($CDCl_3$) δ: 44.1, 55.5, 110.1, 157.7, 161.3. HRMS calcd for $C_{16}H_{22}N_8S_2$ (M^+) 390.14090, found 390.14205.

(Cyclohexylamino)morpholin-4-ylmethane-1-thione (M50). To a solution of morpholine (87 μL, 1 mmol) in anhydrous CH_2Cl_2 was added cyclohexyl isothiocyanate (142 μL, 1 mmol) and Et_3N (70 μL, 0.5 mmol). The reaction mixture was stirred for 3 h. Aminomethylated resin (20 mg, 1.46 mmol/g Novabiochem) was added and the mixture was stirred for another 2 h. The mixture was filtered and then quenched with water. The compound was extracted with CH_2Cl_2, dried over $MgSO_4$ and filtered. The filtrate was concentrated and purified on preparative TLC plate (50% ethyl acetate in hexane) to give **M50** as yellow crystals (127 mg, 56%).

^{1}H NMR ($CDCl_3$) δ: 1.13 (dd, J = 3.5 Hz, 12.0 Hz, 1H), 1.19 (dd, J = 3.5 Hz, 7.4 Hz, 1H), 1.43 (qt, J = 3.2 Hz, 12.3 Hz, 2H), 1.65 (dt, J = 3.5 Hz, 12.1 Hz, 2H), 1.71 (dt, J = 3.6 Hz, 13.5 Hz, 2H), 2.11 (br dd, J = 3.2 Hz, 6.1 Hz, 2H), 3.72–3.75 (m, 4H), 3.77–3.79 (m, 4H), 4.34–4.36 (m, 1H), 5.37 (d, J = 7.0 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ: 24.9, 25.5, 33.0, 47.2, 54.3, 66.1, 171.5. HRMS calcd for $C_{11}H_{20}N_2OS$ (M^+) 228.12964, found 228.12958.

4-Nitrophenyl morpholine-4-carboxylate (M51). To an ice-cooled solution of morpholine (87 μL, 1 mmol) in

anhydrous CH_2Cl_2 was added 4-nitrophenyl chloroformate (100 mg, 0.5 mmol) and Et_3N (140 μL, 1 mmol). The mixture was allowed to warm up to room temperature and stirred for 3 h. The reaction was quenched with water and extracted with CH_2Cl_2. The organic layer was combined, dried over $MgSO_4$ and filtered. The filtrate was concentrated and purified on preparative TLC plate (50% ethyl acetate in hexane) to yield **M51** as white crystal (73 mg, 58%).

^{1}H NMR ($CDCl_3$) δ: 3.59 (br t, J = 4.4 Hz, 2H), 3.69 (br t, J = 4.0 Hz, 2H), 3.76 (t, J = 4.6 Hz, 4H), 7.30 (d, J = 15.3 Hz, 2H), 8.26 (d, J = 15.3 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ: 44.2, 44.9, 66.3, 66.5, 122.2, 125.1, 145.0, 152.3, 156.0. HRMS calcd for $C_{11}H_{12}N_2O_5$ (M^+) 252.07462, found 252.07570.

4-(Naphthylsulfonyl)morpholine (M52). To an ice-cooled solution of morpholine (87 μL, 1 mmol) in anhydrous CH_2Cl_2 was added naphthalene-1-sulfonyl chloride (226 mg, 1 mmol) and Et_3N (140 μL, 1 mmol). The reaction was stirred for 3 h at room temperature. The same work up protocol for **M50** was used. **M52** was isolated as white powder (138 mg, 50%).

^{1}H NMR ($CDCl_3$) δ: 3.16 (br t, J = 4.7 Hz, 4H), 3.69 (br t, J = 4.7 Hz, 4H), 7.55–7.68 (m, 3H), 7.94 (dd, J = 1.1 Hz, 7.5 Hz, 1H), 8.10 (d, J = 8.2 Hz, 1H), 8.21 (dd, J = 1.1 Hz, 7.4 Hz, 1H), 8.77 (d, J = 8.7 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ: 45.6, 66.2, 124.1, 125.1, 126.9, 128.2, 128.9, 129.0, 130.8, 131.8, 134.3, 134.7. HRMS calcd for $C_{14}H_{15}NO_3S$ (M^+) 277.07727, found 277.07636.

Morpholin-4-yl-phenyl ketone (M53). An ice-cooled solution of morpholine (87 μL, 1 mmol), benzoyl chloride (116 μL, 1 mmol) and Et_3N (140 μL, 1 mmol) in anhydrous CH_2Cl_2 was stirred for 10 min before warming up to room temperature. Stirring was continued for another 3 h at room temperature and purification was carried out following the same protocol for **M50** to yield **M53** as translucent syrup (86 mg, 45%).

^{1}H NMR ($CDCl_3$) δ: 3.45 (br s, 2H), 3.54 (br s, 2H), 3.78 (br s, 4H), 7.40–7.45 (m, 5H). ^{13}C NMR ($CDCl_3$) δ: 66.9, 127.1, 128.5, 129.9, 135.2, 170.2. HRMS calcd for $C_{11}H_{13}NO_2$ (M^+) 191.09464, found 191.09745.

Di-morpholin-4-ylmethane-1-thione (M54). A mixture of morpholine (87 μL, 1 mmol), 1,1′-thiocarbonyl diimidazole (87 mg, 0.5 mmol) and Et_3N (70 μL, 0.5 mmol) in anhydrous CH_2Cl_2 was stirred at room temperature for 3 h. The same work up protocol as for **M51** was used. **M54** was isolated as pale yellow powder (67 mg, 62%).

^{1}H NMR ($CDCl_3$) δ: 3.61 (br t, J = 4.8 Hz, 8H), 3.74 (br t J = 4.8 Hz, 8H). ^{13}C NMR ($CDCl_3$) δ: 52.0, 66.4, 174.8. HRMS calcd for $C_9H_{16}N_2O_2S$ (M^+) 216.09325, found 216.09590.

Di-morpholin-4-yl sulfone (M55). An ice-cooled mixture of morpholine (87 μL, 1 mmol), sulfuryl chloride (40 μL, 0.5 mmol) and Et_3N (140 μL, 1 mmol) in anhydrous CH_2Cl_2 was stirred for 10 min before warming up to room temperature. The reaction mixture was stirred for 1 h at room temperature and purified using the same protocol for **M51** to yield **M55** as pale yellow powder (26 mg, 22%).

^{1}H NMR ($CDCl_3$) δ: 3.16 (br s, 4H), 3.87 (br t, J = 4.8 Hz, 4H). ^{13}C NMR ($CDCl_3$) δ: 47.6, 65.2. HRMS calcd for $C_8H_{16}N_2O_4S$ (M^+) 236.08308, found 236.08454.

Expression of E6, E6AP and E6BP as GST-fusion proteins. E6, E6AP (amino acids 391–408) and E6BP-Glutathione *S*-transferase (GST) fusion proteins were prepared as described.[28] For direct use of GST-fusion proteins bound on glutathione–Sepharose beads in the zinc-release assay, the glutathione–Sepharose beads were resuspended in PBS, Tris, pH 8.2. GST-fusion proteins for BIACORE analysis were eluted with elution-buffer (10 mM GSH, 50 mM Tris, PBS, pH 8.2).

Zinc release assays. Release of zinc from HPV-16-E6 was monitored by the change in fluorescence of the zinc-selective fluorophore TSQ ((*N*-6-methoxy-8-quinolyl)-*p*-toluenesulfonamide, Molecular Probes) by published procedures[48,49] as modified by us.[28] In brief, 9 μg (1 μM) recombinant GST-E6 protein, bound to glutathione–Sepharose beads, was incubated with 13 μM compound or 0.3% H_2O_2 in TSQ-assay buffer (10 mM sodium phosphate buffer, pH 7.0, 10% glycerol) for 2 h at room temperature (200 μl total volume in 96-well plates). Immediately after addition of TSQ to a concentration of 100 μM, the increase in fluorescence was measured with a Tecan platereader (355 nm excitation filter and 460 nm emission filter).

BIACORE assays. Binding of GST-E6 to GST-E6BP, GST-E6AP and GST was monitored by surface plasmon resonance (SPR) on a BIACORE 2000 (BIACORE AB) as described.[28] Briefly, purified ligand (GST, GST-E6AP and GST-E6BP) was covalently amine coupled to a CM-5 sensor chip. Typically 6000–10,000 RU of GST, E6BP and E6AP were immobilized on three different flowcells. Aliquots of purified HPV-16 GST-E6 were incubated with either a 400 μM solution of the examined compound or 0.6% (170 mM) H_2O_2 for 2 h at room temperature, and injected at 1 μl/min over the immobilized ligands. Interactions between GST-E6 and the ligands were monitored by the change of resonance signal in arbitrary units (RU). In between each sample, the surfaces were regenerated with a 1-minute pulse of 50 mM NaOH.

Determination of cell viability (WST1 assay). For this study we used three cervical cancer cell lines: HeLa, SiHa, and CaSki, that contain HPV genomes. Additional cell lines studied include: HaCat, an immortalized skin epithelial cell line, MCF7, a mammary epithelial tumor cell line, and HepG2, a liver epithelial tumor cell line. Cell viability was determined as previously described.[28]

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