MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society For Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

MASLINIC ACID ENHANCES IMMUNOGENICITY IN A TIME-DEPENDENT MANNER IN RAJI CELLS

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SHORT COMMUNICATION

History
Received: 18th March 2020
Accepted: 13th April 2020

Keywords:

Maslinic acid; Raji cells; microarray; anti-tumorigenesis, immunogenicity enhancer

Abstract

The survival mechanism of tumourigenic cells has evolved to escape the vigilant scrutiny of immune responses. As such, immediate immune recognition of virus-infected cells is necessary to eradicate and delay or prevent the onset of tumour development. Although maslinic acid has been long identified as a prospective chemopreventive agent, its role as an immunogenicity enhancer is not reported. In Raji cells, maslinic acid significantly induced the expression of various genes in the antigen processing and presentation pathway over 48 hours. This observation suggests a new perspective on the mode of action of maslinic acid in targeting the immunogenicity of Raji cells, thereby preventing the onset of tumorigenesis.

INTRODUCTION

Phytochemicals have a long origin in the history of mankind and many natural compounds have been integrated into the community's healing regime [1]. One of the emerging compounds of interest is maslinic acid, a pentacyclic triterpene which is synthesized from the cytoplasmic acetate/mevalonate pathway and it is highly hydrophobic in nature.

This compound is not only found abundantly in crude pomace olive oils but also in various other dietary sources such as potato tuber (*Coleus tuberosus*) and apple fruit (*Malus* sp.) peel, as well as in different parts of a plant, for examples; the leaves of Eucalyptus (*Eucalyptus viminal*) and flowers of Chinese Trumpet Vine (*Campsisgrandiflora*). Scientific experiments conducted on maslinic acid for almost ten decades have made this compound a common name in the field of cancer chemoprevention. Maslinic acid was found to be a promising agent in preventing the onset of tumourigenesis by targeting various pathways, especially obstructing the NF-κB and MAPK/AP-1 signaling pathways [2]. Many researchers conducted on pentacyclic triterpenes have suggested that the complete pentacyclic ring structure is essential for bioactivities such as tumor suppression [3, 4].

Tumorigenesis is a malignant disease that is notoriously difficult to be eliminated entirely due to the complicated and massive interaction between various signaling pathways. Besides, tumorigenesis is a delicate development where the process takes more than ten years to turn malignant and this window period can often be exploited in numerous ways to hinder the oncogenic transformation of the cells.

Hence, it is ideal to identify a single agent or multiple selective agents that is able to significantly inhibit the cross-talks between multiple signaling networks [5]. However, this method alone is insufficient to address to the complexity of the disease due to the ability of cancer cells in evading immunosurveillance through numerous escape mechanisms such as clonal evolution and the release of T_{reg} factors [6]. Further discoveries have found an alternative solution to intensify the immunogenicity of the tumour cells to the immune system by prompting the tumourigenic cells into expressing its tumor antigens to cytolytic T-cells [7].

In this study, the objective was aimed to confirm if maslinic acid can affect the pathways involved in the immunosurveillance mechanism.

MATERIALS AND METHODS

Human lymphoblastoid cell line, Raji cells were incubated with 3 mM of sodium n-butyrate, 0.05 μM phorbol 12-myristate 13-acetate and 19 μM maslinic acid for 0, 4, 8, 12, 24 and 48 hrs prior to total RNA extraction using Total RNA/Protein extraction kit (Macherey-Nagel, Germany), which is according to the manufacturer's protocol. Concentration and purity of RNA were determined using Nanodrop® UV-Vis Spectrophotometer (Implen, Germany). RNA was used to generate biotin-labeled cDNA with the Applause WT-Amp ST System (NuGEN, CA) kit and the

samples were purified with MinElute Reaction Cleanup kit (Qiagen, Germany) according to the manufacturer's protocol.

The cDNA was hybridized according to Affymetrix GeneChip protocol (Affymetrix, CA, USA), and arrays were scanned with Affymetrix GeneChip 3000 7G (Affymetrix, CA, USA). The experiment was performed in triplicates. A probe-based real-time PCR was carried out using RotorGene thermal-cycler (QIAGEN, Germany). The expression of the selected genes was normalized to a housekeeping gene, GAPDH.

All raw data were processed with GeneSpring GX12 (Agilent Technologies, CA, USA) via the exon expression default analysis settings. The data normalized with the algorithm of RMA16 and tested with one-way ANOVA at a p-value of less than 0.05. The microarray data was made accessible through GEO Series accession number GSE39149 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39149). The gene expression profile of interest was queried against the reference database made available at www.broad.mit.edu/cmap. A cut-off value of 3-folds and a score-value of 1 were set as the criteria for hits identification. Pearson's correlation method was used to average over the drug replicates.

RESULTS AND DISCUSSION

A gene signature analysis was performed on the expression profile constructed from the effects exerted by maslinic acid on Raji cells. 12 hours of incubation with maslinic acid showed a gene expression profile similar to trichostatin A (TSA) and vorinostat, while after 12 hours of incubation, the gene expression profile is significantly comparable to gossypol. TAS, vorinostat and gossypol are recognized for their role in thwarting tumorigenesis whereby both TSA and vorinostat are histone deacetylases that effectively inhibit the activities of cyclin kinases [8].

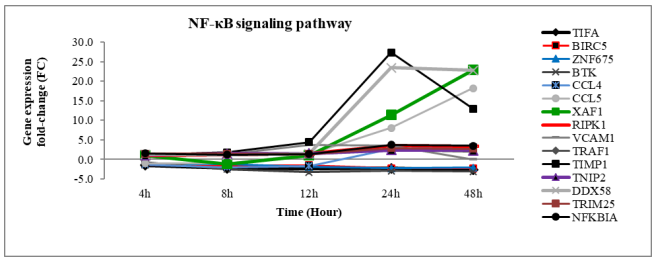

Figure 1. Manipulation of genes involved in the NF-κB signaling pathway had led maslinic acid treated cells into cell cycle arrest and cell death ($p < 0.05$).

On the other hand, gossypol is documented as a BCL-2 inhibitor that significantly suppresses genes involved in the NF-κB pathway [9]. The cellular effects resulted from the treatment with maslinic acid were due to the interaction of the 2ß-hydroxyl group of maslinic acid with the cellular lipid bilayer [10]. The interruption at the Cho-rich membrane domains of the lipid bilayer leads to the modulation of the lipid composition and subsequently affects multiple downstream transcription factors residing within the nucleus [11] that resemble the modulation patterns exhibited by TSA, vorinostat and gossypol. As such, the microarray profile of maslinic acid showed attributes similar to these compounds where maslinic acid also mediates anti-tumorigenesis effects and it was observed to selectively target a majority of genes controlling cellular proliferation and apoptotic cell death within 12 hours of incubation (**Figure 1**). Cell cycle arrest became apparent by 24 hours as genes responsible for the G1/S phase (*CDCA7*, *MYB*), S/G2 phase (*CDK1*, *MAD2L1*, *TYMS*), G2/M (*CCNA2*) and DNA replication (*MCM7*, *PLK4*) were simultaneously down-regulated by at least more than 2.8-fold (**Table 1**).

Table 1. The expression level and the biological function of genes affected by maslinic acid across five time-points. These genes were significantly regulated and hold an important role in different phases of the cell cycle.

Entrez ID	Gene symbol	Gene name	p-value	Time (Hours)					Biological ac
				4	8	12	24	48	
983	CDK1	Cyclin-dependent kinase 1	0.00	-1.6	-2.1	-2.4	-4.2	-5.2	Cell cycle
4085	MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	0.00	-1.6	-2.0	-2.8	-5.3	-7.7	Cell cycle
4176	MCM7	Minichromosome maintenance complex component 7	0.001	-1.5	-2.0	-2.3	-3.3	-4.7	Cell cycle
4609	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	0.00	-2.1	-2.2	-2.1	-1.6	-1.3	Cell cycle arres
4602	MYB	v-myb myeloblastosis viral oncogene homolog (avian)	0.00	-1.5	-2.3	-2.1	-3.1	-2.6	DNA binding
9133	CCNB2	Cyclin B2	0.00	-1.1	-1.1	-1.3	-3.1	-8.2	Cell cycle
890	CCNA2	Cyclin A2	0.00	-1.1	-1.5	-1.9	-3.7	-7.0	Mitotic cell G2/M transitic damage checkp
7298	TYMS	Thymidylate synthetase	0.00	-1.3	-2.0	-2.9	-5.3	-7.2	DNA replicatic
83879	CDCA7	Cell division cycle associated 7	0.00	-1.8	-2.2	-2.4	-2.8	-4.3	Cell proliferati
10733	PLK4	Polo-like kinase 4	0.00	-1.2	-1.4	-2.0	-4.0	-8.1	Positive regul centriole replic
56924	PAK6	p21 protein (Cdc42/Rac)-activated kinase 6	0.00	-1.1	-1.5	-1.4	-3.1	-7.9	Cell proliferati

One of the pathways fueling virally driven cellular proliferation was the NF-κB signaling pathway and the role of this pathway in tumor development is consistently verified by various authors [12, 13]. The data tabulated in Table 2 indicate that maslinic acid may have prevented further NF-κB activation via the up-regulation of IκBα, a key inhibitory protein encoded by *NFKBIA*. The release of NF-κB heterodimer was arrested and consequently avoided the activation of this signaling network as well as its responsive genes like *CYBB*, a transcriptionally activated factor of the Raji cells [14].

Apart from the evident inhibitory effect on the NF-κB signaling pathway [15], this study also demonstrated that an extended incubation with maslinic acid has also led to an intensified immunogenicity response. To evade the immunosurveillance mechanism of the host, EBV has impeded antigen production through the inhibition of proteasomal degradation via *EBNA1* and hence, the latter mechanism is essentially crucial to rectify such actions. In the same way as TSA, maslinic acid was observed to have up-regulated the expression of several components of the antigen presentation machinery (APM) and this event is hypothesized to be coordinated by critical mediators such as IRF1 (**Table 2**) [16]. The functional role of *IRF1* as a tumour suppressor is well established and both *in vitro* as well as *in vivo* studies have reported this gene to be an important moderator of the interaction between tumour cells and the immune system; especially in facilitating the synthesis of APM subunits [17,18]. The results evidently showed that maslinic acid was able to suppress tumourigenesis by arresting the cell cycle of Raji cells at G1 and S/G2 phase and also intercepted immune-evading events via the regulation of multiple genes involved in MHC antigen presentation pathways (**Table 2**). The ability of immune-surveillance evasion also shows the insignificance of EBV infection. EBV is known to weaken the expression of HLA class I tumour antigen-derived peptide complexes on tumour cells. This leads to the prevention of the innate immune system to detect infected cells via MHC class 1 expression. Therefore, cells infected with EBV are continuously produce immortalized cells by creating an autocrine loop. On the contrary, the second half of the gene expression profile has shown a surprising genomic progression of the late events when treated with maslinic acid. During this period, the gene profile shows

down-regulated genes involved in the cell cycle and the up-regulation of several key genes of the MHC-antigen presenting and processing pathway (**Table 2**).

Table 2. The expression level of genes which mediate the cross-talk between tumour cells and the innate defense system.

Entrez ID	Gene symbol	Gene name	Time (Hours)					Biological activity
			4	8	12	24	48	
3105	HLA-A	Major histocompatibility complex, class I, A	-1.1	1.1	1.3	2.0	2.2	MHC class I recep activity
3106	HLA-B	Major histocompatibility complex, class I, B	-1.0	1.1	1.4	2.4	2.6	MHC class I recep activity
3107	HLA-C	Major histocompatibility complex, class I, C	-1.0	1.1	1.3	2.1	2.3	MHC class I recep activity
3135	HLA-G	Major histocompatibility complex, class I, G	-1.1	1.0	1.3	2.0	2.2	MHC class I recep activity
3134	HLA-F	Major histocompatibility complex, class I, F	1.0	1.1	1.1	2.0	2.2	MHC class I recep activity
3133	HLA-E	Major histocompatibility complex, class I, E	1.1	1.2	1.4	3.0	2.8	MHC class I recep activity
3108	HLA-DMA	Major histocompatibility complex, class II, DM alpha	1.1	1.4	1.6	2.0	2.0	MHC class II prot complex
3119	HLA-DQB1	Major histocompatibility complex, class II, DQ beta 1	1.2	1.6	1.5	2.3	2.3	MHC class II recep activity
3123	HLA-DRB1	Major histocompatibility complex, class II, DR beta 1	1.0	1.1	1.4	2.1	1.9	MHC class II prot complex
355	FAS	Fas (TNF receptor superfamily, member 6)	-1.0	-3.1	-3.6	-1.8	-2.4	Induction of apopto by extracellular sign
4033	LRMP	Lymphoid-restricted membrane protein	-1.3	-3.6	-4.9	-3.6	-2.5	Endoplasmic reticul
5698	PSMB9	Proteasome	-1.7	-1.6	-1.3	2.0	2.1	Proteasome comple
6890	TAP1	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	-1.0	-1.0	-1.1	3.7	3.5	Intracellular transp of viral proteins in h cell
3659	IRF1	Interferon regulatory factor 1	1.2	1.1	1.0	4.6	2.7	Sequence-specific DNA bind transcription fac activity
10537	UBD	Ubiquitin D	1.0	-1.0	1.1	3.2	5.0	Protein ubiquitinatic
7124	TNF	Tumor necrosis factor	2.3	2.8	3.5	6.7	3.7	MAPK signal pathway
962	CD48	CD48	-1.4	-3.0	-4.2	-3.0	-1.9	Antigen binding
8832	CD84	CD84 molecule	-1.8	-3.8	-4.8	-4	-4.3	Integral to membran
1536	CYBB	cytochrome b-245, beta polypeptide	-1.3	-2.4	-3.7	-4.5	-6.7	Respiratory burst
4792	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.6	1.2	1.4	3.7	3.5	NF-kappaB binding

The MHC network is critical for the presentation of antigenic peptides on the cell membrane to attract the attention of cytotoxic T lymphocytes. Generally, MHC presentation is categorized under MHC class I and class II molecules. The difference between them is by the length of antigen peptides loaded onto them as well as the antigen processing methods [19]. *PSMB9*, *UBD* and *TAP1* were positively upregulated after 24 hours of treatment with maslinic acid. These genes are categorized under the classical pathway where it usually regulates short half-life antigenic peptides generated from the ubiquitin-proteasome degradation system and provide rapid antigen detection [20]. *TAP1*-encoded protein forms part of the APM components. It translocates the proteosomal substrates into the endoplasmic reticulum where the MHC class I/peptide complexes are later exported onto the cell membrane. Apart from *TAP1*, *HLA-DMA* and *HLA-DQB1*genes that represent MHC class II also up-regulated by 2-folds. However, the number of genes representing MHC class I was more than that in the MHC class II route. Therefore, it is concluded that maslinic acid treatment activates an immune response favouring TAP1 and proteasome-dependent MHC class I antigen processing and presentation pathway.

CONCLUSION

The present study has provided a new perspective of the mechanism of action of maslinic acid on Raji cells in a time-dependent manner and this finding was not previously reported. Therefore, the plausibility of maslinic acid in re-instating the visibility of virally induced B-cells to the innate defense system is proposed in this communiqué.

ACKNOWLEDGMENTS

This work was supported by the Fundamental Research Grant Scheme (FRGS/2/2010/SKK/UTAR/02/1).

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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