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Potential anti-respiratory syncytial virus lead compounds from *Aglaia* species

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Although the global prevalence of respiratory syncytial virus (RSV) infection, especially among infants and young children is on the increase, there are only limited therapeutic options for treatment of this disease. Therefore, the search for novel antiviral inhibitors of RSV has become more intensive. In a pilot screening of eighteen compounds from various *Aglaia* species for anti-RSV activity, we identified dammarenolic acid (ignT1), aglaiol (dupT1) and niloticin (cucT1) as potential anti-RSV compounds, with ignT1 being the most potent. Methylation of ignT1 results in a complete loss of anti-RSV activity. Time of addition studies reveal that both ignT1 and dupT1 target the RSV replication at a post-entry stage, although ignT1 was more potent. Dammarenolic acid (ignT1) was also more cytotoxic than aglaiol (dupT1). By carrying out parallel anti-RSV screening with aphidicolin (a highly cytotoxic diterpenoid) and ignT1, we showed that although aphidicolin was more cytotoxic than ignT1, it had virtually no anti-RSV activity. Therefore, dammarenolic acid, aglaiol and niloticin demonstrate potent anti-RSV activity that should be explored further in the current search for anti-RSV therapeutic agents.

1. Introduction

Respiratory syncytial virus (RSV), which belongs to the *Pneumovirus* genus of the Paramyxoviridae family, is the major cause of acute virus-induced lower respiratory tract disease among infants and young children in both developing and developed countries (Chanock and Parrott 1965; Glezen et al. 1986; Kim et al. 1973; Robertson et al. 2004; Selwyn 1990). Recent studies have also identified RSV as the most common virus in the middle-ear-fluid of children suffering from acute otitis media (Heikkinen et al. 1999; Moyse et al. 2000) and as a significant aetiologic agent in the elderly (Falsey et al. 1992; Mathur et al. 1980; Treanor and Falsey 1999), immunosuppressed patients (such as bone marrow transplant patients) (Englund et al. 1988; Garcia et al. 1997; Martin et al. 1988) and even healthy adults (Dowell et al. 1996; Falsey and Walsh 2000; Murry and Dowell 1997).

Presently, only aerosolized ribavarin (Virazole) and a humanized monoclonal antibody (Synagis) have been clinically approved as treatment and prophylactic options respectively for RSV infection (Prince 2001). However, these are neither cost-effective nor simple to administer (Kneyber et al. 2000). Moreover, the clinical use of ribavarin has been associated with toxicity, which may further limit its future applicability (Lafeuillade et al. 2001; Seetharama and Narayana 2005). Therefore, the development of new anti-RSV agents is most desirable.

Recently, a number of small-molecule inhibitors of RSV infection demonstrating potent *in vitro* and/or *in vivo* properties have been described (Cianci et al. 2004, 2005 Huntley et al. 2002; Kimura et al. 2000; Sudo et al. 2001). One of the most advanced of these compounds, BMS-433771 was sequentially developed from a lead compound (SP-78) (Cianci et al. 2005) and thus aptly demonstrates the usefullness of suitable lead compounds in the search for new chemotherapeutic agents.

Since medicinal plants have consistently served as suitable lead sources for potent antiviral agents (Cowan 1999; De Clercq 2000; Jassim and Naji 2003; Vlietinck and Vanden Berghe 1991), we decided to focus on a series of compounds derived from *Aglaia* species in our search for agents effective against the human respiratory syncytial virus.

Aglaia represents the largest genus within the family Meliaceae and contains more than 100 species (Bohnenstengel et al. 1999; Pannell 1992). It is a woody small or medium-sized tree found mostly in Southeast Asia. Extracts or pure compounds from the various species have been shown to display diverse biological activities ranging from anti-proliferation, anti-inflammatory, fungicidal, bactericidal, anthelminthic and antiviral activity (Bohnenstengel et al. 1999; Lipipun et al. 2003; Perry 1980; Poehland

et al. 1987). Although *Aglaia* species are traditionally used in Southeast Asia and Indo-China for the treatment of various diseases, including ailments related to lower respiratory tract infection and inflammation (Lipipun et al. 2003; Perry 1980), antiviral screening with *Aglaia* species has essentially been limited to Herpes Simplex Virus types 1 and 2 (Lipipun et al. 2003; Poehland et al. 1987). Given that RSV infection has a strong bearing with inflammation of the lower respiratory tract, we decided to explore various compounds from these species for possible anti-RSV activities *in vitro*.

2. Investigations and results

The anti-RSV and cytotoxic activity of 18 pure compounds isolated from various species of the genus *Aglaia* was evaluated in HEp-2 cells. A summary of these activities, including the source from which the compounds were isolated is presented in the Table.

Amongst the 18 compounds screened, only ignT1 (dammarenolic acid), dupT1 (aglaiol) and cucT1 (niloticin) exhibited selective anti-RSV activity. The structural formulae of these compounds are presented in Fig. 1. IgnT1 (isolated from the bark of *Aglaia ignea*) exhibited the strongest activity against RSV followed by dupT1 (isolated from the leaves of *Aglaia duppereana*); cucT1 (isolated from the twigs of *Aglaia cucculata*) exhibited only moderate anti-RSV activity. Interestingly, methylation of ignT1 to yield the methyldammarenolate (ignT1A) resulted in a complete loss of anti-RSV (Fig. 2, Table). Also, apart from ignT1, all other compounds isolated from the bark of *A. ignea* (ignT2-T5) had no anti-RSV activity. Generally, the bioactive compounds inhibited RSV infectivity in a dose-dependent manner (Figs. 2 and 3).

In order to determine the stage in the RSV infection cycle that is targeted by ignT1 or dupT1, a time of addition experiment was carried out. For this, ignT1 (3 μg/ml) or dupT1 (20 μg/ml) were added simultaneously (0 h) or 2 and 4 h after infection to the Hep-2 cells. Infected cells were detected by immunocytochemistry and the percentage of infected cells relative to infected, but untreated control cultures was determined. Significant inhibition of virus replication was observed even when ignT1 or dupT1 was added 4 h after infection (Fig. 4). The activity of ignT1 at all time points was significantly more potent than that of dupT1. These results suggest that ignT1 and dupT1, although to a lesser extent, interfere with a post-

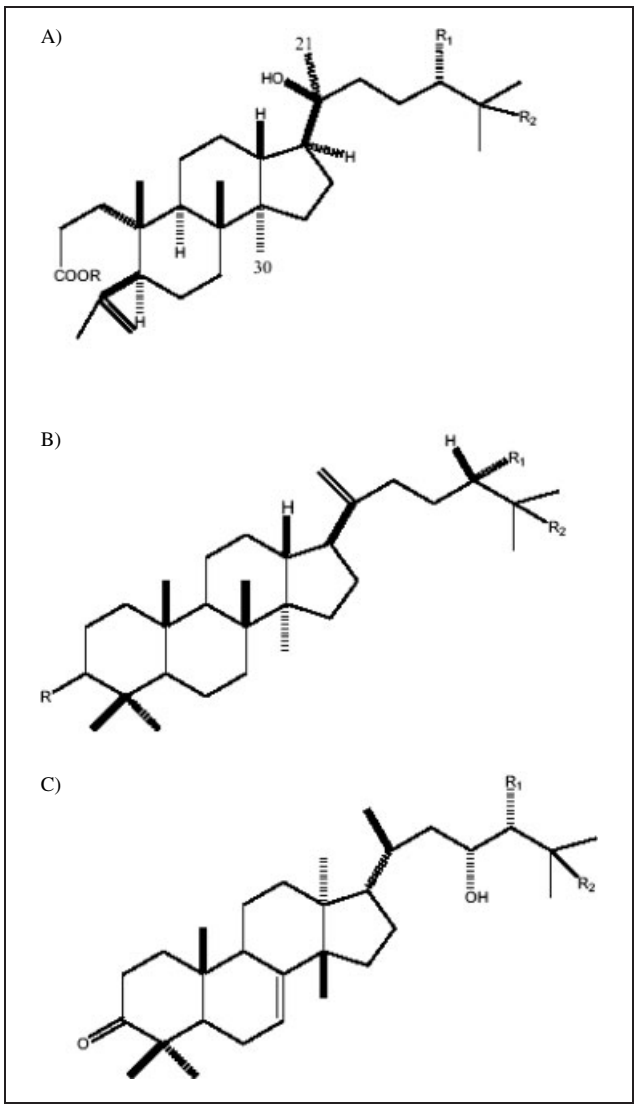

Fig. 1: Structural formulae of anti-RSV compounds isolated from *Aglaia spp*.
A): ignT1 (Dammarenolic acid, isolated from the bark of *Aglaia ignea*)
B): dupT1 (Aglaiol, isolated from the leaves of *Aglaia duppereana*)
C): cucT1 (Niloticin, isolated from the twigs of *Aglaia cucculata*)

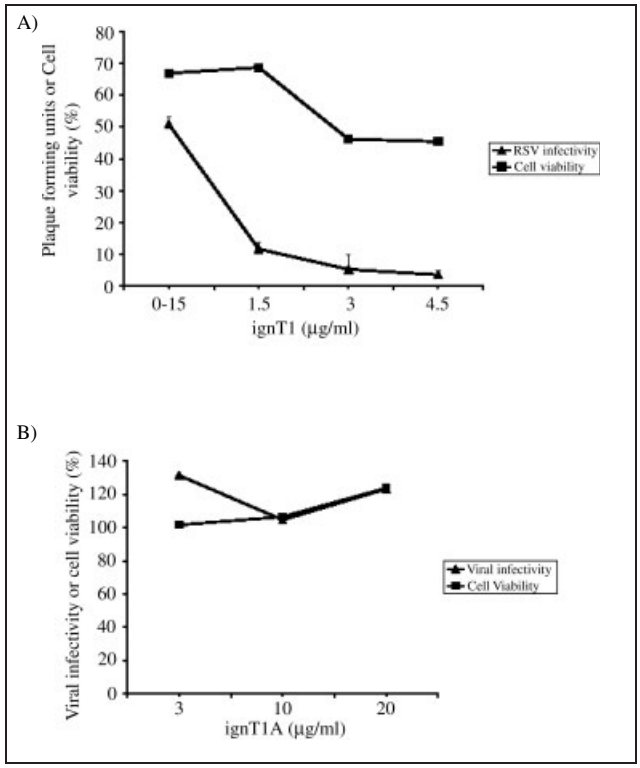

Fig. 2: Inhibition of RSV infectivity by dammarenolic acid (ignT1) but not by the methyldammarenolate (ignT1A)
Various concentrations of ignT1 (A) or ignT1A (B) were preincubated with RSV (long strain) for 30 minute at 37 °C before transducing Hep2 cells to achieve a MOI of 0.01. The anti-RSV activities of the compounds (▲) were determined 48 h latter via an immunocytochemical technique for RSV-induced plaque detection. Toxicity of the compounds to the cell lines incubated with same concentrations of the compounds (■) was evaluated in parallel using the MTT method. The results are expressed as percentage of the activity (plaque forming units for the antiviral evaluation and cell viability for the cytotoxicity evaluation) of cells cultured in the absence of the compounds. The mean and the standard deviation of at least triplicate observations are given

Table: Summary of anti-RSV screening of purified compounds from *Aglaia* spp.

Code	Name	Isolated from	[a]IC_{50} [μg/ml]	[b]TC_{50} [μg/ml]	[c]S.I.
[ignT1]	Dammarenolic acid	*A. ignea* – bark	0.1	2.9	29
[ignT1A]	Methyldammarenolate	*A. ignea* – bark	>40	>40	[d]ND
[ignT2]	(20*S*,24*S*)-20,24-Dihydroxy-3,4-secodammara-4(28),25-diene-3-carboxylic acid	*A. ignea* – bark	>40	10.4	[d]ND
[ignT4]	(20*S*,23*E*)-20,25-Dihydroxy,3,4-secodammara-4(28),23-diene-carboxylic acid	*A. ignea* – bark	>40	70.4	[d]ND
[ignT3]	(23*E*)-(20*S*)-20-hydroxy-25-methoxy-3,4-secodammara-4(28),23-diene-3-carboxylic acid	*A. ignea* – bark	>40	68.4	[d]ND
[ignT5]	Methylester of 20*S*,24-epoxy-25,26,27-trisnor-24-oxo-3,4-seco-4(28)-dammaren-3-carboxylic acid	*A. ignea* – bark	>40	14	[d]ND
[dupT4AB]	Mixture of epimers eichlerianic acid (24(*S*) [8a]) and shoreic acid (24(*R*) [8b])	*A. duppereana* – roots	39.6	12.3	0.3
[dupT5AB]	Mixture of epimers cabraleone (24(*S*)) and ocotillone (24(*R*))	*A. cucculata*- twigs	7.2	7.9	1.1
[dupT1]	Aglaiol	*A. duppereana* – leaves	11.8	168.8	14.3
[dupT2]	24,25-Epoxy-dammar-20-ene-3-one	*A. duppereana* – leaves	21.7	>40	[d]ND
[dupT3]	24,25-Dihydroxy-5α-dammar-20-ene-3-one	*A. duppereana* – leaves	25.5	52.1	2.0
[eupT1]	31-Nor-cycloartenol (29-nor-cycloartenol)	*Aglaia euphoroides* – leaves	>40	>40	[d]ND
[tsaT4]	4α,14-Dimethyl-9,19-cyclocholestan-3β,24α,25-triol	*Aglaia tsangii* – leaves	18.4	19.6	1.1
[tsaT3]	24,25-epoxy-cycloartan-3-ol	*Aglaia tsangii* – leaves	30.1	63.2	2.1
[cucT1]	Niloticin	*A. cucculata* – twigs	15.8	66.8	4.2
[cucT2]	Piscidinol A	*A. cucculata* – twigs	12.8	17.7	1.4
[tsaT1]	Lupeol	*Aglaia tsangii* – leaves	21.2	25.7	1.2
[tsaT2]	Lupeone	*Aglaia tsangii* – leaves	18	14.8	0.8

[a] Concentration of compound (μg/ml) that inhibits RSV infectivity by 50%
[b] Concentration of compound (μg/ml) that inhibits viability of target cells (HEp2) by 50%
[c] Selectivity index (SI) = TC_{50}/IC_{50}
[d] Not determined (ND), because observed activity was not dose-dependent
Experiments were performed at least thrice

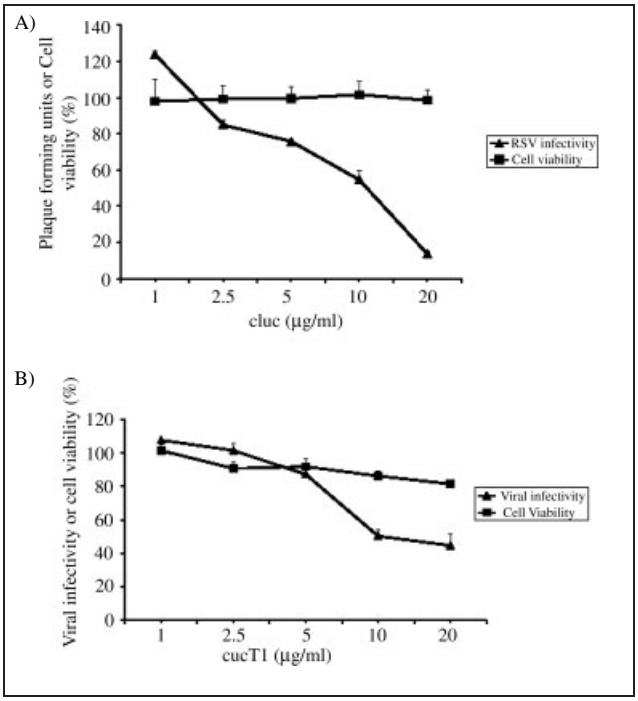

Fig. 3: Inhibition of RSV infectivity by aglaiol (dupT1) and niloticin (cucT1)
Various concentrations of dupT1 (A) or cucT1 (B) were preincubated with RSV (long strain) for 30 min at 37 °C before transducing Hep2 cells to achieve a MOI of 0.01. The anti-RSV activities of the compounds (▲) were determined 48 h latter via an immunocytochemical technique for RSV-induced plaque detection. Toxicity of the compounds to the cell lines incubated with same concentrations of the compounds (■) was evaluated in parallel using the MTT method. The results are expressed as percentage of the activity (plaque forming units for the antiviral evaluation and cell viability for the cytotoxicity evaluation) of cells cultured in the absence of the compounds. The mean and the standard deviation of at least triplicate observations are given

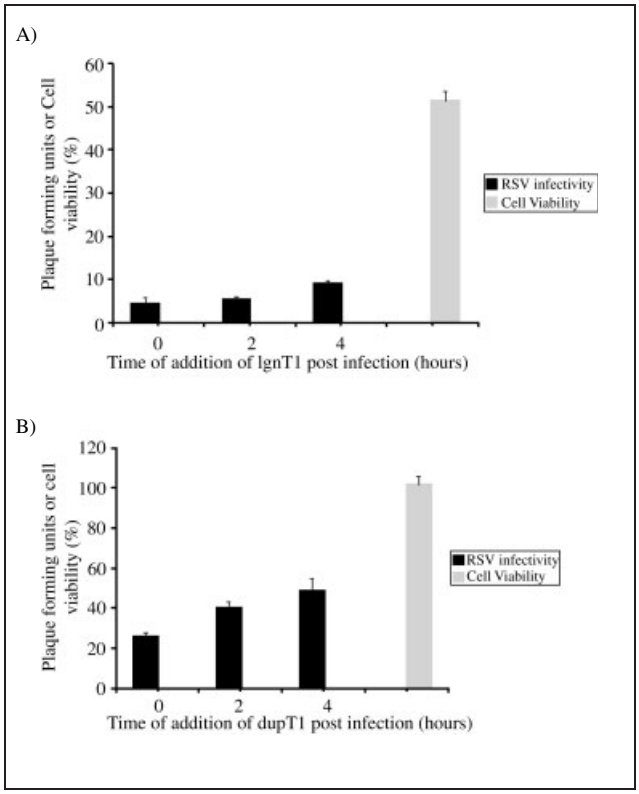

Fig. 4: Kinetics of RSV inhibition by ignT1 (A) and dupT1 (B)
RSV (long strain) was added simultaneously with 3 μg/ml of ignT1 or 10 μg/ml of dupT1, or added 2 and 4 h post infection to HEp2 cells. Two days after infection, virus titer (expressed as percentage of the plaque forming units of cells cultured in the absence of the compounds) was determined by a modified plaque reduction assay, employing an immunocytochemical technique (black bars). The cytotoxicity of the drugs was determined in parallel using the MTT method (gray bars). The mean and the standard deviation of triplicate observations are given

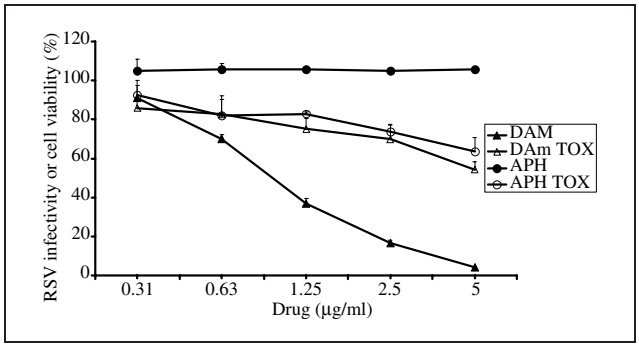

Fig. 5: Comparative analysis of the cytotoxicity and inhibition of RSV infectivity by ignT1 and aphidicolin
For the RSV inhibition studies, various concentrations of Dammarenolic acid (ignT1), DAM (▲) or Aphidicolin, APH (●) were preincubated with RSV (long strain) for 30 minute at 37 °C before transducing HEp2 cells to achieve a MOI of 0.01. The anti-RSV activities of the compounds were determined 48 h latter via an immunocytochemical technique for RSV-induced plaque detection. Toxicity of DAM, DAM TOX (△) or APH, APH TOX (○) to the cell lines was evaluated in parallel using the MTT method. The results are expressed as percentage of the activity (plaque forming units for the antiviral evaluation and cell viability for the cytotoxicity evaluation) of cells cultured in the absence of the compounds. The mean and the standard deviation of at least triplicate observations are given

entry step of RSV replication. Cell viability was unaffected by dupT1, but reduced to 50% by ignT1 at the concentrations that exhibited post-entry inhibition. Therefore, to find out whether the antiviral effect of ignT1 was cell mediated, we carried out parallel anti-RSV screening with aphidicolin (a diterpene which strongly inhibits cellular DNA polymerase and hence cell proliferation) and ignT1 (Fig. 5). Although aphidicolin inhibited cell viability slightly more than ignT1, it demonstrated no anti-RSV activity (Fig. 5). Therefore, ignT1 exhibit specific anti-RSV effect that is not cell mediated.

3. Discussion

The primary focus of the present study was to identify *Aglaia*-derived compounds with anti-RSV activity for possible development as therapeutic agents. Based on this preliminary screening study, the potential applicability of three *Aglaia*-derived compounds (dammarenolic acid, aglaiol and niloticin) as anti-RSV agents has been demonstrated. These compounds, phytochemically designated as triterpenes, are amenable to unlimited structural mordifications and thus represent potential anti-viral lead compounds. Traditionally, triterpenes have served as leads for the development of potent chemotherapeutic agents, including anti-cancer and anti-HIV agents (Aiken and Chen 2005; Curreli et al. 2005; Dargan and Subak-Sharpe 1992; Fujioka et al. 1994; Nishino et al. 1988). For instance, after the identification of the triterpenes, betulinic acid and dihydrobetulinic acid, by Lee, Fujioka and colleagues as the antiviral compounds present in the leaves of *Syzigium claviflorum* (Fujioka et al. 1994), several synthetic modifications, especially at the C3 and C28 positions of the betulinic acid scaffold has resulted in the generation of anti-HIV compounds with increased potency and diverse modes of action (Aiken and Chen 2005; Evers et al. 1996; Kanamoto et al. 2001). Therefore, it is possible that structural modification of these *Aglaia*-derived compounds could also lead to the generation of more potent anti-RSV compounds.

Time-of-addition studies revealed that both ignT1 and dupT1 inhibit RSV replication at a post-entry stage, with ignT1 being significantly more potent than dupT1. This post-entry inhibition of viral replication could suggest that the inhibitors target any of the viral replicative enzymes, possibly the RNA polymerase. This is partly consistent with the reported biological activity of triterpenoids, many of which have been shown to be broad spectrum DNA polymerase inhibitors (Mizushina et al. 2000; Sun et al. 1996). However, a specific target for the inhibitors can only be defined with much precision after thorough enzyme inhibition studies (which were not the focus of the present study).

Based on the observed cytotoxic effect of ignT1 on the target cell line, one might be tempted to conclude that the observed potent antiviral effect is cellularly mediated. However, a reference plant derived diterpenoid compound (aphidicolin), which was at the concentration used about twice as cytotoxic as ignT1 demonstrated virtually no anti-RSV activity. Although this does not completely rule out the possibility of the involvement of other cellular components (e.g. cellular RNA polymerases), the high selectivity index of ignT1 for the RSV inhibition (about 29) further points to a specific antiviral effect. Besides, ribavarin which is the only currently approved anti-RSV therapeutic agent exhibits much more toxicity resulting from its effect on cellular RNA and DNA polymerases (Lafeuillade et al. 2001; Prince 2001; Seetharama and Narayana 2005).

We also observed that methylation of ignT1 resulted in a complete loss of anti-RSV as well as cytotoxicity. This remarkable loss of activity could be related to an interaction of the polar carboxylic group of dammarenolic acid with a potential target molecule of the virus. In the case of the methylated derivative this group is chemically masked and hence the interaction is nullified. However, this hypothesis needs to be further confirmed.

Given that the present need for novel anti-RSV compounds is dire, it would be worthwhile to further explore these *Aglaia*-derived compounds (dammarenolic acid, aglaiol and niloticin) as lead sources from which more potent anti-RSV compounds could be developed. Most of the substances at the advanced stages of development are mainly fusion (entry) inhibitors. Therefore, ignT1 and dupT1, with post-entry inhibitory activities may represent novel plant-derived post-entry anti-RSV agents.

4. Experimental

4.1. Virus and cell

All tissue culture reagents were obtained from Invitrogen (Karlsruhe, Germany). Respiratory Syncytial Virus (RSV) long strain was obtained from Brunhilde Schweiger of Robert Koch Institut of Berlin, Germany. Monolayer cultures of HEp-2 cells were grown in D-5, consisting of Dulbecco's modified Eagle Medium (DMEM) with high glucose, 2 mM L-glutamine (Glu) and supplemented with 5% v/v heat-inactivated foetal calf serum (FCS) and a mixture of penicillin (100 U/ml) and streptomycin (100 µg/ml) (Pen-Strep). Stocks of the RSV long strain were propagated on 85% confluent HEp-2 monolayers in DMEM supplemented with 2% FCS, Pen-Strep, and Glu. Cells were infected at a multiplicity of infection (MOI) of 0.01 and viral titers were determined 48 h post-infection by immunostaining using RSV-antigen-specific monoclonal antibodies as previously described (Ternette et al. 2007).

4.2. Plant compounds

The compounds used in this study have been isolated from various species of the genus *Aglaia* (see Table). Details on the isolation and spectroscopic structure elucidation will be reported elsewhere.

4.3. Plaque reduction immunocytochemical assay

The anti-viral effect of the compounds against RSV was evaluated by a modified plaque reduction assay, employing an immunocytochemical technique for plaque detection. HEp-2 cells were plated in triplicates into 96-well plates at 6000 cells/well and incubated over-night. The compounds were first solubilized in DMSO and various concentrations were prepared in D-5 such that the final DMSO concentration did not exceed 0.5%. These various concentrations were pre-incubated with equal volumes of the virus (200 µl) for 30 min at 37 °C. Culture medium from the cell monolayers was replaced with 100 µl of the above virus/drug mixtures in triplicates (MOI of 0.01) and incubated for 48 h at 37 °C +5% CO_2. Control wells contain virus alone at the same MOI in D-5 (containing 0.5% DMSO) but without drugs.

After the 48 h incubation, the number of plaque forming units (pfu) in drug-treated and untreated HEp-2 cells was determined by immunocytochemical staining with a monoclonal antibody to the RSV-P protein (3C4) as described previously (Ternette et al. 2007). After removal of the supernatant, cells were fixed for 10 min with 80% ethanol, allowed to dry in air before re-hydrating with PBS-T (phosphate-buffered saline: 10 mM sodium phosphate, 150 mM NaCl, 0.05% v/v Tween 20, pH 7.2) for 5 min. Thereafter, cells were incubated with 1 : 250 dilution in PBS-T of3C4 antibody for 45–60 min at 37 °C. After washing three times with PBS-T, cells were similarly incubated with the 2° antibody (peroxidase-conjugated rabbit antimouse IgG, P0260 (Dako, Germany) diluted 1 : 400 in PBS-T. Cells were again washed three times with PBS-T followed by incubation with the red-colour staining AEC substrate (10 ml phosphate citrate pH 5.0, 200 µl AEC and 10 µl hydrogen peroxide) for 30 min at 37 °C. Supernatants were discarded and 100 µl of water was added into each well. Reddish-brown plaques showing fused cells were enumerated microscopically. Each single value of the triplicates was expressed as percent of the mean of triplicates of control cultures (infected with same MOI of virus in the absence of the drugs) and the mean and standard deviation of the percent values was calculated for each triplicate.

4.4. Cytotoxicity

The cytotoxicity of test compounds to HEp-2 cells was always evaluated in parallel with the antiviral effect using the MTT assay. HEp-2 cells were similarly seeded onto a 96-well plate at a concentration of 6000 cells/well and a volume of 200 µL per well. Same concentrations of the test compounds used for the antiviral screening described above were applied to culture wells in triplicate. D-5 containing 0.5% DMSO was used as the "no-drug" control. After incubation at 37 °C under 5% CO_2 for 2 days, a solution of MTT (3 mg/ml, 50 µl per well) was added to each well and further incubated at 37 °C + 5% CO_2 for 1 h to allow formazan production. After this time, medium was removed and 200 µl 0.04 N HCl + 10% SDS was used to dissolve the resulting blue formazan crystals in living cells. The optical density was determined at 550 nm using a multi-well microtiter plate reader (Tecan, Austria). Each single value of the triplicates was expressed as percent of the mean of triplicates of the "no-drug" control cultures and the mean and standard deviation of the percent values was calculated for each triplicate. The concentration of 50% cellular toxicity (TC_{50}) of the test compounds was calculated by simple regression analysis.

4.5. Time-of-addition studies

Time-of-addition experiments were carried out with two of the compounds (ignT1 and dupT1) that displayed appreciable anti-RSV activity (selectivity index > 10). HEp-2 cells plated at a density of 6000 cells/well in 96-well plates were inoculated in triplicates with virus/drug mixture to achieve an MOI of approximately 0.01 (time zero) and incubated at 37 °C + 5% CO_2. Additionally, triplicate wells were also inoculated with same MOI of virus and incubated for 2 h (time 2 h.p.i) or 4 h (time 4 h.p.i) at 37 °C + 5% CO_2. After the designated incubation period, wells were washed with PBS and replaced with D-5 medium containing ignT1 (3 µg/ml) or dupT1 (20 µg/ml). Control wells were replaced with 0.5% DMSO in D-5 medium (without drugs). Plates were further incubated at 37 °C + 5% CO_2 for 48 h before estimation of plaque titer by the immunoflourescent technique described above. Cytotoxicity of the compounds to HEp-2 cells was estimated in parallel.

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