Biotechnology Journal International

21(2): 1-10, 2018; Article no.BJI.40802
ISSN: 2456-7051
(Past name: British Biotechnology Journal, Past ISSN: 2231–2927, NLM ID: 101616695)

Low-dose Sekikaic Acid Modulates Host Immunity and Protects Cells from Respiratory Syncytial Virus Infection

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Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/BJI/2018/40802
Editor(s):
(1) Chung-Jen Chiang, Department of Medical Laboratory Science and Biotechnology, China Medical University, Taiwan.
Reviewers:
(1) Geetha Ramakrishnan, Sathyabama University, India.
(2) I. Nyoman Suartha, Udayana University, Indonesia.
(3) A. Papazafiropoulou, Tzaneio General Hospital of Piraeus, Greece.
(4) Audu Lamidi Isah, National Hospital Abuja, Nigeria.
Complete Peer review History: http://www.sciencedomain.org/review-history/24387

Original Research Article

Received 16th February 2018
Accepted 24th April 2018
Published 30th April 2018

ABSTRACT

Introduction: Investigations of *Ramalina farinaceae* and its isolated compounds had reportedly shown appreciable anti-RSV activity albeit within a slim therapeutic window. Sekikaic acid represent one of such compounds. In this present study, sekikaic acid in very low dose was evaluated as a cell restorative and immunomodulatory factor.
Methodology: The effect of sekikaic acid on the cell viability of RSV-infected HEp2 cells was investigated. Further assay for cellular immune response in primed mouse splenocytes was established by cell culture and flow cytometry. Splenocytes were treated with graded concentrations of sekikaic acid (6.25-25 µg/ml) and screened for their effect on the expression of IFNγ and IL-2 and T-lymphocytes markers $CD4^+$ and $CD8^+$ using intracellular cytokine staining and Fluorescence-activated cell sorting (FACS) analysis.
Results: Results reveal that sekikaic acid protects RSV-infected Hep 2 cells from infection-induced cytopathology. Moreover, sekikaic acid displayed some degree of immune-regulatory activity in the primed mouse splenocytes increasing the proportion of $CD8^+/IFN\gamma^+$ (70.95-73.8%) and $CD4^+/ IFN\gamma^+$

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(3.44-4.13%) T lymphocytes when compared to the cells in untreated controls 2.75% ($CD4^+$) and 69.35% ($CD8^+$) respectively. Interleukin-2 (IL-2) expression and signalling by T-Lymphocytes was selective but pronounced for CD4+ cells activation (sekikaic acid 2.67-3.6%, control 0.44%). Similar scenario was recorded for Intracellular IL-2 secretion by the T-lymphocytes.

Conclusion: Low-dose sekikaic acid protects RSV infected cells and lead to immune lymphocytes proliferation. This recorded T-lymphocyte-specific immune-modulatory property may contribute to explain in part the dynamics associated with the overall antiviral effect of Sekikaic acid, and may also find relevance as a necessary cellular immune response precursor to infection-associated disease management.

Keywords: RSV; Sekikaic acid; antiviral; cellular immunity; cytokine; T-Lymphocytes; cell viability.

1. INTRODUCTION

Human respiratory syncytial virus (RSV) is a member of the *Paramyxoviridae* family, and more than any other virus, causes acute upper and lower respiratory tract infections in infants, young children, and adults [1,2]. Following the outcome of many studies it is well known that the immune response to RSV infection is not protective, therefore RSV infections reoccur throughout adulthood. Regular outbreaks of infection due to RSV are well characterized and predictable, occurring between October and May each year with peak occurrences in January and February. RSV, a leading viral agent of pediatric respiratory tract disease worldwide has also gained recognition as a significant cause of morbidity and mortality in the elderly and severely immunocompromised individuals [3].

Available data has shown that RSV on average has been responsible for about 17% of acute respiratory infections in children admitted to hospital in the developing countries [4]. Equally data from studies carried out in Africa have reported the influence of malnutrition on the prevalence of RSV [5,6]. In areas with tropical or subtropical climates and seasonal rainfall, RSV outbreaks are frequently associated with the rainy rather than the colder season [4]. RSV epidemics occur yearly during winter and early spring in temperate climates and during the rainy season in some tropical climates [7].

Inspite of the increasing global prevalence and importance of RSV infection worldwide, vaccine development nonetheless appear severely hampered due to persitently difficult obstacles. Therapeutic options are acutely limited, and are of little or no benefit. Ribavirin as the only commercially available agent used to treat RSV infection present excruciating challenges bordering on efficacy and toxicity concerns, as well as the very long treatment regimen required for its delivery by aerosol inhalation [8,9,10]. A number of small-molecule inhibitors of RSV have been identified, but to date none are clinically approved [7,11]. Retrospectively, it has been known that potent anti-viral agents for certain other types of viral infection have been previously harnessed from medicinal plants, therefore, medicinal plants continually pose a caveat for discovery of suitable lead sources for potent anti-viral agents [12,13]. Accordingly the RSV field has not been spared these on-going efforts [14,11]. Developing anti-RSV compounds from phytomedicine remains a worthwhile venture.

Our work group had previously reported the isolation of Sekikaic acid screened against RSV as a potent antiviral hit although in high concentration relative to the toxicity range [15]. In this present paper I report the utilization of sekikaic acid in very low dose as a cell restorative in RSV-infected conditions, and the outcome of immune modulatory activity as an antiviral compound-induced host regulatory response.

2. MATERIALS AND METHODS

2.1 Medicinal Plant Collection

The lichen was collected in Nigeria, and identified by Mr. A.O Ozioko of International Centre for Ethnomedicine and Drug Discovery, Nsukka (InterCEDD). A voucher specimen (No. InterCEDD/1567) was deposited at the herbarium of InterCEDD.

2.2 Extraction and Isolation

The isolation of sekikaic acid from the lichen *Ramalina farinaceae* has been described [15]. Briefly, the air-dried lichen of *Ramalina farinaceae* (100 g) were powdered, and extracted with methanol (400 ml×4) four times at 40°C (3

h×4). After filtration, the methanol in the filtrate was removed under vacuum to give a brown residue (12.5 g), which was suspended in 400 ml distilled water, and partitioned against ethyl acetate (EtOAc) (200 ml×6) to yield a EtOAc extract (7.9 g) after removal of the solvent. The EtOAc extract (7.7 g) was subjected to further elaborate standard isolation procedures to yield the pure drug sekikaic acid. The pure sekikaic acid, a phenolic compound is a colourless amorphous solid with molecular weight of 417.1 [15].

2.3 Human Cell Lines and Human Respiratory Syncytial Virus (HRSV)

Human larynx epidermoid (HEp-2) was used to culture HRSV (Long strain: ATCC VR-26). Cells were propagated in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/ streptomycin antimicrobials. HRSV was propagated in DMEM with 0.5% FCS and antimicrobials 1% penicillin/streptomycin. Viral titer was determined and expressed as plaque forming units per ml (pfu/ml) [16]. Virus was stored at -80°C until use.

2.4 Cytotoxicity

The cytotoxicity of the extracts was evaluated using the MTT assay [17]. HEp-2 and Vero cells were seeded onto a 96-well plate at a concentration of 6000 cells/well and a volume of 200 µl of D-5 (5% FCS DMEM medium) per well. The extracts were solubilized in 0% FCS DMEM medium containing DMSO to give 100, 50, 25, 12.5, 6.25 µg/ml extract such that the final DMSO concentration in cell culture did not exceed 0.6%. D-5 containing 0.1% DMSO was used as the "no drug" control. After incubation at 37°C under 5% CO_2 for 2 days, a solution of MTT (5 mg/ml, 50 µl per well) was added to each well and further incubated at 37°C + 5% CO2 for 1 h to allow for formazan production. After this time, medium were removed and 200 µl of 10% SDS solution (pH 4.7) was used to dissolve the resulting blue formazan crystals in living cells overnight. The optical densities were determined at 550 nm using a multi-well microtiter plate reader (Tecan, Austria). Each single value of the triplicates were expressed as percent of the mean of triplicates of the "no drug" control cultures and the mean and standard error of the percent values were calculated for each triplicate. The concentration of 50% cellular toxicity (TC_{50}) of the test compounds were calculated by simple regression analysis.

2.5 Protection from Viral-induced Cytopathic Assay

Hep-2 cell cultures were prepared in 96-well plastic plates (Falcon Plastics, Oxnard, Ca.). After 24 hrs of incubations at 37°C in a CO_2 incubator, culture medium was removed from the confluent monolayer cells. Virus suspension (containing virus at MOI of 3 or 5 together with 100 µl of D5 medium containing the appropriate concentrations of the test compounds were added. As the virus control, similar virus suspensions in medium without compounds were added. The plates were incubated at 37°C in a humidified CO_2 atmosphere (5% CO2), for 5-6 days after which cytopathic effect (CPE) was observed. The virus induced CPE was determined by MTT assay as described above in cytotoxicity assay. The reduction of virus multiplication was calculated as % of virus control:

Percentage (%) CPE= (OD Mock – OD herb+virus / OD Mock – OD virus control) × 100

The concentration reducing CPE by 50% in respect to virus control was estimated from graphic plots using the regression statistic, and was defined as 50% inhibited or protective effective concentration (EC_{50}).

2.6 Immune Modulatory Studies

2.6.1 Animals

Female BALB/c mice (6–8 weeks old, 22–28 g) used in the experiment were obtained from Janvier (Le Genest-ST-Isle, France). Mice were maintained on standard livestock pellets (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) and had unrestricted access to drinking water. The animal use and care practice adopted in this study adhered strictly with the ethical guidelines stipulated under the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (EEC Directive 86/609/EEC), 1986.

2.6.2 Preparation of mouse splenocytes

Mouse splenocytes were prepared according to standard procedure [18]. The mice were sacrificed by CO2 asphyxiation, andaseptically

removed spleens were collected into ice-cold Hanks' Balance Salt Solution (HBSS, Gibco, Darmstadt, Germany). Preparation of single cell suspension was done by gently dispersing straining the cells through 70 mM nylon strainer. Further, Red blood cells (RBCs) were lysed in 2 ml ACK lysis buffer (Lonza, Walkersville, MD) for 2 min, and the reaction was stopped in 8 ml of HBSS. The cells were washed and suspended in R-10 (RPMI 1640 medium supplemented with 10% heatin activated foetal calf serum (FCS), 50 mM 2-mercaptoethanol (Gibco, Darmstadt, Germany), 100 U/mL penicillin and 100 mg/mL streptomycin).

2.6.3 T-Lymphocytes Intracellular Cytokine Staining

Intracellular cytokines staining were conducted following previously established protocols [19,20]. Splenocytes (1.5 x 10^6 cells per well) in R10 medium were plated in 96-well round bottom plates. Splenocytes were stimulated by sekikaic acid for 24 h with varying concentrations 25, 12.5, and 6.25 µg/ml respectively per well at 37°C. Positive controls were splenocytes stimulated with Lipopolysaacharides (LP) or phorbol myristate acetate (30 ng/ml) and ionomycin (500 ng/ml). For the negative control, cells were cultured in R10 medium alone containing DMSO. Antibodies used were sourced from BD Biosciences. Cells were washed with PBS/bovine serum albumin/azide after stimulation, and Fc (II/III) receptors were blocked with a mixture of anti-CD16 and anti-CD32 antibody. Afterwards, cells were labelled with either allophycocyanin (APC)-conjugated anti-CD8 or anti-CD4 antibodies (BD Biosciences, San Jose, CA). Following washing with PBS cells were fixed in 2% paraformaldehyde, followed by permeabilization with 0.5% saponin in PBS/bovine serum albumin/azide buffer. Intracellular cytokines gamma interferon (IFN-gamma) was detected with a phycoerythrin-conjugated anti- IFN-PE antibody while IL-2 was detected with anti-IL-2-FITC (BD Biosciences, San Jose, CA). FACS Calibur instrument using Cell Quest Pro software (BD Biosciences) was used for analyses of cells. Reanalyses were carried out using Flowjo (TreeStar Inc., Ashland OR).

2.7 Statistical Analysis

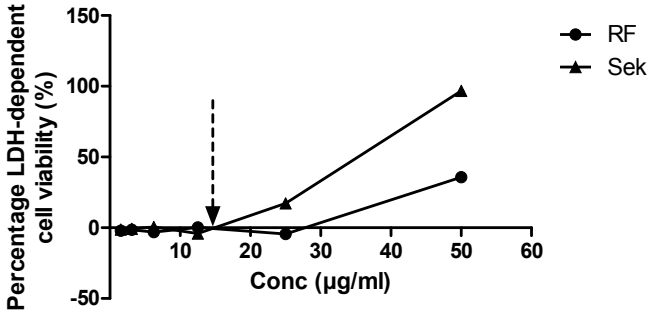
Results were expressed as mean ± standard error of mean (S.E.M). Percentage of control (infection rate; %) was calculated from the plaque counts of extracts-treated groups divided by that of viral control. Data were analyzed by Graphpad Prism Software (Graphpad Software Inc., La Jolla, CA) using ANOVA. Differences between means of the treated groups and control were considered significant at $P<0.05$.

3. RESULTS

3.1 Sekikaic Acid Demonstrates Cell Tolerability when Measured by an LDH Enzyme Release-dependent Approach

Sekikaic acid demonstrates tolerability when measured in an LDH-release-dependent cytotoxic assay (Fig. 1). At up to 15µg/ml of sekikaic acid the incident Hep2 cell showed a 100% viability (dashed Arrowhead position), and 82.5% at 25 µg/ml, and 50% viability at up to 35µg/ml of the compound.

Hep2 cells were incubated with various concentrations of Sekikaic acid (Sek) and *Ramalina farinaceae* (RF). After corresponding



LDH-dependent cell viability profile of Sekikaic acid

Fig. 1. Effect of Sekikaic (Sek) acid on Hep2 cell viability by LDH release-dependent assay

48hrs of incubation alongside the infectivity assay, plates were centrifuged and supernatant collected. The supernatant was subsequently mixed with LDH-enzyme cocktail (Roche ®) incubate at room temperature in the dark for 30 minutes. Coloured complex formed from enzymes interaction with substrate is measured at 550nm for absorbance. The results are expressed as percentage of the activity (cell viability) of cells cultured in the absence of the compounds. The mean and the standard deviation of at least triplicate observations are given.

3.2 Cell Protective Property of Sekikaic acid from RSV-induced Cytopathology

Sekikaic acid and RF demonstrated some cell protective effect from RSV-induced cytopathology in Hep 2 cells. The evaluation for protection from viral-induced cell cytopathic effects assay was done. The results showed cell protection capacities with EC_{50} values of 15.78 and 22.47 µg/ml for Sekikaic acid and RF respectively.

3.3 Effect on Cytokines Stimulation and Secretion in T-lymphocytes Subsets

Sekikaic acid appears to upregulate the subsets of largely interferon gamma (INF-g) expressing CD4+ and CD8+ T Lymphocytes population, and to a lesser extent interleukin 2 (IL-2) secreting T-Lymphocytes populations (Figs. 2-4). Flourescent activated cell sorting (FACS) data show simple increases of CD4+ and CD8+ T cell clones consequent to sekikaic acid stimulation. From 6.25 µg/ml to 25 µg/ml doses of sekikaic acid incremental expression and detection of INF-g ranged from 3.44 – 4.13% (sekikaic acid-treated CD4+ cells) and 2.75% (for untreated

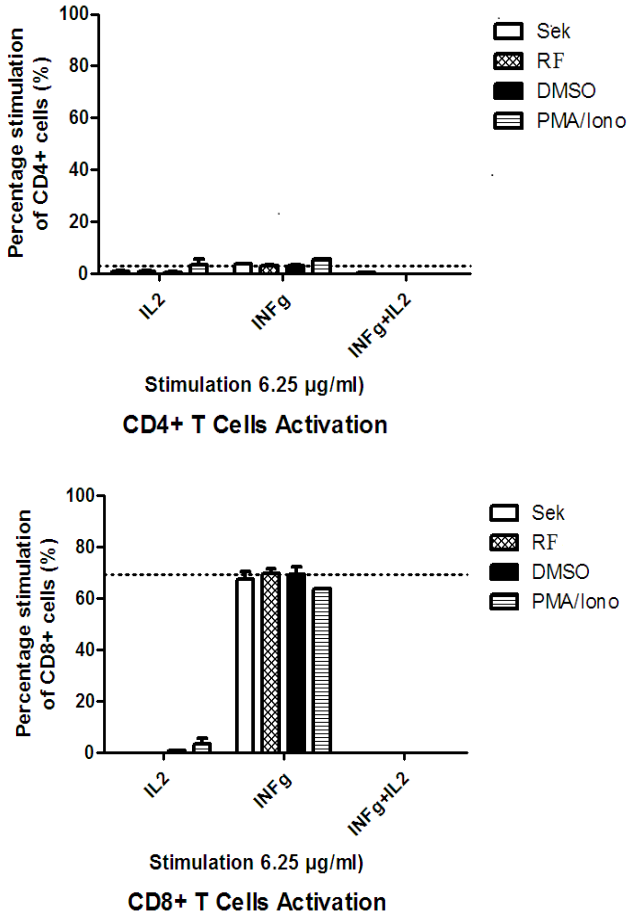


Fig. 2. Effect of 25 µg/ml sekikaic acid dose on T-Lymphocytes Cytokine secretion

Intracellular cytokine secretion in activated CD4+ T-lymphocytes (upper panel) and CD8+ T-lymphocytes (lower panel) secreting interferon-gamma (IFN-γ) and interleukin-2 at 6.25 µg/ml sekikaic acid dose. Shown also are cells double positive for IFN-γ and IL-2

controls). $CD8^+$ T-Lymphocytes were not similarly affected except for a marginal increase recorded only for 12.5 -25 µg/ml dose regimen (sekikaic acid-treated 70.95-73.8%, control 69.35%). Detection of IL-2-expressing T-Lymphocytes occurred for $CD4^+$ cells at sekikaic acid dose regimen 12.5 µg/ml (sekikaic acid 3.6%, control 0.44%), and 25µg/ml (sekikaic acid 2.67%, control 0.44%) respectively.

4. DISCUSSION

Search for novel anti-RSV compounds with better therapeutic efficacy and safety margins than ribavirin is currently a hotbed of research focus in the RSV antiviral field. This is largely predicated on well-known precedents that potent anti-viral agents have previously been harnessed from medicinal plants [21,22,23]. As a result of these ongoing efforts a plethora of potential anti-respiratory syncytial virus (anti-RSV) substances have been uncovered in various investigations; however some are too cytotoxic to be developed as clinically useful agents [7,24,25]. Accordingly, given the consistency of reports of anti-respiratory syncytial substances with such recorded tight therapeutic window, the continued screening of existing medicinal plants for promise

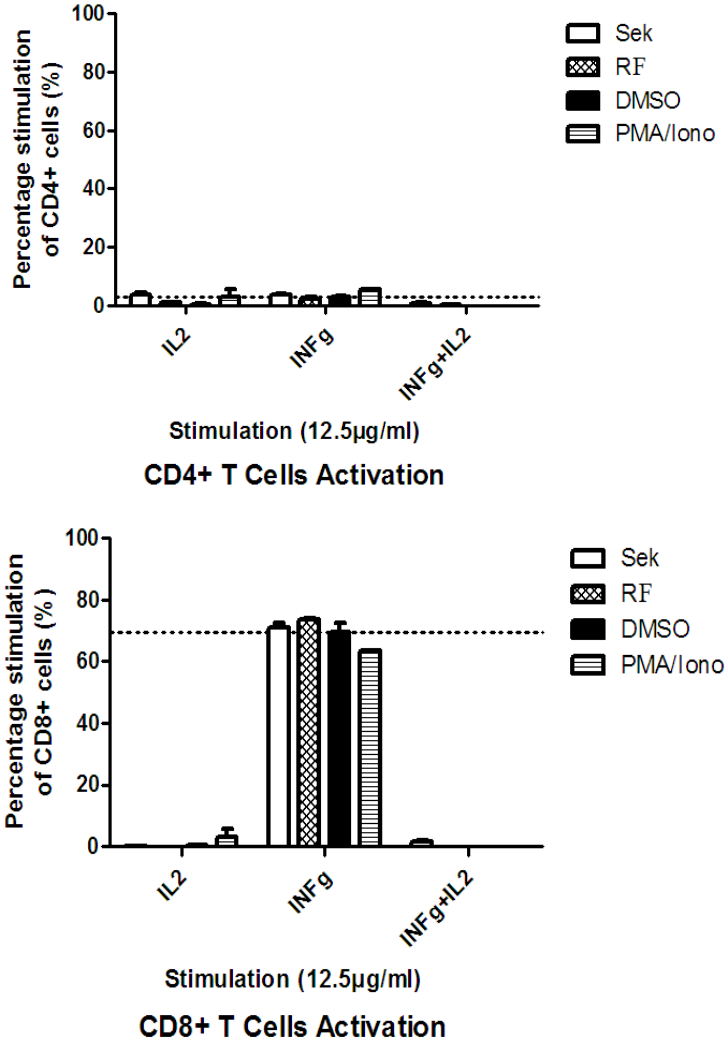


Fig. 3. Effect of 12.5 µg/ml sekikaic acid dose on T-Lymphocytes Cytokine secretion

Intracellular cytokine secretion in activated $CD4^+$ T-lymphocytes (upper panel) and $CD8^+$ T-lymphocytes (lower panel) secreting interferon-gamma (IFN-γ) and interleukin-2 at 12.5 µg/ml sekikaic acid dose. Shown also are cells double positive for IFN-γ and IL-2

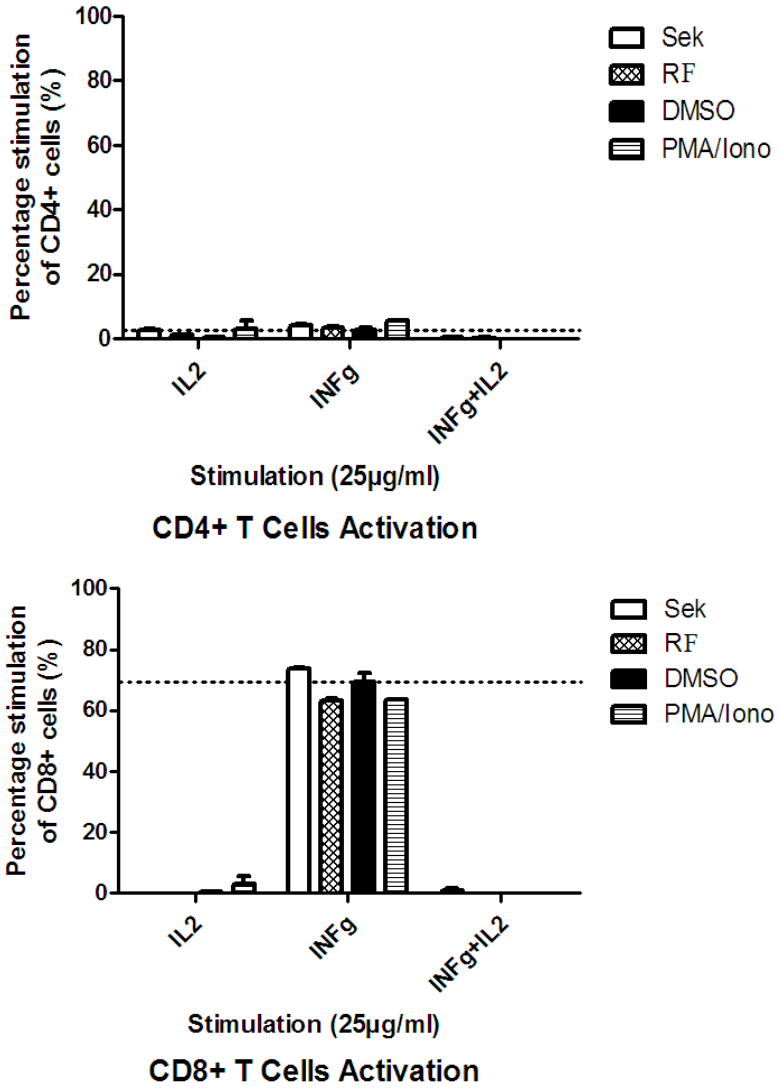


Fig. 4. Effect of 25 µg/ml sekikaic acid dose on T-Lymphocytes Cytokine secretion

Intracellular cytokine secretion in activated $CD4^+$ T-lymphocytes (upper panel) and $CD8^+$ T-lymphocytes (lower panel) secreting interferon-gamma (IFN-γ) and interleukin-2 (IL-2) at 25 µg/ml sekikaic acid dose. Shown also are cells double positive for IFN-γ and IL-2

of therapeutically useful anti-RSV compounds remain strongly imperative. Therefore, I embarked on reporting the investigations of extract of RF and its associated compound sekikaic acid which had earlier shown profound anti-RSV activity [15]. Sekikaic acid was found to inhibit RSV even at a late viral replication stage. However, the mechanism of antiviral activity remains to be fully elucidated. The outcome of this present study seek to further x-ray this mechanistic explanation by exploring other closely related biological activity of both sekikaic acid and RF. First, I analyzed the cell enhancing effect of both sekikaic acid and RF towards Hep2 cells by employing the lactate dehydrogenase (LDH) enzyme secretion system which records the release of LDH as a marker for cell viability status [26]. Applying this measurement, sekikaic acid and RF were found to promote restorative status of Hep2 cells at low doses. This evaluation is important to factorize into the overall picture of utility for sekikaic acid as a putative RSV inhibitor given that the absence of cell restorative property may become an elimination criterion for considerable antiviral candidates. Several promising antiviral candidates were not successful due to problems bordering on cytotoxicity [7,24,25].

Moreover, by carefully administering sekikaic acid and RF to Hep2 cell culture previously infected with RSV inoculum, the protection and recovery effect of the compound on the Hep2 cell could be observed and measured. Such recovery and restorative effect furnish sufficient data to support the use of the compound such as sekikiac acid not only as a treatment but also as a palliative protective device. RSV-induced cell cytopathy represents a major arm of the respiratory disease associated with RSV infection [27,28]. Obviously, protection of infected cells from the lethal effect of virus presence and propagation would definitely ameliorate the disease impact and progression. This property has been effectively measured on the basis of cell viability status of infected cells [29]. This study reports that both Sekikaic acid and RF were capable of protecting viral-infected cells from the virus-induced cytopathy at EC_{50} values of 4.63 and 51.76 µg/ml respectively. Therefore, both agents at quite low dose concentration may promote the profound recovery of RSV-infected cells from the pathological effects of any established infection.

In addition to this, an attempt made at modulating the immune status of the host may further compliment the sekikaic acid-induced cell restoratve and antiviral effect. Immunomodulatory compounds of natural product origin with potential to alter immune system through their direct action on immune cells or their effect on the signalling molecular components of the immunological synapse represent a natural approach to immunotherapy. Thus, the use of immunomodulatory substances of phyto origin have been shown to be effective immunotherapy and offer advantage over orthodox treatments given its potential towards avoidance of complications associated with say, cytokine therapy [30,31,32]. Therefore, sekikaic acid was evaluated for its modulatory effect on $CD4^+$ and $CD8^+$ Lymphocytes in the context of their expression pattern for INF-g and IL-2 type 1 cytokines mileu. After stimulation of spleen-resident T-lymphocytes, expressed cytokines were intracellularly localised following treatment of cells with 2µM Monensin entrapment factor [19]. The $CD4^+$/$CD8^+$ cells loaded with entrapped cytokines were made permeable, stained with definition antibody markers and subjected to FACS analyses to characterize T-Lymphocytes types and the nature of expression cytokines. From FACS analysis it could be observed that at 6.25 µg/ml dose of sekikaic acid there was increase only in the population of INF-g-expressing $CD4^+$ cells (sekikiac acid 3.44%, control 2.75%). No such observation was made for $CD8^+$ T-Lymphocytes subset. Similarly, a notable increase was defined for INF-g-expressing $CD4^+$ cells (sekikiac acid 3.74%, control 2.75) while a marginal increase is shown for $CD8^+$ cells (sekikiac acid 70.95%, control 69.35%) at 12.5 µg/ml sekkaic acid dose point. In the case of 25 µg/ml dose of sekikaic acid, a more pronounced upregulation was recorded for INF-g-expressing $CD8^+$ cells (sekikiac acid 73.8%, control 69.35), and to a lesser extent with $CD4^+$ cells (sekikiac acid 4.13%, control 2.75%). In all, IL-2-expressing $CD4^+$ or $CD8^+$ cells were almost not detected except for $CD4^+$ cells where probounced differential is recorded at sekikaic acid dose regimen 12.5 µg/ml (sekikaic acid 3.6%, control 0.44%), and 25µg/ml (sekikaic acid 2.67%, control 0.44%) respectively. Doubtless, escalation of relevant T-Lymphocytes subsets ($CD4^+$ and $CD8^+$) may well represent a critical cellular response to ameliorate the effects of invasion by a foreign pathogen. These cell types may directly engage this pathological cascade or drive the resolution of the invasiveness through cytokines expression which in turn leads to recruitment of more and diverse immune cells to resolve infection [33,34]. And in this case the sekikaic acid-induced immunological response translates to a slight up-regulation of INF-g secretion at all doses applied, and to a lesser extent, IL-2 release. These two cytokines represent a type I cytokine exrpession scenario; a condition that prime for antiviral conditioning and a host of intracellularly-mediated events [27,28]. This observations portend utility against not only tumor-associated loci, but possible widespread antiviral applications. Given this outcome, the usefulness of sekikaic acid against the repiratory syncytial virus is no doubt favourable. Moreover, safe outcomes arising from usage is accommodated since low dose of the drug could not only recruite necessary immune cells to the site of infection through INF-g/Il-2 signalling, but could also protect challenged cells from the lethal effects of RSV infection.

5. CONCLUSION

In the current study the antiviral and imunomodulatory effects of sekikaic acid and RF have been evaluated. The findings revealed the potential utility of sekikaic acid as future antiviral, cell restorative, and immunemodulatory agent for further development against the respiratory syncytial virus.

ETHICAL APPROVAL

As per international standard or university standard ethical approval has been collected and preserved by the authors.

ACKNOWLEDGEMENT

Author wish to acknowledge the DAAD and ETF scholarship awards for doctoral study in Germany. And also staff of Department of Molecular and Medical Virology, Ruhr University Bochum for technical assistance.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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