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Short Communication

Lopinavir shows greater specificity than zinc finger ejecting compounds as a potential treatment for human papillomavirus-related lesions

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ABSTRACT

Non-surgical, antiviral treatment options are desirable for HPV-related lesions within the genitourinary and upper digestive tract. We compared the toxicity of three zinc finger-ejecting (ZFE) compounds (4,4-dithiodimorpholine, azodicarbonamide, and diamide) to the HIV protease inhibitor lopinavir using HPV-positive SiHa, CaSki, HeLa, ME180, and HPV-negative C33A cervical carcinoma cell lines as well as primary human foreskin keratinocytes (PHFKs). Colorimetric growth assays revealed selective toxicity when treated with lopinavir. All carcinoma cell lines, except CaSki, were sensitive to 20 μM lopinavir whereas primary PHFKs were highly resistant. In contrast, 4,4-dithiodimorpholine was uniformly toxic to all cells tested while azodicarbonamide and diamide showed no effect at all. It is concluded that lopinavir may be an attractive candidate to treat pre-cancerous and cancerous HPV-positive lesions.

1. Introduction

Cervical cancer pathogenesis is mediated by human papillomavirus (HPV)-encoded oncoproteins E6 and E7. These viral proteins deregulate cell cycle and apoptosis pathways, eventually leading to malignant transformation (Wise-Draper and Wells, 2008). E6 binds to the E6-associated protein ubiquitin–protein ligase (E6AP) to form an ubiquitin ligase complex that binds to and targets the pro-apoptotic protein p53 for degradation (Scheffner et al., 1990). In addition to target p53, E6 binds to and regulates other cellular proteins involved in apoptosis, genomic stability and cell polarity (Pim and Banks, 2010). Previous studies have also documented that expression of E7 without E6 promotes apoptosis in cells with a functional p53 pathway (Aguilar-Lemarroy et al., 2002; Stöppler et al., 1998; Jones et al., 1997). Thus, inhibiting E6 alone as an antiviral therapy may eliminate HPV-positive carcinoma cells. The two zinc fingers of the E6 protein play an important functional role in transformation since mutations within these domains inhibit the interaction between E6 and its cellular targets such as the E6AP and the E6-binding endoplasmic reticulum calcium-binding protein (E6BP/ERC-55) Dalal et al., 1996; Nakagawa et al., 1995.

Several different antiviral approaches have been documented that either inhibit E6 directly or its related functions. An organic disulfide 4,4-dithiodimorpholine (DTDM) was identified as a potential lead compound that ejects zinc ions from the zinc finger domains of E6 thus significantly altering that protein's structure and function (Beerheide et al., 1999, 2000). DTDM also reduced cell viability associated with increased p53 expression and PARP cleavage in the HPV-positive cell lines CaSki, SiHa and HeLa but not in the HPV-negative cell lines HaCat, MCF7 and HT3. However, although DTDM inhibited cell viability and induced apoptosis, its effect was modest as the concentration used was high (50 or 100 μM). The compound azodicarbonamide (Azo) was investigated as another E6 zinc finger inhibitor that targets Cys-Cys-His-Cys zinc finger domains of the nucleocapsid p7 protein (Beerheide et al., 1999). Although Azo promoted *in vitro* release of zinc from E6, there was no effect on the binding of E6 to E6AP or E6BP or on the viability of HPV-positive cell lines (Beerheide et al., 1999). *In silico* chemical mapping of the E6-binding LxxΦLsh motif, found in E6 target proteins, was used to identify compounds with similar structural characteristics (Liu et al., 2004; Baleja et al., 2006). The rationale was that these would competitively inhibit the binding of E6 to its cellular targets. Several compounds were identified

Abbreviations: HPV, human papillomavirus; PHFK, primary human foreskin keratinocyte.

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by this approach although the outcome was not ideal since concentrations as high as 500 μM were needed for activity (Baleja et al., 2006).

Efficient anti-HPV therapies are lacking. As a potential solution, the HIV protease inhibitor lopinavir (Rice et al., 1997) has been shown to stabilize the p53 protein and induce apoptosis in HPV16-positive SiHa cells (Hampson et al., 2006). Lopinavir has also been shown to inhibit the proteasome (Piccinini et al., 2002) which is consistent with the anti-HPV effects observed in our previous study (Hampson et al., 2006). We have extended these studies on lopinavir by comparing its effects to ZFE compounds using a range of cervical carcinoma cell lines in addition to primary human foreskin keratinocytes (PHFKs).

2. Materials and methods

2.1. Cell lines and tissue culture

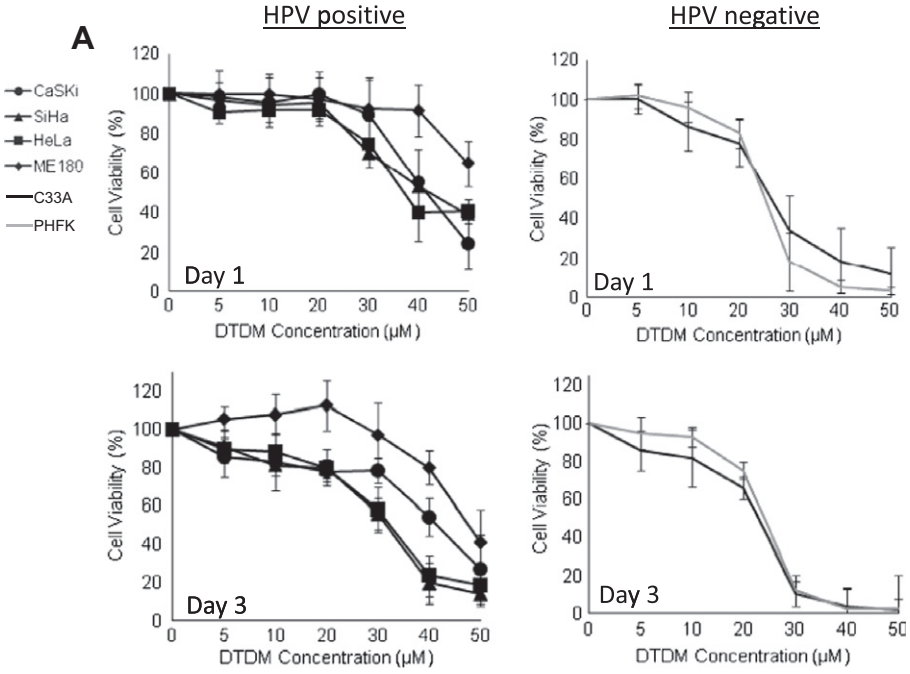
Cervical cancer-derived cell lines HeLa, CaSki, SiHa, ME180 and C33A (ATCC, Rockville, MD, USA) were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma–Aldrich, Oakville, ON, CA) with 10% heat-inactivated fetal bovine serum (FBS, PAA Laboratories, Etobicoke, ON, CA) and 1% antibiotic/antimycotic (Gibco, Grand Island, NY, USA) and incubated at 37 °C with 5% CO_2. CaSki and SiHa are HPV16-positive, HeLa are HPV18-positive, ME180 are HPV39-positive and C33A are devoid of HPV. PHFKs isolated from normal neonatal foreskin were incubated in PHFK growth medium (Cell Applications, San Diego, CA, USA) at 37 °C with 5% CO_2.

2.2. ZFE compounds and lopinavir

4,4-dithiodimorpholine (DTDM; CAS: 103-34-4), azodicarbonamide (Azo; CAS: 123-77-3), diamide (CAS: 10465-78-8) (all from Sigma–Aldrich) and lopinavir (Abbot Laboratories, Chicago, IL, USA) were used from 100 mM DTDM, 200 mM Azo, 20 mM Diamide and 20 mM lopinavir stock solutions.

2.3. Cell viability

Two thousand cells were seeded per well in a 96 well plate, incubated for 24 h and then treated with DTDM, Azo, Diamide or lopinavir. Cell viability was determined after treatment by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium



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Cell Type	LD50 (μM)		
	24 hr	48 hr	72hr
PHFK	25.1	25.8	24.0
C33A	26.4	28.9	22.9
SiHa	42.3	35.1	31.6
HeLa	37.2	36.2	32.3
CaSki	41.8	44.1	41.5
ME180	>50	>50	47.7

Fig. 1. (A–B) 4,4-dithiodimorpholine (DTDM) negatively affects cell viability of tumor and non-tumor cells. Cells negative for HPV (PHFK and C33A), and positive for HPV 16 (CaSki, SiHa), HPV 18 (HeLa) and HPV39 (ME180) were treated with increasing concentration of DTDM. Cell viability was measured 24, 48 and 72 h later by the MTT assay and expressed as a mean percent average relative to buffer treated cells (±SD, experiment replicates $n = 3$ for cancer cell lines, $n = 2$ for PHFKs) (A). The LD50's are indicated in the table (B).

bromide) assay: 20 μL of 5 mg/ml MTT (Sigma–Aldrich) dissolved in Dulbecco's phosphate buffered saline (PBS) (Sigma–Aldrich) was added to each well containing 200 μL of media and incubated for 4 h. Media/MTT was then aspirated and the formazan product was solubilized in 100 μL dimethyl sulfoxide (DMSO) (Fisher Scientific, Waltham, MA, USA). Absorbance values at 570 nm with background subtraction at 650 nm were obtained with the PowerWave XS microplate spectrophotometer (Bio-Tek, Winooski, VT, USA).

2.4. Statistical analysis

For calculating statistically significant differences, one-way ANOVA and the Dunnett's Multiple Comparison Test were used. Values $p < 0.05$ were considered to be significant.

3. Results

3.1. ZFE's compounds are equally toxic to PHFKs and cervical carcinoma cell lines

Previous studies investigated the effects of compounds that inhibit zinc binding to the E6 protein on CaSki, SiHa and HeLa cells (Beerheide et al., 1999, 2000). Since relatively high concentrations of DTDM inhibited cell viability and induced apoptosis, this prompted us to further investigate DTDM efficacy at lower concentrations and with a larger panel of cells, including PHFKs, which represent an ideal HPV-negative, non-tumorigenic control cell type. Cervical carcinoma cell lines used in these experiments included CaSki, SiHa, HeLa, ME180 and HPV-negative C33A.

All cells were treated with a single dose (5–40 μM) of DTDM and viability was measured 1, 2 and 3 days later by the MTT assay. At all time points, ME180 was more resistant to 30 and 40 μM DTDM relative to other cervical carcinoma cell lines. CaSki cells were slightly more resistant to DTDM when compared to SiHa and HeLa after 3 days at 40 μM (Fig. 1A) whereas HPV-negative C33A and control PHFKs were uniformly sensitive at 30 μM. The LD50's for the response of cells to DTDM are shown in Fig. 1B with similar results observed after 4 and 6 days (data not shown). These experiments demonstrated that DTDM had no preferential toxicity against tumor cells or PHFKs. Control PHFKs were the most sensitive to DTDM which indicates that that this compound may non-specifically inhibit zinc finger proteins other than E6.

The ZFE Azo has been previously shown to have no effect on cervical carcinoma cell lines (Beerheide et al., 1999) although the tetra-methylated form of Azo (diamide) has not been previously tested in an HPV context. Diamide compounds have previously been suggested as drugs for systemic malignancies such as leukemia (Landis-Piwowar et al., 2006). We postulated that the additional methyl groups of diamide may improve cell penetration. Yet, similar to Azo, no significant effect on cell viability was observed in cells treated for up to 3 days with 5–40 μM of diamide (Fig. 2A and B) and similar results were seen with both Azo and diamide after 1 and 2 days (data not shown).

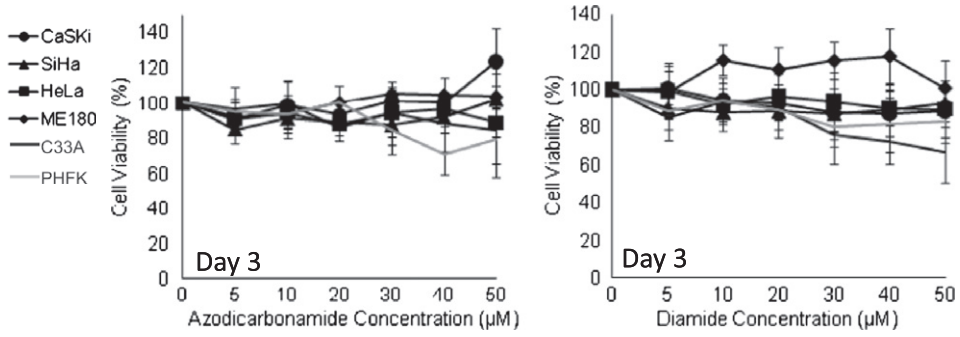


Fig. 2. Azodicarbonamide and diamide do not affect cell viability of tumor or non-tumor cells. Cells negative for HPV (PHFK and C33A), and positive for HPV 16 (CaSki, SiHa), HPV 18 (HeLa), and HPV39 (ME180) were treated with increasing concentration of azodicarbonamide and diamide. Cell viability was measured 72 h later by the MTT assay and expressed as a mean percent average relative to buffer treated cells (±SD, experiment replicates $n = 3$ for cancer cell lines, $n = 2$ for PHFKs) for cells treated with Azodicarbonamide or with diamide.

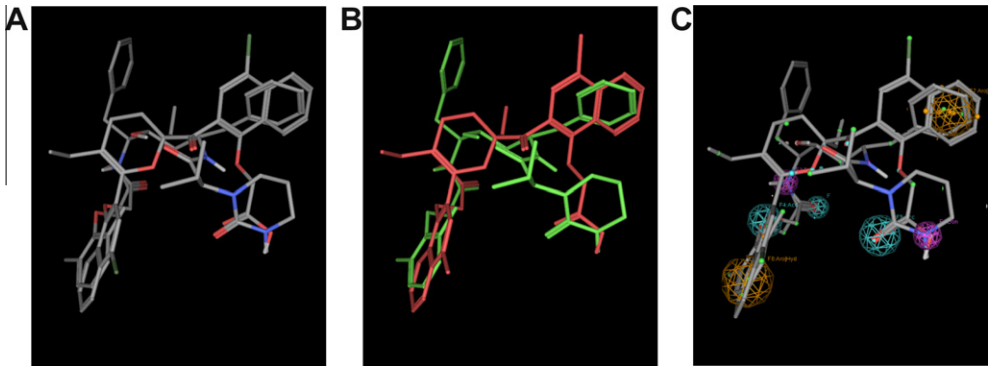
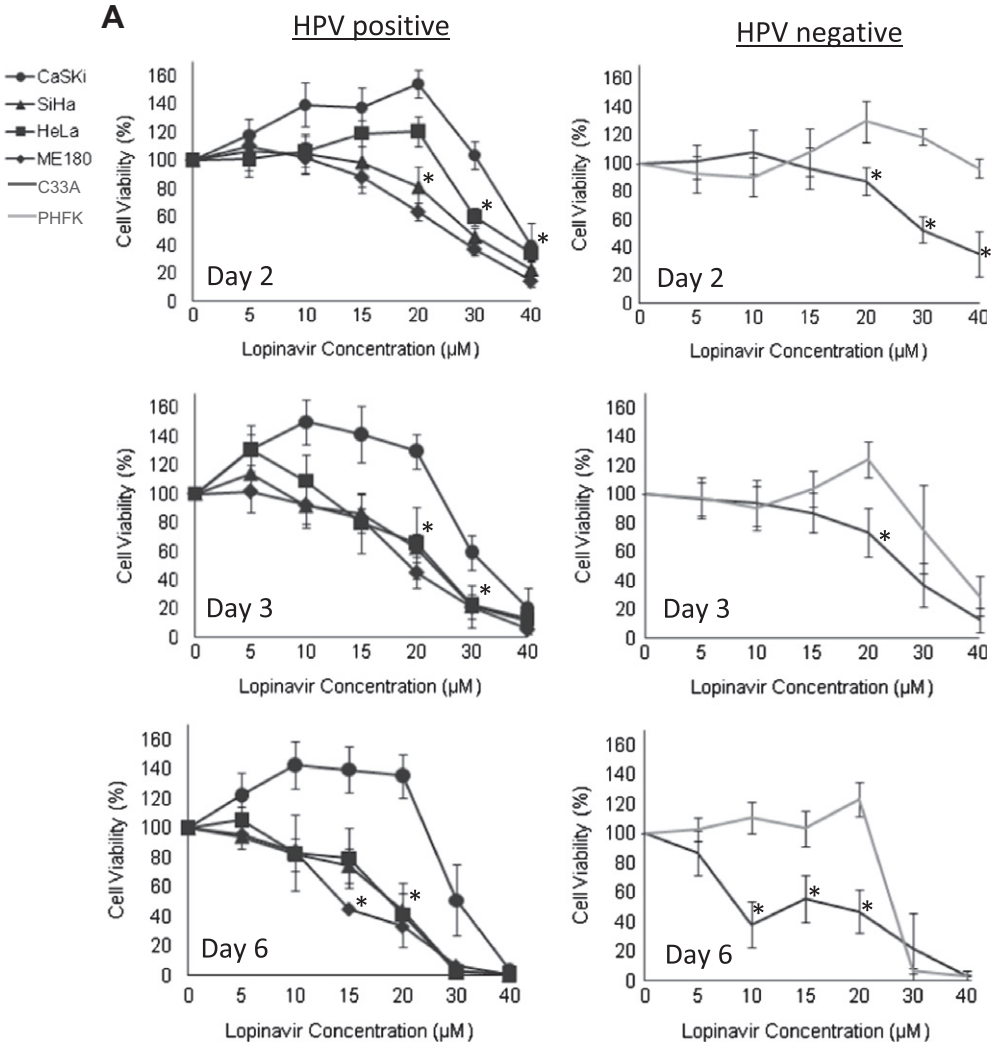


Fig. 3. (A–C) Flexible alignment of lopinavir and the compound NSC83143. (A) Alignment of lopinavir and the compound NSC83143 using the default parameters of the flexible alignment module of MOE2008 (Chemical Computing Group) non-polar hydrogens removed for clarity. (B) Alignment of lopinavir (green) and NSC83143 (red). (C) Pharmacophore features in common with the two structures with the proposed alignment. Blue spheres: hydrogen bond acceptors, purple spheres: hydrogen bond donors, orange spheres: aromatic or hydrophobic regions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Lopinavir kills cervical cancer-derived cell lines but spares PHFKs

We have shown that the anti-retroviral drug lopinavir inhibits HPV16 E6-mediated p53 degradation as evidenced by the induction of apoptosis in the HPV-positive cervical carcinoma cell line SiHa (Hampson et al., 2006). Moreover, our recently published data demonstrated that E6/E7-immortalized keratinocytes are considerably more sensitive to lopinavir than their mortal parental counterpart (Batman et al., 2011) indicating that immortalized cells per se are susceptible to lopinavir killing. Interestingly, a three dimensional alignment (MOE 2008, Chemical Computing Group) showed a good overlap between lopinavir and a compound previously shown to inhibit the E6-E6AP interaction (NSC83143) Baleja et al., 2006. This suggested a common binding site for both of these



B

Cell Type	LD50 (μM)		
	2 days	3 days	6 days
PHFK	>40	35.5	26.3
C33A	31.6	26.45	18.2
SiHa	28.8	23	19
HeLa	33.9	23.7	18.85
CaSki	38.4	32.35	30.2
ME180	25.2	19.4	14.4

Fig. 4. (A–B) Lopinavir affects cell viability of tumor and non-tumor cells differently. Cells negative for HPV (PHFK and C33A), and positive for HPV 16 (CaSki, SiHa), HPV 18 (HeLa), and HPV39 (ME180) were treated with increasing concentrations of lopinavir. Cell viability was measured 2, 3 and 6 days later by the MTT assay and expressed as mean percent average relative to buffer treated cells (±SD, experiment replicates $n = 3$ for cancer cell lines, $n = 3$ for PHFKs) (A). The LD50's are indicated in the table (B).

pharmacophores (Fig. 3A–C) and provided a possible explanation for the mechanism of action of lopinavir against HPV16 E6 in SiHa cells (Hampson et al., 2006). In light of these previous studies the toxicity of lopinavir (5–40 μM) was evaluated against the four HPV-positive and one HPV-negative cervical carcinoma cell lines and PHKFs (Fig. 4A). After two days exposure to 40 μM lopinavir, control PHFKs were still resistant whereas CaSki and HeLa cells were resistant up to 30 and 20 μM lopinavir, respectively. SiHa, ME180 and C33A were the most sensitive and all of these showed a significant decrease in cell viability with exposure to 20 μM lopinavir. After 3 and 6 days exposure to 20 μM lopinavir, the viability of SiHa, HeLa, ME180 and C33A was significantly lower than that of PHFKs and CaSki although for concentrations >20 μM, cell viability was significantly decreased for all the cell types tested. The LD50's for the response of cells to lopinavir are shown in Fig. 4B.

4. Discussion

Unlike the ZFE's DTDM, Azo and diamide, the protease-inhibitor lopinavir has selective toxicity against non-tumor and tumor cells of epithelial origin. Of the five tested cervical cancer cell lines, four were responsive to lower doses of lopinavir, whereas most significantly, control PHFKs were highly resistant. This toxicity profile indicates that lopinavir could provide an attractive anti-viral and anti-cancer treatment regimen for a number of HPV-related premalignant and malignant lesions within the genitourinary and upper digestive tract. Furthermore, in support of this new indication we have also recently shown that lopinavir up-regulates expression of the interferon-inducible antiviral protein ribonuclease L in HPV immortalized and transformed cells (Batman et al., 2011).

HPV-negative C33A cells were also sensitive to lower doses of lopinavir than control PHFK's, indicating that its selective toxicity is not exclusively restricted to HPV-transformed or -immortalized cells. This is in concordance with the observation that PHFK's immortalized by ectopic expression of hTert were sensitive to lower doses of lopinavir (<20 μM) than parental PHFKs (Gavin Batman, Ian Hampson and Lynne Hampson, unpublished results).

How can the resistance of normal PHFKs and CaSki carcinoma cells to lopinavir be explained? Selective inhibition of the proteasome can have different effects on the induction of apoptosis depending on the cell type under study (Liu et al., 2007) and it is becoming clear that the proteasome plays an important role in controlling the apoptotic process being pro-apoptotic in most cell types studied (Orlowski, 1999). However, some cells are prevented from undergoing proteasome inhibitor-induced apoptosis although the mechanism for this effect is not yet clear. It has been suggested that this could be associated with the ability of cells to enter a quiescent state or result from the differential activity of the pro-apoptotic c-Jun NH_2-terminal kinase (JNK) Yu et al., 2004. Another possibility with regard to CaSki cells is the increased levels of viral gene expression. CaSki cells contain up to 600 integrated HPV16 copies per cell (Shibata et al., 1988) whereas SiHa cells contain only one or two (Friedl et al., 1970). This results in higher levels of E6 expression in CaSki cells which may quantitatively impact on drug efficiency (Batman et al., 2011).

Our results have clear implications for the potential use of lopinavir as a topical treatment for HPV-related lesions and may benefit HIV-positive patients in whom HPV-induced lesions is a serious problem. Lopinavir could also be useful for the treatment of HPV-unrelated malignancies. This is not without precedent since several HIV protease inhibitors have been shown to be active against various types of cancer (Bernstein and Dennis, 2008). However, careful pre-clinical studies followed by phase 1 clinical trials are necessary to prove lopinavir's use within an HPV context.

Conflicts of interest statement

Research grade lopinavir was provided to the Hampson group as a gift from Abbot Laboratories, Chicago, Illinois, USA and subsequently provided to the Zehbe group via a Material Transfer Agreement.

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