A Study of 3-Substituted Benzylidene-1,3-dihydro-indoline Derivatives as Antimicrobial and Antiviral Agents

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3-Substituted benzylidene-1,3-dihydro-indoline derivatives were tested for their *in vitro* antibacterial activity against the Gram-negative bacteria *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, and the Gram-positive bacteria *Bacillus subtilis*, *Staphylococcus aureus*, and for their their *in vitro* antifungal activity against *Candida krusei* and *Candida albicans*. The minimum inhibitory concentration (MIC) values were determined by the 2-fold serial dilution technique in Mueller Hinton broth and Sabouraud dextrose agar using antibacterial and antifungal assays, respectively. For comparison of the antimicrobial activity, rifampicin, ampicillin trihydrate, gentamicin sulfate, and ofloxacin were used as reference antibacterial agents, and fluconazole and amphotericin B were employed as reference antifungal agents. The most active compound **10** showed notable inhibition against *Bacillus subtilis, Staphylococcus aureus*, and *Candida krusei*. Compounds **1** and **6** were found slightly effective against *Klebsiella pneumoniae* and *Escherichia coli*. In addition, compounds **13** and **14** showed inhibition against *Bacillus subtilis* and *Staphylococcus aureus*. Indole derivatives were also tested *in vitro* for replication of the HepAD38 cell line and compared with lamivudine (3TC, L-2′,3′-dideoxy-3′-thiacytidine). The IC_{50} values of the compounds were found to be >1000 μM against HBV except for compound **13** which exhibited activity with an IC_{50} value of 500 μM.

Key words: Antibacterial and Antifungal Activity, Inhibition of HBV, Indole-2-thione Derivatives, Indole-2-one Derivatives

Introduction

Heterocyclic indole compounds represent several important classes of compounds with biological activities. The most important of these are anti-inflammatory, anticonvulsant, antidepressant, antihistamine, antitubercular, antidiabetic, anthelmintic, antiallergic, antiviral, antibacterial, and antifungal activities. In several studies indole derivatives have been found to be associated with antimicrobial and antifungal activities. Among them, haloindoles, ethyl-3-indolylacrylate, 5-bromo-3-(2-cyanovinyl)indole and 3-(2-nitrovinyl)indole (Whitehead and Whitesitt, 1974), 3-acyl-4,7-dihydroxy indoles (Malesani *et al.*, 1974), 1-(4-phenyl)- and (1-naphthyl-4*H*-1,2,4-triazole-5-thion-3-yl)indoles (Tsotinis *et al.*, 1997), some indole-containing derivatives of thiosemicarbazide and their cyclic 1,2,4-triazole and 1,3,4-thiadiazole analogues (Varvaresou *et al.*, 2000), several indole derivatives fused with heterocyclic compounds such as 4*H*-pyrano[2,3-*f*]-indole, benzotetrahydrocyclohept[1,2-*b*]indole, and 1-triazolylethylbenz[*g*]indole (Macchia *et al.*, 1996; Gadaginamath and Kavali, 1999; Bhovi and Gadaginamath, 2005), and 1-morpholino-3-carbethoxy-5-hydroxy-2-methylindole (Donawade and Gadaginamath, 2005) were found to be active against a large number of microorganisms. More recently, 38 indole carboxamide and propanamide compounds were screened for their antimicrobial, antifungal and antiviral activities and some of them were found to be active against *Candida albicans* (Ölgen *et al.*, 2008).

Several indole dione and indole-2-one derivatives were also investigated as antimicrobial and antifungal agents. Isatin *N*-Mannich bases were tested for their antiviral, antimicrobial and antifungal activities and some of them were found to be highly active (Varma *et al.*, 1975). A study

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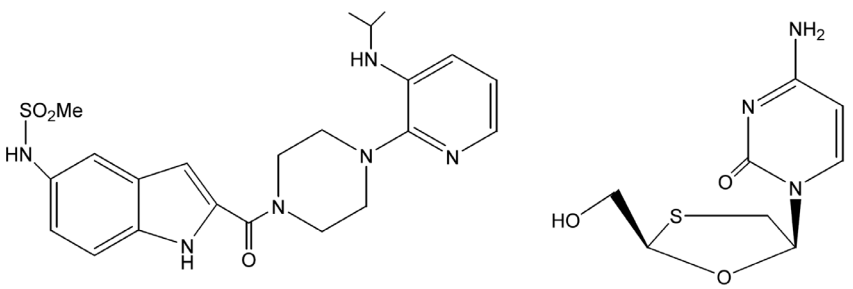
on isatin derivatives showed that substituted isatin-beta-thiosemicarbazones and isatin-beta-hydrazonothiazoline derivatives have potent antimicrobial and antiviral activities (Omar *et al*., 1984). Another study also showed that some organotin(IV) complexes with isatin and *N*-alkyl-isatin bisthiocarbonohydrazones exhibit good antibacterial activity (Bacchi *et al*., 2005).

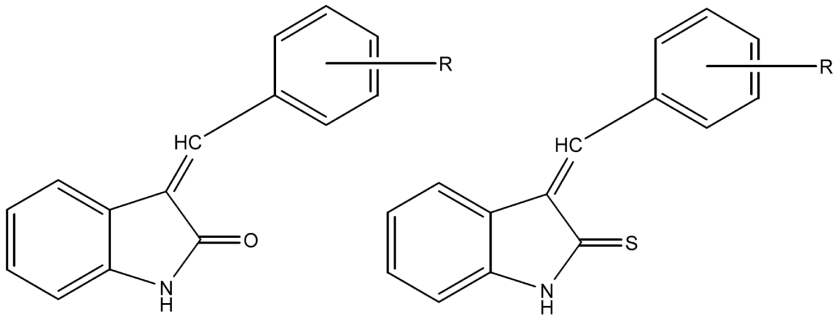
Variously substituted indole-2,3-dione hydrazone derivatives were tested for their antimicrobial activities especially towards *Salmonella typhi*, *Streptococcus beta haemoliticus*, *Mycobacterium paratuberculosis* 607, *Aspergillus niger, Candida albicans,* and *Saccharomyces cerevisiae* (Piscopo *et al.*, 1986a, b, 1987). 1-Substituted aminomethyl-3-cyclohexylthiosemicarbazone-2-indolinones were tested against several microorganisms and fungi, and the majority of the compounds exhibited promising antibacterial and antifungal activities (Singh and Jha, 1989). Benzylideneindolone derivatives showed potent antimicrobial activity against *Staphylococcus aureus* in μM ranges (Angell *et al.*, 2004). Some spiro[indoline]-based heterocycles such as spiro[3*H*-indole-3,4′-pyrano(3′,2′-*d*)oxazole] and spiro[3*H*-indole-3,4′-pyrazolo(3′,4′-*b*)pyrano(3′,2′-*d*)oxazole] derivatives revealed very high activity against Gram-positive (*Bacillus subtilis* and *Bacillus megatherium*) and Gram-negative (*Escherichia coli*) bacteria and against fungi (*Aspergillus niger* and *Aspergillus oryzae*) (Abdel-Rahman *et al*., 2004). Among the potent diorganosilicon(IV) complexes of indole-2,3-dione derivatives it was shown that diphenylsilicon complexes of 1,3-dihydro-3-[2(4-nitrophenyl)-2-oxoethylidene]-2*H*-indole-2-one-hydrazinecarbothioamide demonstrate potent antifungal and antibacterial activities (Singh and Nagpal, 2005). In addition, 1*H*-indole-4,7-diones were reported as potent inhibitors of *Candida krusei*, *Cryptococcus neoformans*, and *Aspergillus niger* (Ryu *et al.*, 2007).

Several indole derivatives were also studied for their antiviral activities. Among them, 5-chloro-3-(phenylsulfonyl)indole-2-carboxamide derivatives were found to be HIV-1 reverse transcriptase inhibitors with IC_{50} values in the nM range (Williams *et al.*, 1993). An indole-containing compound, delavirdine (Fig. 1), was shown to be a HIV-1 reverse transcriptase inhibitor and approved as a commercial anti-HIV drug named, Rescriptor® (Romero *et al.*, 1996). *N*-Alkyl-substituted indolocarbazoles were reported to be potent inhibitors of the human cytomegalovirus in nM concentrations (Slater *et al.*, 2001). A study showed that ethyl-6-bromo-5-hydroxy-1*H*-indole-3-carboxylate derivatives are potent influenza virus inhibitors (Zhao *et al.*, 2004). Some tetracyclic indole derivatives were reported as HCV inhibitors with IC_{50} values less than 5 μM (Conte *et al.*, 2006). Recently, *N*-morpholinoacetyl-2-phenyl-indole-6-carboxylic acid was determined as a potent inhibitor of subgenomic hepatitis C virus replication with an IC_{50} value of 0.127 μM (Harper *et al.*, 2005). A structurally similar compound, *N*-methyl-2-phenyl-3-cyclohexyl-indole-6-carboxylic acid was reported by another research group to be an allosteric inhibitor of the HCV virus with an IC_{50} value of 0.009 μM (Beaulieu *et al.*, 2006). Oxindole derivatives were also investigated as antiviral agents and some of them were found to be potent HIV-1 non-nucleoside reverse transcriptase inhibitors (Jiang *et al.*, 2006).



Delavirdine Lamivudine

Fig. 1. Chemical structures of the non-nucleoside reverse transcriptase inhibitor delavirdine and the nucleoside inhibitor lamivudine.



1 (Z)-4'-N$(CH_3)_2$
2 (Z)-3',4'-Cl
3 (Z)-3'-F
4 (Z)-2'-Cl,5'-NO_2
5 (E)-4'-OCH_3
6 (Z)-3'-OH,4'-OCH_3
7 (Z)-2'-OH
8 (Z)-4'-N$(CH_3)_2$
9 (Z)-3',4'-Cl
10 (Z)-3'-F
11 (Z)-2'-Cl,5'-NO_2
12 (E)-4'-OCH_3
13 (E)-3'-OH,4'-OCH_3
14 (Z)-2'-OH

Fig. 2. Chemical structures of 3-substituted benzylidene-1,3-dihydro-indolin-2-one and 3-substituted benzylidene-1,3-dihydro-indolin-2-thione derivatives.

Prompted by these findings about antimicrobial and antiviral activities of several indolinone derivatives, we investigated herein the antimicrobial, antifungal and antiviral activities of 3-substituted benzylidene-1,3-dihydro-indoline-2-one derivatives and their modified congeners 3-substituted benzylidene-1,3-dihydro-indoline-2-thione (Fig. 2) which were previously evaluated as tyrosine and protein kinase inhibitors (Olgen *et al.*, 2005, 2007).

Experimental

Materials

Mueller Hinton agar (MHA), Mueller Hinton broth (MHB) and Sabouraud dextrose agar (SDA) from Merck (NJ, USA), RPMI-1640 medium with L-glutamine and 3-[*N*-morpholino]-propanesulfonic acid (MOPS) from Sigma (St. Louis, MO, USA), Falcon 96-well microplates from BD Biosciences (NJ, USA), Biohit transfer pipette from Laborgerätebörse (Burladingen, Germany), rifampicin from Koçak (Istanbul, Turkey), ampicillin trihydrate from Paninkret Chemical Company (Pinneberg, Germany), gentamicin sulfate from Deva (Istanbul, Turkey), ofloxacin from Zhejiang Huangyan East Asia Chemical Company (Zhejiang, China), fluconazole from Nobel (Istanbul, Turkey), amphotericin B from Bristol Myers Squibb (NY, USA), and ethanol and dimethylsulfoxide (DMSO) from Riedel de Haen (Seelze, Germany) were used.

Microorganisms

Isolates: Klebsiella pneumoniae isolate [has extended spectrum beta lactamase (ESBL) enzyme], *Pseudomonas aeruginosa* isolate (resistant to gentamicin), *Escherichia coli* isolate (has ESBL enzyme), *Bacillus subtilis* isolate (resistant to ceftriaxon), *Staphylococcus aureus* isolate [resistant to methicillin (MRSA)] and *Candida albicans* isolate (biofilm positive) were used for the assay.

Standard strains: Klebsiella pneumoniae RSHM 574 (Refik Saydam Hygiene Center Culture Collection), *Pseudomonas aeruginosa* ATCC 25853 (American Type Culture Collection), *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Candida al-*

bicans ATCC 10231, *Candida krusei* ATCC 6258 were used.

Antimicrobial activity

Tests of standard strains of *Klebsiella pneumoniae* RSHM 574, *Pseudomonas aeruginosa* ATCC 25853, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231, *Candida krusei* 6258 and clinical isolates of these microorganisms that are known to be resistant to various antimicrobial agents were included in the study. The resistance of the clinical isolates was determined by the Kirby Bauer disk diffusion method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) (2006a). Standard powders of rifampicin, ampicillin trihydrate, gentamicin sulfate, ofloxacin, fluconazole, and amphotericin B were obtained from the manufacturers. Stock solutions were prepared in DMSO for ofloxacin, in methanol for rifampicin, in phosphate-buffered saline (PBS), pH 8, for ampicillin trihydrate, and distilled water for gentamicin sulfate, fluconazole, and amphotericin B. All bacterial isolates were sub-cultured in MHA plates and incubated overnight at 37 ºC. All *Candida* isolates were sub-cultured in SDA plates at 35 ºC for 24–48 h. The microorganisms were passaged at least twice to ensure purity and viability. The solutions of the newly synthesized compounds and standard drugs were prepared at concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.8, 3.9, 1.95, 0.98, 0.48, 0.24, 0.12, 0.06 μg/ml in the wells of microplates by diluting in the liquid media. Bacterial susceptibility testing was performed according to the guidelines M100-S16 of Clinical and Laboratory Standards Institute (CLSI) (2006b). The bacterial suspensions used for inoculation were prepared at 10^5 cfu/ml by diluting fresh cultures at MacFarland 0.5 density (10^7 cfu/ml) (McFarland, 1907). Suspensions of the bacteria at 10^5 cfu/ml were inoculated to a 2-fold diluted solution of the compounds. The bacteria amount was found to be 10^4 cfu/ml in the wells after inoculations. MHB was used for diluting the bacterial suspensions and for 2-fold dilution of the compounds. DMSO, water, PBS, pure microorganisms, and pure media were used as controls. A 10-μl bacteria inoculum was added to each well of the microdilution trays. The trays were incubated at 37 ºC in a humid chamber, and MIC endpoints were read after 24 h of incubation. All organisms were tested in triplicate in each run of the experiments. The lowest concentration of the compounds that completely inhibited the the macroscopic growth was determined and minimum inhibitory concentrations (MICs) were reported.

All *Candida* isolates were sub-cultured in SDA plates, incubated at 35 °C for 24–48 h prior to antifungal susceptibility testing, and passaged at least twice to ensure purity and viability. Susceptibility testing was performed in RPMI-1640 medium, buffered with L-glutamine, pH 7, and MOPS, and culture suspensions were prepared according to the guideline CLSI M27-A of Clinical and Laboratory Standards Institute (2006c). The yeast suspensions used for inoculation were prepared at 10^4 cfu/ml by diluting fresh cultures at McFarland 0.5 density (10^6 cfu/ml) (McFarland, 1907). Suspensions of the yeast at 10^4 cfu/ml were inoculated to a 2-fold diluted solution of the compounds. The yeast amount was found to be 10^3 cfu/ml in the wells after inoculations. A 10-μl yeast inoculum was added to each well of the microdilution trays. The trays were incubated at 35 ºC in a humid chamber, and MIC endpoints were read after 48 h of incubation. All organisms were tested in triplicate in each run of the experiments. The lowest concentration of the compounds that completely inhibited the macroscopic growth was determined and MIC values were reported.

Antiviral activity

The *in vitro* replication ability of the HepAD38 cell line (was kindly gifted by Raymond F. Schinazi, Emory University, Atlanta, USA) was tested in the presence of the novel compounds synthesized. 24-Well plates were seeded with $5 \cdot 10^4$ HepAD38 cells in growth medium containing DMEM/Ham's F12 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 IU/ml penicillin/50 μg/ml streptomycin mix, 400 μg/ml G418, and 0.3 μg/ml tetracycline. In order to initiate viral replication, the growth medium was changed with the assay medium by the withdrawal of tetracycline. 2 d post seeding, the cells were fed with fresh assay medium alone to test the replication efficiency or with assay medium containing increasing concentrations (0.1, 1, 10 and 100 μM) of the compounds. The supernatant of the cells fed with only fresh assay medium was collected every

day during 5 d and the supernatant of the cells fed with the drug-containing assay medium was collected at the end of the fifth day. Viral DNA extraction was performed using "QIAamp DNA Mini Kit" (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. The HBV activity of the compounds was measured with a real-time PCR method using hybridization probes and "Fast Start DNA Hybridization Kit" (Roche Diagnostics, Indianapolis, USA). Two independent experiments were performed for each drug and the results compared with those of lamivudine.

Results and Discussion

The *in vitro* antibacterial activities of all synthesized compounds were assayed against the Gram-negative bacteria *Klebsiella pneumoniae* RSHM 574, *Pseudomonas auroginosa* ATCC 25853, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* isolate, *Pseudomonas aeruginosa* isolate, *Escherichia coli* isolate (ESBL), and the Gram-positive bacteria *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* isolate, *Staphylococcus aureus* isolate. The compounds were also evaluated for their antifungal activity against *Candida albicans* ATCC 10231, *Candida krusei* ATCC 6258 and *Candida albicans* isolate. The MIC values were calculated by the 2-fold serial dilution technique in MHB and SDA for the antibacterial and antifungal assay, respectively. For comparison of the antimicrobial activity, rifampicin, ampicillin trihydrate, gentamicin sulfate, and ofloxacin were used as the reference antibacterial agents, and fluconazole and amphotericin B were used as the reference antifungal agents. The MIC values of the compounds are given in μg/ml in Table I.

The most active compound, 3-(3′-fluorobenzylidene)-1,3-dihydro-indoline-2-thione (**10**), showed notable inhibition (15.62–62.5 μg/ml) against *Bacillus subtilis, Staphylococcus aureus,* and *Candida krusei*. Although **10** showed the largest activity spectrum, inhibited Gram-positive bacteria and fungi, its oxo congener **3** did not exhibit the similar antimicrobial spectrum. Compounds **1** and **6** were found slightly effective against the Gram-negative bacteria *Klebsiella pneumoniae* and *Escherichia coli* with MIC values between 31.5 to 62.5 μg/ml. In addition, the antibacte-

Table I. MIC values of indole derivatives (in μg/ml).

Compound	A	B	C	E	F	G	H	I	K	L	M	N	P
1	125	250	250	250	250	62.5	125	62.5	250	250	125	125	250
2	250	500	500	500	500	125	250	125	500	500	250	250	250
3	250	500	500	500	500	125	250	125	500	500	250	500	500
4	250	500	500	500	500	125	250	125	500	500	250	500	500
5	250	500	500	500	500	125	250	125	500	500	250	250	250
6	250	500	500	500	500	62.5	250	31.25	500	500	250	250	250
7	250	500	500	500	500	125	250	125	500	500	250	250	250
8	250	500	500	500	250	125	250	125	500	500	250	250	250
9	125	250	250	125	125	125	250	125	125	125	125	125	62.5
10	250	125	500	15.62	31.25	125	125	125	15.62	31.25	125	250	62.5
11	125	125	250	250	125	62.5	125	125	250	250	125	250	250
12	250	500	500	500	250	125	250	250	500	>500	250	500	500
13	250	250	500	125	125	125	250	250	62.5	62.5	250	500	500
14	250	250	500	125	125	125	125	250	62.5	62.5	250	250	250
Ampicillin	15.62	>500	>15.62	0.48	1.9	0.48	>500	3.9	0.48	0.48	–	–	–
Gentamicin	7.8	62.5	15.62	0.12	7.8	0.24	31.25	0.48	0.24	0.48	–	–	–
Rifampicin	7.8	>500	3.9	3.9	0.9	1.9	>500	1.9	0.12	0.06	–	–	–
Ofloxacin	3.9	62.5	7.8	3.9	1.9	0.12	62.5	0.12	0.12	0.12	–	–	–
Fluconazole	–	–	–	–	–	–	–	–	–	–	1.9	0.48	15.62
Amphotericin B	–	–	–	–	–	–	–	–	–	–	0.48	0.24	1.9

A, *Klebsiella pneumoniae* isolate; B, *Pseudomonas aeruginosa* isolate; C, *Escherichia coli* isolate; E, *Bacillus subtilis* isolate; F, *Staphylococcus aureus* isolate; G, *Klebsiella pneumoniae* RSHM 574; H, *Pseudomonas aeruginosa* ATCC 25853; I, *Escherichia coli* ATCC 25922; K, *Bacillus subtilis* ATCC 6633; L, *Staphylococcus aureus* ATCC 25923; M, *Candida albicans* ATCC 10231; N, *Candida albicans* isolate; P, *Candida krusei* ATCC 6258.

rial activity of compounds **13** and **14** against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* was expressed by MIC values of 62.5 μg/ml. Moreover, **11** showed slight inhibition (62.5 μg/ml) of the Gram-negative bacterium *Klebsiella pneumoniae*. None of the compounds had considerable inhibition against *Candida* species except for **9** and **10**, which showed inhibition of *Candida krusei* with MIC values of 62.5 μg/ml. The standard drugs exhibited MIC values in the range of 0.06–62.5 μg/ml against the microorganisms and fungi tested.

There is no certain relationship between the structure and activity of the compounds. Although the most active compound **10** has lipophilic fluoro substitution on the benzene ring at position 3 of the indole scaffold, the other active compounds have polar substituents such as $N(CH_3)_2$, OH and OCH_3. However, the other halogen-substituted compounds 3-(3′,4′-dichloro-benzylidene)-1,3-dihydro-indoline-2-thione (**9**) and 3-(2′-chloro-5′-nitro-benzylidene)-1,3-dihydro-indoline-2-thione (**11**) slightly inhibited *Candida krusei* and *Klebsiella pneumoniae*, respectively. These results showed that no direct evidence between the antimicrobial activity and lipophilicity of the tested compounds is found. Perhaps the reason for activity differences is that the geometric orientation of the isomers (*Z*- or *E*-) causes diverse activity patterns. An attempt to generalize the relation of activity to the chemical structure of the compounds leads to the following conclusions: the small structural differences result in great differences of the antimicrobial activity, and the same substitution pattern on indole-2-one and -2-thione may cause diverse inhibition profiles of microorganisms including the type of microorganisms. Based on the present study, **10** might be of interest for designing indole-2-thione derivatives as a new hit for compounds with antimicrobial activity. Variously substituted hydrazone indole-2,3-dione derivatives were studied by Piscopo *et al.* (1987) and they reported that bromo-substituted aromatic ring-containing compounds were found to be the most active against microorganisms. Our next goal is to investigate the role of halogen atoms attached at different positions of the aromatic ring of oxindole and thioindole derivatives for the evaluation of antimicrobial activity. Moreover, our future design of antimicrobial agents should be also orientated towards further hydrophilic and lipophilic analogues of indole-2-one and -2-thione to clarify the effects of physicochemical properties on the antimicrobial activity. Keeping the favourable substituted aromatic ring at position 3 as well as the substitution on the indole nucleus, especially five positions will be investigated.

Since some indole-2-one derivatives presented potent antiviral activity and it was important to test our compounds for their potential as anti-HBV drugs, the HBV activity of the compounds was measured with a real-time PCR method using hybridization probes and "Fast Start DNA Hybridization Kit" according to a previously published protocol (Bozkaya *et al.*, 2005). Two independent experiments were performed for each drug and the anti-HBV activity of lamivudine (Borgia and Gentile, 2006) (Fig. 1) was used as the positive control. The IC_{50} values of the compounds were found >1000 μM against HBV, except **13** [3-(3′-hydroxy-4′-methoxy-benzylidene)-1,3-dihydro-indoline-2-thione] which showed activity with an IC_{50} value of 500 μM. The IC_{50} value of lamivudine was detected as 0.29 μM at the same reaction conditions. Some results from literature show that more ionized compounds or compounds containing the H-bond-donating groups such as NH_2, COO^- and CH_2OH containing compounds are better inhibitors of the HBV virus (Beaulieu *et al.*, 2006; Thakur *et al.*, 2006). The introduction of these types of functional groups on the indole unit might lead to the discovery of unexploited interactions with the protein and further improvements of the overall potency. Polar substituents might be also favourable for 3-substituted benzylidene-1,3-dihydro-indoline-2-one and -2-thione derivatives to develop new antiviral agents. Our future aim to design new antiviral indole derivatives will be based on searching the effects of both lipophilic and hydrophilic substituents for antiviral activities.

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