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Original Article

In vitro Activity of Novel 1,3-Oxazole Derivatives against Human Papillomavirus

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Abstract

Background: Chemotherapeutic approaches to the control of HPV infection suffer from a lack of specificity. For most existing HPV inhibitors, the weak antiviral effects observed in cellular assays suggest that further improvements in selecting targets, in drug potency, and in bioavailability and cell uptake are required. **Objective:** To synthesize novel 1,3-oxazole derivatives and define their antiviral activities against the human papillomavirus (HPV) *in vitro*. **Methods:** Determination of transient replication of an HPV-11 in transfected HEK293 cells, and HPV-18 DNA amplification in an organotypic squamous epithelial raft culture of primary human keratinocytes (PHKs), and cytotoxicity assays were used. **Results:** Bioassays showed that the synthesized compounds 2, 4, 5, and 9 exhibited potent antiviral activity against low-risk HPV-11 (IC50 = 1.7–9.6 μM) in a transient DNA replication assay and exhibited low cytotoxicity in HEK293 cells compared to cidofovir (CDV), antiviral agent in clinical use. Selectivity indices of compounds 4 and 5 were 20–40 times greater than that of CDV. However, compounds 4 and 9 did not exhibit a significant antiviral effect against high-risk HPV-18 infections in organotypic epithelial raft cultures. Although prophylactic HPV vaccines are now available to protect against primary infections by the seven genotypes most commonly found in cervical, penile, anal and oro-pharyngeal cancers (HPV16, 18, 31, 33, 45, 52, and 58) and two genotypes (HPV6 and 11) that cause benign anogenital warts and laryngeal papillomas, they do not protect against infections by other HPV types. Moreover, individuals already infected with HPV will not benefit from the vaccines. Thus, the need for antiviral agents to treat HPV-associated diseases remains great, but few options currently exist. **Conclusions:** We show that substituted 1,3-oxazole derivatives are a promising structure class of chemical compounds for the development of antiviral drugs against HPV lesions.

Keywords: 1,3-oxazole, antiviral discovery, HEK293 cells, organotypic epithelial raft cultures, papillomavirus DNA replication, primary human keratinocytes

Introduction

Antibiotics are effective since they act against bacterial structures that are not present in eukaryotic cells or they selectively inhibit essential activities such as protein translation based on the differences between microbial and eukaryotic ribosomes. With respect to antiviral agents, prophylactic vaccinations to stimulate host immune responses against virus particles are very successful. In contrast, research into antiviral drugs for existing infections by human papillomaviruses (HPVs) remains in relatively early stages. Agents that target host cell enzymes and pathways on which HPVs depend have the possibility of off-target effects with toxicities. It is difficult to find active molecules that selectively inhibit HPV replication or destroy virus-infected cells but leave the uninfected host cells and tissues without damage.

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Nonetheless, opportunities remain as there are modified biochemical activities in virus-infected cells that offer potential therapeutic targets. The antiviral drug should interfere with a virus or host function such that the virus cannot replicate or, alternatively, the agent induces apoptosis of virus-infected cells only. An ideal drug should have high aqueous solubility, good stability, and efficient uptake into cells infected with the virus. All papillomavirus infections are in cutaneous or mucosal epithelia. Most primary lesions can be addressed topically, locally, and at safe and effective doses without resorting to

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oral or intravenous delivery. Local treatment avoids a potential problem with toxicity to liver and other internal organs. It should not be cytotoxic, carcinogenic, allergenic, mutagenic, and teratogenic although some toxicity of an antiviral drug may be acceptable. Unfortunately, many inhibitors that are effective *in vitro* fail to be effective *in vivo*.

Specificity against virus replication is a key issue in chemotherapy. Because of the close interaction between viral reproduction and normal cellular metabolism, it was originally thought difficult to interrupt the viral life cycle without adversely affecting the host cell metabolism. However, it is now clear that several events associated with viral replicative amplification either do not occur in normal uninfected cells or are controlled by virus-specified enzymes that differ structurally and functionally from the corresponding host cell enzymes. Until recent years, there were rather few chemotherapies for HPV infections.[1] For example, cidofovir (CDV) (its active metabolite, CDV diphosphate) marketed as Vistide is an acyclic nucleoside phosphonate which nonspecifi cally blocks viral replication because of more effi cient conversion of the prodrug to the active precursor in virus-infected cells or by selective inhibition of a viral DNA polymerase.[2] Cidofovir also inhibits human polymerases, but this action is 60–80 times weaker than its actions on viral DNA polymerases.[3] Topical gel application or direct intralesional injections of CDV may have some efficacy (complete and permanent remissions) against HPV lesions *in vivo*.[3] Other small molecule inhibitors of HPV lesions with some selectivity but not absolute anti-HPV specificity include injectable interferon-alpha, the interferon inducer imiquimod, sinecatechins (Veregen), podophyllotoxin (a topoisomerase II inhibitor, marketed as Condylox), and some other natural products.[4]

Substituted oxazole derivatives can exert various biological effects such as antibacterial, antifungal, anticancer, antitubercular, and anti-inflammatory activities.[5-8] Some DNA binding activity of oxazoles has been demonstrated.[9] Various substituted oxazoles have significant antiviral activity.[10]

One of the major conditions for evaluating compounds with antiviral activity against HPV is the choice of suitable *in vitro* models. Such assay systems should allow the monitoring of both cytotoxicity and HPV DNA replication in the presence of antiviral agents. HPVs show a high degree of tropism for cutaneous or mucosal squamous epithelial cells. Individual HPV genotypes display specificities for anatomic site that lead to distinct histopathologies of the associated lesions. Organotypic epithelial raft culture systems support all the stages of squamous differentiation and enable the complete HPV life cycle.[11] Raft cultures of primary human keratinocytes (PHKs) harboring complete viral genomes have been a core format for HPV investigations. However, raft culture experiments have complexities and labor intensiveness that preclude screening a large number of chemical inhibitors. For higher throughput assays, transformed HEK293 cells are easy to grow in culture and to cotransfect with plasmid DNAs expressing HPV E1 and E2 replication proteins or carrying the viral replication origin to test for transient amplification of the origin-containing plasmids.

The present study is an exploratory investigation of anti-HPV activity by novel 1,3-oxazole derivatives designed and synthesized in Kyiv, with all the cytotoxicity and efficacy tests performed at UAB. The evaluation was carried out under the auspices of the National Institute of Allergy and Infectious Diseases (USA) and its Collaborative Antiviral Testing Group.

Methods

Synthesis of 1,3-oxazole derivatives

A series of 1,3-oxazole derivatives [Table 1] was designed and synthesized according to published procedures, as noted.

Compound 1 was synthesized following a procedure described in the literature.[12] Yield: 87%. Colorless powder, m.p. 115–116°C (EtOH). IR (KBr, ν_{max}, cm^{-1}): 1162, 1346 (SO_2). ^1H NMR (400 MHz, DMSO-d_6): δ 0.96 (s, 3H, CH_3), 2.38 (s, 2H, CH_2), 2.55 (s, 4H, $2CH_2$), 3.31 (s, 4H, $2CH_2$), 7.57 (s, 3H, ArH), 7.62 (s, 3H, ArH), 8.00 (s, 2H, ArH), 8.11 (s, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6): 12.33, 46.59, 51.70, 52.00, 125.97, 126.12, 127.08, 129.10, 129.22, 129.83, 131.14, 132.26, 134.04, 152.32, 159.82. EI-MS, m/z: 398 $[M + H]^+$. Found, %: C, 63.55%; H, 5.75%; N, 10.25%; S, 8.11%. Calc. for $C_{21}H_{23}N_3O_3S$: C, 63.46%; H, 5.83%; N, 10.57%; S, 8.07%.

Compound 2 was synthesized following a procedure described in the literature.[13] Yield: 87%. Colorless powder, m.p. 130–131°C (EtOH). IR (KBr, ν_{max}, cm^{-1}): 1161, 1352 (SO_2). ^1H NMR (400 MHz, DMSO-d_6): δ 0.85 (d, J_{HH} = 6.0 Hz, 3H, CH_3), 0.96–1.12 (m, 1H, CH), 1.48–1.71 (m, 4H, $2CH_2$), 2.49–2.55 (m, 1H, CH), 2.85–2.83 (m, 1H, CH), 3.58–2.67 (m, 2H, CH_2), 7.57 (s, 3H, ArH), 7.63 (s, 3H, ArH), 8.00 (s, 2H, ArH), 8.11 (s, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6): 19.11, 24.82, 30.81, 31.94, 46.86, 53.23, 126.00, 126.18, 127.08, 129.10, 129.12, 129.84, 131.09, 132.23, 134.73, 151.81, 159.71. EI-MS, m/z: 383 $[M + H]^+$. Found, %: C, 65.91%; H, 5.87%; N, 7.45%; S, 8.48%. Calc. for $C_{21}H_{22}N_2O_3S$: C, 65.95%; H, 5.80%; N, 7.32%; S, 8.38%.

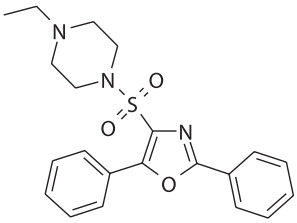
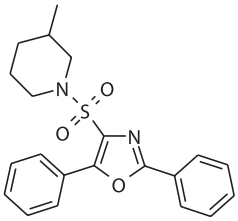
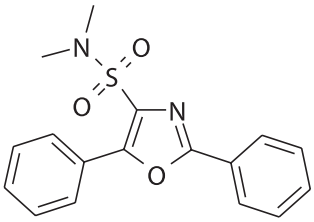
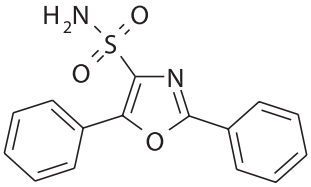
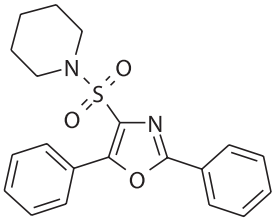
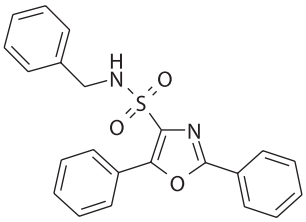
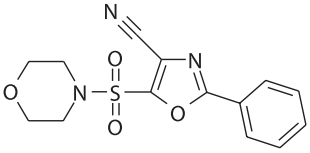
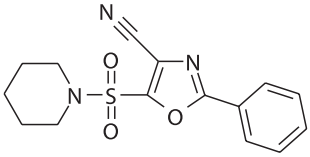
Compounds 3-6 has been described in the literature.[12]

Compounds 7 and 8 have been described in the literature.[13]

Compound 9 was synthesized following a procedure described in the literature.[14] Yield: 72%. Colorless powder, m.p. 227–228°C (EtOH). IR (KBr, ν_{max}, cm^{-1}): 1174, 1377 (SO_2); 2253 (CN); 3262, 3360 (NH_2). ^1H NMR (500 MHz, DMSO-d_6): δ 7.65–7.72 (m, 3H, ArH), 8.05 (s, 2H, ArH), 8.75 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): 111.46, 114.93, 124.66, 127.63, 130.11, 133.57, 156.18, 162.75. EI-MS, m/z: 248 $[M - H]^-$. Found, %: C, 47.95%; H, 2.87%; N, 16.99%; S, 12.87%. Calc. for $C_{10}H_7N_3O_3S$: C, 48.19%; H, 2.83%; N, 16.86%; S, 12.86%.

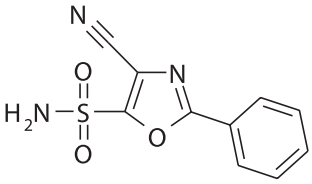
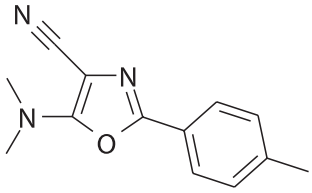
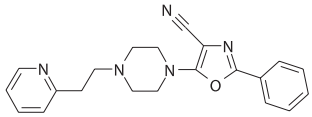
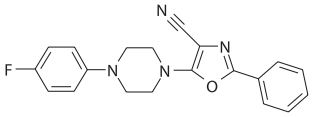
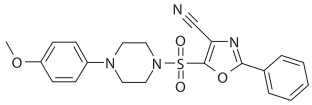
Compound 10 was synthesized following a procedure described in the literature.[14] Yield: 72%. Colorless powder, m.p. 140–141°C (EtOH). IR (KBr, ν_{max}, cm^{-1}): 1638; 2201 (CN). ^1H NMR (400 MHz, DMSO-d_6): δ 2.36 (s, 3H, CH_3), 3.17 (s, 6H,

Table 1: 1,3-oxazole derivatives synthesized

Compound number	Structural formula	Name
1		1-[(2,5-diphenyl-1,3-oxazol-4-yl) sulfonyl]-4-ethylpiperazine
2		1-[(2,5-diphenyl-1,3-oxazol-4-yl) sulfonyl]-3-methylpiperidine
3		*N*, *N*-dimethyl-2,5-diphenyl-1,3-oxazole-4-sulfonamide
4		2,5-diphenyl-1,3-oxazole-4-sulfonamide
5		1-[(2,5-diphenyl-1,3-oxazol-4-yl) sulfonyl] piperidine
6		*N*-benzyl-2,5-diphenyl-1,3-oxazole-4-sulfonamide
7		5-(morpholin-4-ylsulfonyl)-2-phenyl-1,3-oxazole-4-carbonitrile
8		2-phenyl-5-(piperidin-1-ylsulfonyl)-1,3-oxazole-4-carbonitrile

Contd...

Table 1: Contd...

Compound number	Structural formula	Name
9		4-cyano-2-phenyl-1,3-oxazole-5-sulfonamide
10		5-(dimethylamino)-2-(4-methylphenyl)-1,3-oxazole-4-carbonitrile
11		2-phenyl-5-[4-(2-pyridin-2-ylethyl) piperazin-1-yl]-1,3-oxazole-4-carbonitrile
12		5-[4-(4-fluorophenyl) piperazin-1-yl]-2-phenyl-1,3-oxazole-4-carbonitrile
13		5-{[4-(4-Methoxyphenyl) piperazin-1-yl] sulfonyl}-2-phenyl-1,3-oxazole-4-carbonitrile

2CH_3), 7.32 (d, J_{HH} = 7.5 Hz, 2H, ArH), 7.74 (d, J_{HH} = 7.5 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6): 21.51, 38.86, 84.54, 117.24, 123.52, 125.62, 130.14, 140.59, 150.28, 161.22. EI-MS, m/z: 228 [M + H]$^+$. Found, %: C, 68.55%; H, 5.61%; N, 18.45%. Calc. for $C_{13}H_{13}N_3O$: C, 68.71%; H, 5.77%; N, 18.04%.

Compound 11 was synthesized following a procedure described in the literature.[14] Yield: 72%. Colorless powder, m.p. 94–95°C (EtOH). IR (KBr, ν_{max}, cm^{-1}): 1617; 2216 (CN). ^{1}H NMR (400 MHz, $CDCl_3$): 2.73 (s, 4H, 2CH_2), 2.89 (s, 2H, CH_2), 3.07 (s, 2H, CH_2), 3.68 (s, 4H, 2CH_2), 7.15 (s, 1H, ArH), 7.27 (s, 1H, ArH), 7.47 (s, 3H, ArH), 7.65 (s, 1H, ArH), 7.88 (s, 2H, ArH), 8.56 (s, 1H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6): 35.32, 46.56, 51.77, 57.97, 86.23, 117.21, 121.85, 123.68, 125.84, 126.05, 129.63, 130.94, 136.89, 149.44, 150.80, 160.75. EI-MS, m/z: 360 [M + H]$^+$. Found, %: C, 70.10%; H, 5.88%; N, 19.80%. Calc. for $C_{21}H_{21}N_5O$: C, 70.18%; H, 5.89%; N, 19.48%.

Compound 12 was synthesized following a procedure described in the literature.[14] Yield: 70%. Colorless powder, m.p. 139–140°C (EtOH). IR (KBr, ν_{max}, cm^{-1}): 1625; 2214 (CN). ^{1}H NMR (400 MHz, DMSO-d_6): 3.28 (s, 4H, 2CH_2), 3.75 (s, 4H, 2CH_2), 7.05–7.12 (m, 4H, ArH), 6.90 (s, 3H, ArH), 6.90 (s, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6): 46.46, 48.78, 86.52, 115.81, 116.03, 116.06, 116.59, 118.49, 118.57, 125.89, 126.05, 126.17, 129.65, 130.99, 147.93, 150.94, 160.78. EI-MS, m/z: 349 [M + H]$^+$. Found, %: C, 68.70%; H, 4.97%; N, 16.31%. Calc. for $C_{20}H_{17}FN_4O$: C, 68.95%; H, 4.92%; N, 16.08%.

Compound 13 was synthesized following a procedure described in the literature.[13] Yield: 75%. Colorless powder, m.p. 189–190°C (EtOH). IR (KBr, ν_{max}, cm^{-1}): 1162, 1379 (SO_2); 2246 (CN). ^{1}H NMR (500 MHz, DMSO-d_6): 3.17 (s, 4H, 2CH_2), 3.51 (s, 4H, 2CH_2), 3.68 (s, 3H, OCH_3), 6.83 (d, J_{HH} = 8.0 Hz, 2H, ArH), 6.90 (d, J_{HH} = 8.0 Hz, 2H, ArH), 7.65–7.71 (m, 3H, ArH), 8.07 (s, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6): 45.97, 49.94, 55.67, 111.33, 114.81, 118.32, 118.82, 124.98, 127.93, 129.98, 133.57, 145.07, 150.93, 154.08, 164.21. EI-MS, m/z: 425 [M + H] +. Found, %: C, 59.37%; H, 4.75%; N, 13.01%; S, 7.56%. Calc. for $C_{21}H_{20}N_4O_4S$: C, 59.42%; H, 4.75%; N, 13.20%; S, 7.55%.

Primary assay: Transient replication of an human papillomavirus-11 replication origin-containing plasmid in transfected HEK293 cells

In this assay, vectors for expression of an HPV genotype-matched set of viral E1 protein (a replicative DNA helicase) and E2 protein (a DNA binding protein that recruits the E1 helicase to the HPV origin of replication) along with an HPV ori-containing plasmid are cotransfected into HEK293 cells, a human embryonic kidney epithelial cell line transformed by adenovirus *in vitro*, as described.[15] Cells were cultured in the absence or presence of the test compounds at 1, 10, and 100 μM added 4 h posttransfection for a total exposure of 44 h. Low-molecular-weight DNA was harvested 2 days posttransfection and digested with restriction endonuclease Dpn1 and exonuclease III to remove transfected plasmid DNA that had not replicated. The Dpn 1-resistent replicated DNA was then subjected to real-time quantitative polymerase chain reaction (qPCR) analyses in triplicate. Two controls

were performed. One was to omit the E1 expression vector to provide a background amount of undigested and unreplicated DNA. The other, positive control was treatment with the known inhibitor CDV. As embryonic kidney cells may have a different susceptibility to an agent compared to human foreskin fibroblast (HFF), a toxicity assay based on cell viability at the time of harvest on day 2 was performed alongside each transient replication assay in HEK293 cells. More than $1 \cdot 10^5$ cells were scored in a BioRad Automatic Cell Counter, with a determination of the total numbers, number of trypan blue stained (dead) cells, and % of dead cells.

To determine whether each compound has antiviral activity which exceeds its level of toxicity in HEK293 cells, the concentration of each compound at which virus replication was inhibited by 50 percent (effective concentration, EC_{50}), and the concentration of drug cytotoxic for 50% of the cells (CC_{50}) was calculated by regression analysis. A selectivity index was calculated according to CC_{50}/EC_{50}. CDV was tested for relative comparison.

Secondary assay: Human papillomavirus-18 DNA amplification in an organotypic squamous epithelial raft culture of primary human keratinocytes freshly prepared from neonatal foreskins

PHKs harboring whole genomic HPV-18 plasmid replicons were developed into organotypic cultures in the absence or presence of test compounds (selected from the primary assay) at several concentrations for different durations. In such raft cultures, viral DNA amplification usually takes place between day 10 and day 14 after lifting the dermal equivalent (collagen with embedded fibroblasts) and the keratinocyte monolayer seeded on it to the liquid medium/air interface. Test compounds were added to the tissue culture media at three concentrations from day 6 through day 13. The media and test compounds were refreshed every other day. To label host cell DNA replication, bromodeoxyuridine was added to the culture medium at 100 µg/ml for the final 12 h before harvesting on day 13. Total viral DNA copy numbers/per cell were analyzed by real-time qPCR with HPV-specific primer pairs. Inhibition was expressed as % of viral DNA copies relative to the untreated cultures. In addition, fluorescence *in situ* analyses of amplified viral DNA were performed in formalin-fixed, paraffin-embedded raft culture sections. Cell DNA replication and tissue histology reveal possible toxicity.

Cytotoxicity assays

Every antiviral assay included a parallel cytotoxicity assay with the same cells used for the virus-infected cells: the same cell number, the same drug concentrations, and the same incubation times to provide the same drug exposure. To ensure that the cytotoxicity of all compounds could be compared directly, we also performed a standard neutral red uptake cytotoxicity assay for all compounds in confluent HFF cell line with a 7 day incubation period. HFF cells were seeded into 96-well tissue culture plates at a 2.5×10^4 cells/well in standard growth medium. After 24 h of incubation, the medium was replaced with Modified Eagle Medium containing 2% fetal bovine serum, and

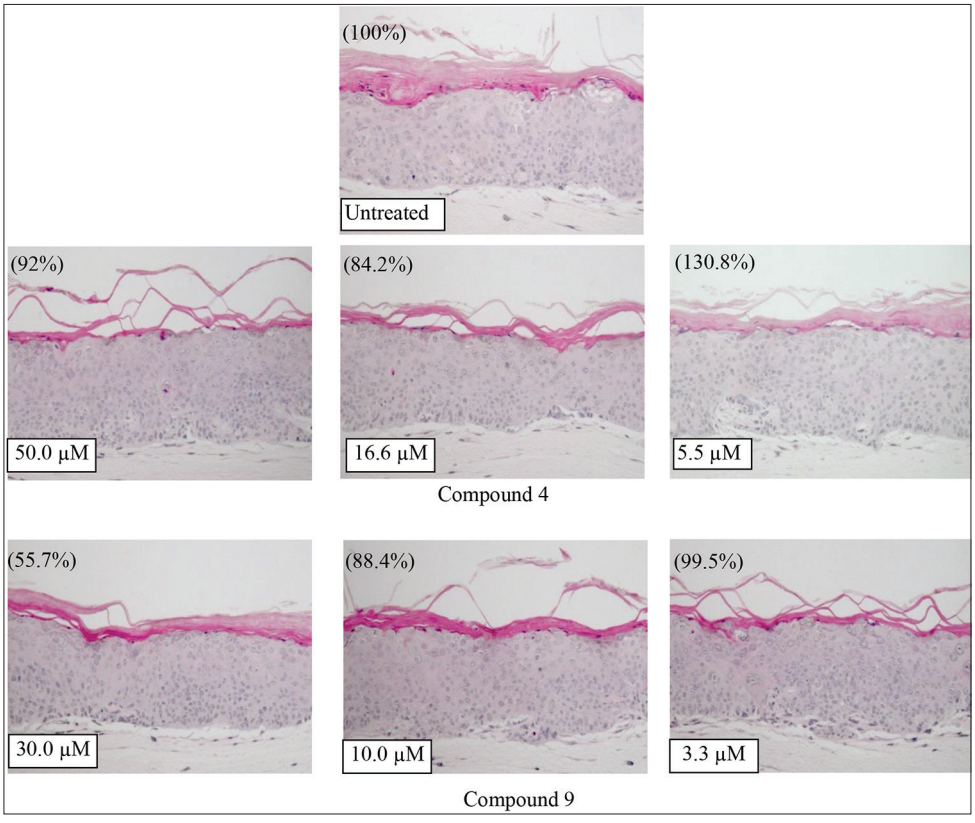


Figure 1: H and E staining of HPV-18-containing PHK raft cultures treated over days 6-13 (relative viral DNA copy number, in %). Histology was normal for all the treated cultures.

Table 2: Activity of oxazole derivatives against human papillomavirus-11 DNA amplification in HEK293 cells and their cytotoxicity in HEK293 and human foreskin fibroblast cells

Compound number	HEK293				HFF
	EC_{50}	EC_{90}	CC_{50}, TB	SI_{50}	CC_{50}, NR
1	24.53	41.26	>100.00	>4	>300.00
2	6.13	28.05	>100.00	>16	>300.00
3	21.94	31.11	>100.00	>5	>300.00
4	4.91	52.02	>100.00	>20	>300.00
5	2.43	20.86	>100.00	>41	>300.00
6	25.51	42.91	>100.00	>4	>300.00
7	18.22	33.21	42.19	2	6,24
8	47.28	70.53	>100.00	>2	28,58
9	8.44	20.69	>100.00	>12	38,63
10	23.81	31.51	>100.00	>4	107,64
11	18.68	30.18	>100.00	>5	144,10
12	39.41	57.91	>100.00	>3	>300.00
13	13.02	17.93	>100.00	>8	163,89
Cidofovir	148.00	>200.00	>200.00	>1	>300.00

Compound concentrations are in μM. EC: Effective concentration, CC_{50}: 50% cytotoxic concentration, SI_{50}: 50% selectivity index, NA: Not available, NR: Neutral red, TB: Tuberculosis, HFF: Human foreskin fibroblast

Table 3: The influence of 1,3-oxazol derivatives on human papillomavirus-18 DNA amplification in organotypic squamous epithelial raft cultures of primary human keratinocytes

HPV-18, qPCR (relative DNA copy number)				
Compound	**EC_{50}**	**EC_{90}**	**CC_{50}**	**SI_{50}**
4	>50.00	>50.00	>50.00	1
9	>30.00	>30.00	>30.00	1
Cidofovir	148	>200.00	>200.00	>1

Compound concentrations are in μM. Based on tissue histology, there was no discernable toxicity at any of the concentrations tested. HPV: Human papillomavirus, EC: Effective concentration, CC_{50}: 50% cytotoxic concentration, SI_{50}: 50% selectivity index, qPCR: Quantitative polymerase chain reaction

Table 4: Quantitative polymerase-chain reaction for relative human papillomavirus-18 DNA copy number/cell in primary human keratinocyte raft cultures (as percentage of untreated cultures)

Compound	Concentration (μM)	Copy number (%)
4 (treatment of HPV18 cultures over days 6-13)	50	92.04
	16.6	84.29
	5.5	130.83
9 (treatment of HPV18 cultures over days 6-13)	30	55.76
	10	88.41
	3.3	99.59
HPV18 control culture harvested on day 13	No drug	100.00
PHK	No drug	0.00

HPV: Human papillomavirus, qPCR: Quantitative polymerase chain reaction, PHK: Primary human keratinocyte

the compounds to be tested were added to the first row. Then, 5-fold serial dilutions were used to generate a series of compound concentrations with a maximum of 300 μM. Assay plates were incubated for 7 days, then 100 μl of a 0.66 mg/ml neutral red solution in phosphate-buffered saline (PBS) was added to each well and the plates incubated for 1 h. Excess dye was removed by rinsing with PBS. Cell monolayers were completely suspended by shaking and the dye that was internalized by viable cells was solubilized in PBS supplemented with 50% ethanol and 1% glacial acetic acid. The optical density was determined in a multichannel spectrophotometer (Packard Spectra Count™, USA) at 550 nm. The optical densities were directly related to the percentages of viable cells, and compound cytotoxicities were interpolated from the experimental data. The CC_{50} of the test compound was defined as the concentration that reduced the absorbance of cells with test compounds to 50% that of controls.

Results

The influence of the 1,3-oxazol sulfonamide derivatives on transient replication of HPV origin-containing plasmid was first evaluated in transfected HEK293 cells [Table 2]. All compounds exhibited anti-replication activity. The order of decreasing activity was: 5 > 4 > 2 > 9 > 13 > 7 = 11 > 3 > 10 > 1 > 6 > 12 > 8. The compounds tested are more active than CDV in this assay.

Compounds 4 and 9, which showed activity against HPV-11 in the primary *in vitro* antiviral assays, were chosen for the secondary assays. The effects of compounds 4 and 9 on HPV-18 DNA amplification in a squamous epithelial raft culture of PHKs were investigated, using real-time quantitative PCR to determine HPV-18 DNA copy numbers in PHK raft cultures.

In contrast to the HPV-11 in HEK293 cells, the cytotoxicity and the ECs of both compounds for HPV-18 were the same in PHK raft cultures [Table 3]. In addition, DNA replication in PHK raft cultures containing the transfected HPV-18 genome

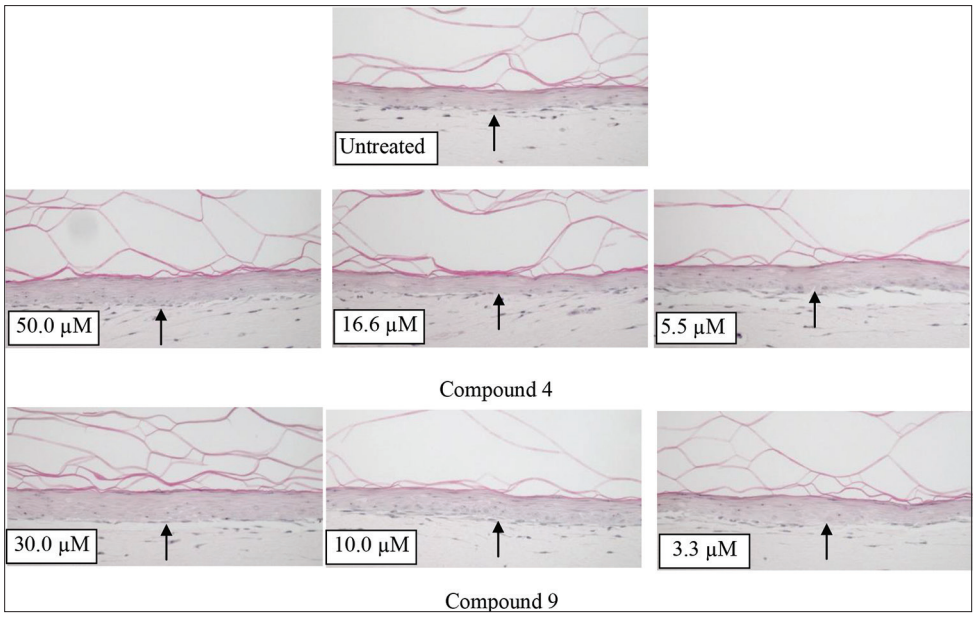


Figure 2: Histology was normal for all the treated cultures, with no apparent effect on squamous differentiation. Arrowheads point to basal cells.

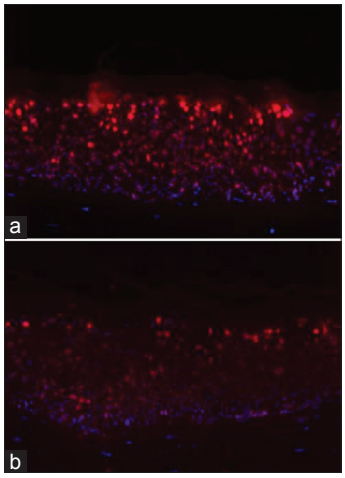


Figure 3: Fluorescence *in situ* hybridization to detect human papillomavirus-18 DNA amplification in raft cultures containing human papillomavirus-18 whole genomic plasmids. (a) Untreated control culture; (b) culture treated with compound 9 (30 µM). Tissue sections were probed for human papillomavirus-18 DNA content. Nuclei were stained with DAPI/4,6-diamidino-2-phenylindole (blue). Magnification scale: ×10. Fluorescent *in situ* hybridization was performed on untreated and compound 9 treated raft cultures.

was essentially resistant to the inhibitory effect of these compounds [Table 4]. Histology was normal for all the treated cultures with [Figure 1] without visible effect on squamous differentiation [Figure 2]. These qPCR data are consistent with analyses of HPV-18 DNA amplification by fluorescence in situ hybridization [Figure 3].

Discussion

Chemotherapeutic approaches to the control of HPV infection suffer from a lack of specificity. Papillomaviruses encode a single replication enzyme, the E1 DNA helicase, but they do not encode a DNA polymerase and other essential replicative enzymes and must therefore rely on host cell replication machinery. Moreover, the tight association of the HPV infection program with cellular pathways makes it difficult to identify chemotherapeutic agents with appropriate selectivity. For most existing HPV inhibitors, the weak antiviral effects observed in cellular assays suggest that further improvements in selecting targets, in drug potency, and in bioavailability and cell uptake are required.

Molecular studies have identified the HPV E1 and E2 proteins as essential for viral DNA replication.[16] These investigations were carried out by expressing E1 and E2 proteins from vectors and observing transient replication of plasmids containing an HPV origin of replication while such model systems provide a convenient assay system for molecular characterization and elucidation of the intermolecular interactions required for viral DNA replication, they do not address replication in the context of the entire viral genome in the differentiating squamous epithelium. Thus, these systems cannot identify compounds that do not act directly on E1, E2, or their formation of the prereplication complex at the origin. Decreased replication of HPV-11 origin-containing plasmids in the presence of the new 1,3-oxazole derivatives as compared to replication in the absence of the drug suggested an antiviral activity of this class of molecules.

Autonomous replication of intact HPV-6, HPV-11, and HPV-18 genomes was reported.[17] Their study demonstrates that the full-length genomic DNAs of HPV-11 and HPV-18 are capable of replicating transiently after transfection into several different squamous cell carcinoma cell lines. However, this cellular milieu is significantly distinct from the differentiating squamous epithelium on which the viral productive cycle depends. Furthermore, there is no assurance that the viral genes are properly expressed in carcinoma cell lines.

HPV infects the basal or parabasal layers of keratinocytes in squamous epithelia through wounds that penetrate deep into

the epithelium. This basal layer is capable of proliferating and providing cells for renewal of the upper epithelial layers. As keratinocytes depart the parabasal layer and are pushed into the upper strata, they become progressively more differentiated. The replication of the HPV genome and synthesis of HPV capsid proteins are tightly integrated with this squamous differentiation program, so that daughter virions are produced in the uppermost differentiated keratinocytes and mature in and are shed with the desquamating layers of the epithelium. The organotypic raft culture model provides a dynamic real-time process of epithelial differentiation, in which the viral reproductive cycle can be studied. Cell lines capable of maintaining HPV plasmids in organotypic culture have been used to study the effects of two commercial preparations of interferon and an antisense oligonucleotide on HPV replication.[18,19] It is not likely that productive programs of high- and low-risk mucosotropic HPVs differ fundamentally. Nonetheless, there might well be differences in the persistence of viral genomes in infected host cells.[20] For example, organotypic epithelial raft cultures are currently limited by their lack of biological parameters such as immune and vascular systems. At present, the reasons for the difference in the anti-HPV effect of 1,3-oxazole derivatives in HEK29 cells versus the HPV-18 containing PHK raft cultures are not understood. Perhaps, the compounds are metabolized differently in the two distinct culture systems. This discrepancy points to the importance of the raft cultures in verifying putative anti-HPV compounds.

Conclusion

The interesting activities of oxazole derivatives clearly extend to include the inhibition of origin-dependent replication of HPV11 while this study was unable to confirm the antiviral activity of this series of compounds in raft cultures, it does provide useful information on specific analogs with antiviral activity *in vitro* that can be further modified to identify more active inhibitors.

Disclosures

All the authors contributed substantially adequately in the conception and conduct of the work to qualify for authorship.

Conflicts of interest

There are no conflicts of interest.

Compliance with ethical principles

The study was conducted under the auspices of the National Institute of Allergy and Infectious Diseases (USA) and its Collaborative Antiviral Testing Group.

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