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The Farnesyltransferase Inhibitors Tipifarnib and Lonafarnib Inhibit Cytokines Secretion in a Cellular Model of Mevalonate Kinase Deficiency

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ABSTRACT: The shortage of geranylgeranyl-pyrophosphate (GGPP) was associated to an increased IL-1β release in the autoinflammatory syndrome mevalonate kinase deficiency (MKD), a rare inherited disease that has no specific therapy. Farnesyltransferase inhibitors (FTIs) act at the end of mevalonate pathway. Two FTIs, Tipifarnib (Tip) and Lonafarnib (Lon), were therefore evaluated as possible therapeutical choices for the treatment of MKD. FTIs could lead to a redirection of the limited available number of mevalonate intermediates preferentially to GGPP synthesis, eventually preventing the uncontrolled inflammatory response. The effect of Tip and Lon on intracellular cholesterol level (ICL) and on proinflammatory cytokines secretion was evaluated in a cellular model of MKD, chemically obtained treating RAW 264.7 cells with lovastatin (Lova) and alendronate (Ald). The combination of FTIs with the isoprenoid geraniol (GOH) was also tested both in this model and in monocytes isolated from MKD patients. Tip and Lon proved to revert the ICL lowering and to significantly reduce the lipopolysaccharide-induced cytokines secretion in Ald-Lova -RAW 264.7 cells. This anti-inflammatory effect was amplified combining the use of GOH with FTIs. The effect of GOH and Tip was successfully replicated in MKD patients' monocytes. Tip and Lon showed a dramatic anti-inflammatory effect in monocytes where mevalonate pathway was chemically or genetically impaired. **(*Pediatr Res* 70: 78–82, 2011)**

In the familial mevalonate kinase deficiency (MKD, OMIM: *251170), the second enzyme of the mevalonate pathway [mevalonate kinase, MK/mevalonate kinase gene (NM_000431) (*MVK*)] is mutated and shows a reduced activity, leading to a shortage of downstream compounds. In particular, the lack of geranylgeranyl-pyrophosphate (GGPP) results in an augmented caspase-1-dependent IL-1β secretion that is the major cytokine responsible for the inflammatory systemic effects observed in MKD patients (1,2). These subjects present periodic fever attacks associated with lymphadenopathy, abdominal, articular, and cutaneous involvement, and, in most severe cases, also neurological impairment (3). Despite the many efforts done in the past decades to elucidate the molecular events linking the mevalonate pathway impairment to the inflammatory clinical phenotype, no specific treatment has been yet developed for MKD.

We and other authors have recently proposed natural exogenous isoprenoids (NEIs) as a possible therapeutic approach for MKD. These compounds, because of their isoprenoid structure, are supposed to enter the mevalonate pathway and to bypass the biochemical block, reconstituting the pathway and limiting the shortage of GGPP (2,4,5) (Fig. 1).

With similar results, the farnesyltransferase inhibitor (FTI) Manumycin A (ManA) was recently used in our cellular and murine model of MKD (6). FTIs are a class of experimental drugs that target protein FT with the downstream effect of preventing the proper functioning of Ras protein, and they have been studied as anticancer agents but have not been definitely approved yet (7,8). In our opinion, FT inhibition in MKD cells lead to a redirection of the limited available number of GGPP molecules preferentially to geranylgeranylation, thereby preventing the activation of caspase-1 and production of mature IL1β (Fig. 1).

Taking into account all these findings, and in particular those obtained with ManA, we decided to investigate the effect of two novel FTIs, Tipifarnib (Tip) and Lonafarnib (Lon) (9,10), on the inflammation induced by the inhibition of the mevalonate pathway, in the hypothesis to find out an alternative use for these anticancer drugs in the treatment of MKD (Fig. 1).

Tip and Lon were first tested in a cellular model of MKD obtained treating the murine monocytic cell lines RAW 264.7 with two known inhibitors of the mevalonate pathway, alendronate (Ald) (11) and lovastatin (Lova) (12), and then in monocytes isolated from two MKD patients. The cellular model obtained with the combined use of Ald and Lova aims

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Abbreviations: Ald, alendronate; **FTIs,** farnesyltransferase inhibitors; **GGPP,** geranylgeranyl-pyrophosphate; **GOH,** geraniol; **ICL,** intracellular cholesterol level; **Lon,** Lonafarnib, Sarasar; **Lova,** lovastatin; **LPS,** lipopolysaccharide; **ManA,** Manumycin A; **MK,** mevalonate kinase (E.C. 2.7.1.36); **MKD,** mevalonate kinase deficiency; **MTT,** 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide; ***MVK,*** mevalonate kinase gene (NM_000431); **NEIs,** natural exogenous isoprenoids; **Tip,** Tipifarnib, Zarnestra

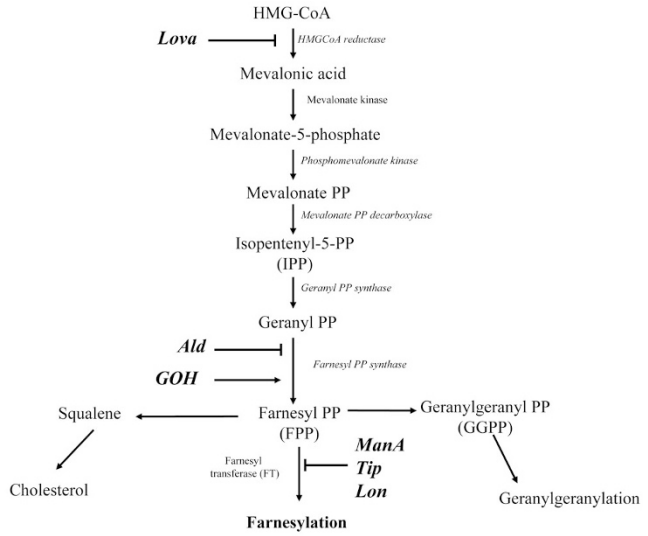

Figure 1. Schematic representation of mevalonate pathway. Compounds used in the experiments are indicated along the pathway in *bold* characters.

to reproduce as faithfully as possible the condition of block metabolic pathway of cholesterol. The results were compared with those obtained with NEIs for their anti-inflammatory action that has been recently described in these models (6,13): finally, we evaluated the additive effect of FTIs and NEIs.

MATERIALS AND METHODS

Chemicals. Lipopolysaccharide (LPS, *E. coli*-serotype 055:B5, 1 mg/mL stock in H_2O), Ald (30 mM), geraniol (GOH, 6 M; Euphar group s.r.l., Piacenza, Italy), and Lova (50 mM) were dissolved in saline solution. ManA (10 μM), Tip (Tip, 20 mM), and Lon (16.5 mM) were dissolved in DMSO so that the final concentration of DMSO would not exceed 0.1%. Tip (Tip, R115777, Zarnestra) and Lon (SCH66336, Sarasar) were kindly provided by Prof G. Martinelli (Institute of Hematology 'L and A Seràgnoli', University of Bologna, Bologna, Italy). All the reagents were from Sigma Chemical Co.-Aldrich (Milan, Italy), except where differently specified.

RAW 264.7 cell culture. RAW 264.7 cells (murine monocyte/macrophage cell line) were cultured in DMEM supplemented with 10% fetal bovine serum with 100 μM Ald and 20 μM Lova for 20 h and then with 10 μg/mL LPS for additional 24 h. At the end of the incubation period, the supernatants were collected for cytokine evaluation, while the cells were pelleted for the quantification of intracellular cholesterol. In the experiments, GOH (100 μM), ManA (10 μM), Tip (5 μM), or Lon (5 μM) were added together with Ald and Lova.

MKD patients and monocyte isolation. Monocytes isolated from the peripheral blood of two MKD subjects (patient 1, P1: 4 y old, male, *MVK*: S135L/V377I; patient 2, P2: 10 y old, male, *MVK*: I268T/V377I), through standard protocol, were cultured at a cell density of 2×10^5 cells/well in RPMI 1640 containing 10% fetal bovine serum (Euroclone, Milan, Italy). Cells were incubated with 10 μM GOH for 20 h, or 5 μM Tip for 1 h, or their combination. Cells were then treated with 1 μg/mL LPS for supplementary 24 h. At the end of the incubation period, the supernatants were collected for IL1β assay. Written informed consent was obtained from patients' parents, according to the protocol of the ethical board of Institute of Children Health "Burlo Garofolo" (Trieste, Italy; n.185/08, 19/08/2008).

Determination of cytokines release (IL1β, IL18, and TNFα). IL1β, IL18, and TNFα concentrations were determined in the cell culture medium (supernatant) by ELISA kits according to manufacturer's protocols, and the amount of cytokines expressed as picograms per milliliters (Endogen Human IL-1β ELISA Kit; Pierce Biotechnology Inc., Rockford, IL).

Determination of intracellular free cholesterol. The amount of free cholesterol was determined with the Amplex red cholesterol assay kit according to the manufacturer's instructions (Molecular Probes, Invitrogen), and the amount of cholesterol expressed as micrometers (14,15).

Evaluation of cytotoxicity. Tip, Lon, ManA, and GOH toxicity were evaluated with the MTT (3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) assay according to the method of Mosmann (16). Briefly, 2×10^5/mL RAW 264.7 cells or human monocytes were incubated with 10 to 200 μM Ald, 5 to 40 μM Lova, 1 to 50 μg/mL LPS, 1 to 20 μM Tip, 1 to 20 μM Lon, 1 to 20 μM ManA, and GOH 10 to 100 μM alone and in combination, for 3 d; before adding MTT reagent.

Statistical analysis. All results are expressed as the mean ± SE. Statistical significance was calculated using one-way ANOVA, followed by Tukey multiple comparison test. p values less than 0.05 were considered significant. Statistical analysis was performed using the GraphPad Prism software version 5 (GraphPad Software, San Diego, CA).

RESULTS

Tip (1–20 μM) or Lon (1–20 μM) (9,17,18) were added to the cellular model of MKD obtained treating RAW 264.7 cells simultaneously with 100 μM Ald and 20 μM Lova. Ald + Lova lead to a significantly reduction of intracellular cholesterol level (ICL; 10.43 ± 1.12 μM) when compared with untreated cells (14.40 ± 0.72 μM) as expected being inhibitors of mevalonate pathway; this inhibition was amplified in the presence of LPS (Ald + Lova + LPS: 7.88 ± 0.28 μM). In Figure 2, the effect of 5 μM Tip and 5 μM Lon was reported as minor dose with maximum results. We tested several concentration of both compounds and finally we chose the dose of 5 μM according to indication in literature data. The DMSO used as solvent for the two compounds did not alter any of the results (data not showed).

Tip and Lon were able to significantly revert the diminishing of ICL (9.70 ± 0.17 μM and 9.76 ± 0.40 μM, respectively) compared with Ald + Lova + LPS. These results were comparable with those obtained with ManA (9.53 ± 0.56 μM) and GOH (9.6 ± 0.69 μM).

The secretion in the cell culture medium of proinflammatory cytokines IL1β, IL18, and TNF were significantly reduced in Ald-Lova-LPS cells treated with Tip and Lon (Table 1). In Figure 3, the effect of Tip (5 μM) and Lon (5 μM) on IL1β reduction was reported as exemplificative of the three cytokines. Similar reductions (Tip: 44.3%; Lon: 46%) were obtained with the natural isoprenoid GOH (54.3%) or with another FTI, ManA (32.3%; Fig. 3). The cytokine release to

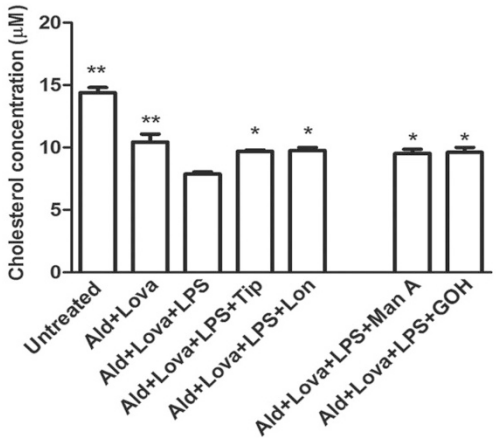

Figure 2. Tipifarnib and Lonafarnib rescue the levels of intracellular cholesterol in Ald-Lova-LPS RAW 264.7 cells. Cells were incubated with 100 μM alendronate (Ald) and 20 μM lovastatin (Lova) for 20 h, and then with 10 μg/mL LPS for supplementary 24 h. *Bars* represent the means ICL (μM) ± SE of three independent experiments. *$p < 0.05$ significantly different from ICL in Ald + Lova + LPS-treated cells. **$p < 0.01$ significantly different from ICL in Ald + Lova + LPS-treated cells.

Table 1. *Effect of Tipifarnib (Tip), Lonafarnib (Lon), Manumycin A (ManA), and Geraniol (GOH) on LPS-induced cytokines secretion in Ald-Lova-LPS RAW 264.7 cells*

	Ald + Lova + LPS	Ald + Lova + LPS + Tip	*p*	Ald + Lova + LPS + Lon	*p*	Ald + Lova + LPS + ManA	*p*	Ald + Lova + LPS + GOH	*p*
IL-1β (pg/mL)	5506 ± 675.2	3291 ± 401.7	<0.01*	1567 ± 243.1	<0.01*	2673 ± 305.8	<0.01*	2673 ± 305.8	<0.05†
IL-18 (pg/mL)	1297 ± 135.2	790.5 ± 112.5	<0.01*	628 ± 49.47	<0.01*	611.6 ± 6.3	<0.01*	611.6 ± 6.3	<0.01*
TNF-α (pg/mL)	7260 ± 329.1	3456 ± 78.42	<0.01*	1690 ± 10.15	<0.01*, <0.05†	2329 ± 195.2	<0.01*	2329 ± 195.2	<0.01*

Data were representative of three distinct experiments. Mean concentration and SE are reported.

* Significantly different from cytokine concentration in Ald-Lova-LPS cells.

† Significantly different from cytokine concentration in Ald-Lova-LPS cells.

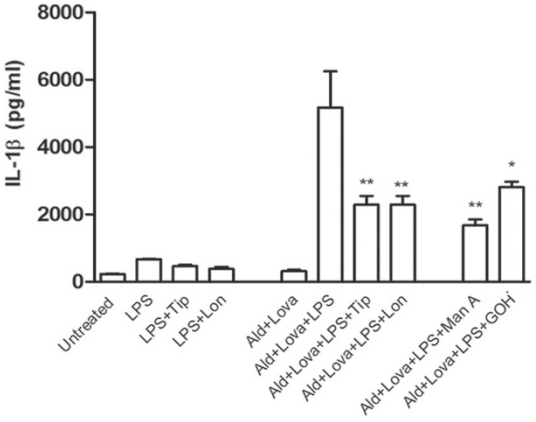

Figure 3. Tipifarnib and Lonafarnib are able to lower the IL-1β secretion in Ald-Lova-LPS RAW 264.7 cells. Cells were incubated with 100 μM alendronate (Ald) and 20 μM lovastatin (Lova) for 20 h, and then with 10 μg/mL LPS for supplementary 24 h (Ald + Lova + LPS). FTIs (10 μM ManA, 5 μM Tip, 5 μM Lon) and GOH (100 μM) were added simultaneously with Ald and Lova. *Bars* represent the mean concentration (pg/mL) ± SE of three experiments. *$p < 0.05$ significantly different from cytokine concentration in Ald-Lova-LPS cells. **$p < 0.01$ significantly different from cytokine concentration in Ald-Lova-LPS cells.

the untreated cells (230 ± 20 pg/mL) and in presence of Ald and Lova (316 ± 20 pg/mL) alone can be seen in the Figure 3. Tip and Lon were able to rescue the IL-1β secretion compared with LPS-treated cells even if not in a statistically significant way (Fig. 3). When GOH was used together with Tip and Lon, the effect of the two FTIs on the IL1β, IL18, and TNF secretion was amplified (Table 2) as reported in Figure 4 for IL1β.

Tip + GOH and Lon + GOH significantly diminish IL1β secretion (277.1 ± 92.1 pg/mL and 421.9 ± 122.5 pg/mL, respectively) when compared with Ald + Lova + LPS-treated cells (5519 ± 1497 pg/mL; $p < 0.05$) as well as to Tip (2291 ± 453.2 pg/mL; $p < 0.05$), Lon (2305 ± 359.8 pg/mL; $p < 0.05$), or GOH (2811 ± 271.1 pg/mL; $p < 0.05$) alone (Fig. 4). A similar additive anti-inflammatory effect was also observed using GOH combined with ManA (Fig. 4).

The combination Tip + GOH resulted significantly more effective than Lon + GOH ($p < 0.001$), and so we decided to test Tip, GOH, and Tip + GOH on LPS-induced IL-1β secretion in monocytes isolated from two MKD patients. In MKD patient 1 (P1), Tip was more effective in diminishing IL-1β secretion compared with LPS-treated cells alone or in combination with 10 μM GOH even if not in a statistically significant way (Tip + LPS 107.8 ± 32.8 pg/mL; Tip + GOH + LPS 92.36 ± 0.47 pg/mL *versus* LPS 171.9 ± 11.6 pg/mL: p; Fig. 5*A*). This effect was registered also in MKD patient 2 (P2): Tip and Tip + GOH significantly reduced LPS-induced IL-1β secretion in (P2) (Tip + LPS 273.3 ± 9.2 pg/mL, Tip + GOH + LPS 188.2 ± 20.5 pg/mL *versus* LPS 1068 ± 92.7 pg/mL: $p < 0.001$; Fig. 5*B*). Tip and Lon, at the concentration used, did not result cytotoxic when evaluated with the MTT assay (data not shown).

DISCUSSION

The inhibition of farnesyltransferase is nowadays a common target for antineoplastic drugs because of the consequent reduction of farnesylated protein such as small GTP proteins of oncogene Ras family (19). Recently, we reported the use of the FTI ManA as a possible choice for the treatment of the hereditary rare and still orphan disease MKD (6). The hypothesized mechanism of FTIs is principally related to the redirection of GGPP to geranylgeranylation (6).

In this study, we decided to investigate the effect of other two FTIs, the Tip and Lon, that are currently used in clinical trial (20,21) in a MKD cellular model because in our opinion, they could be more easily and rapidly included in the treatment of other inflammatory diseases.

In this cellular model, the combination of Ald and Lova induced an expected and significant reduction in intracellular cholesterol, similarly to what shown in mice serum level (22), and let us using this parameter to verify the changes in mevaloante pathway induced by different compounds (isoprenoids or FTIs).

Tip and Lon were able to revert the decrease of intracellular cholesterol induced by Lova and Ald (Fig. 2; inhibitors of HMG-CoA-red and farnesyl-pyrophosphate synthase, respectively, as shown in Fig. 1), demonstrating that these compounds, acting on FT, could modulate the outcome of the residual intermediates of mevalonate pathway below farnesylpyrophosphate synthase driving them through the other two main exits, cholesterol biosynthesis and geranylgeranylation.

Moreover, these FTIs reduced proinflammatory cytokines secretions either in MKD cellular model (Fig. 3) and in monocytes isolated from MKD patients (Fig. 5), emphasizing the hypothesis that the reequilibration of GGPP production could ameliorate the inflammatory state because of a biochemical or a familial unpaired mevalonate pathway (6). Similar results were previously obtained through the use of exogenous isoprenoids, such as GOH, farnesol, and geranylGOH (4,13),

Table 2. *Geraniol (GOH) enhances the anti-inflammatory effect of farnesyltransferase inhibitors (Tipifarnib, Lonafarnib, and Manumycin A)*

	Ald + Lova + LPS + GOH	Ald + Lova + LPS + Tip	Ald + Lova + LPS + GOH + Tip	P1	P2
IL-1β (pg/mL)	2811 ± 157.1	3291 ± 401.7	310.6 ± 72.37	$p < 0.05$	$p < 0.05$
IL-18 (pg/mL)	730.6 ± 17.77	790.5 ± 112.5	132.5 ± 25.76	$p < 0.05$	$p < 0.05$
TNF-α (pg/mL)	1841 ± 44.84	3456 ± 78.42	446.1 ± 3.45	$p < 0.01$	$p < 0.01$, $p < 0.05$
	Ald + Lova + LPS + GOH	Ald + Lova + LPS + Lon	Ald + Lova + LPS + GOH + Lon	P3	P4
IL-1β (pg/mL)	2811 ± 157.1	1567 ± 243.1	321.9 ± 18.92	$p > 0.05$	$p > 0.05$
IL-18 (pg/mL)	730.6 ± 17.77	628 ± 49.47	95.60 ± 5.3	$p < 0.01$	$p < 0.05$
TNF-α (pg/mL)	1841 ± 44.84	1690 ± 10.15	777.3 ± 0.54	$p < 0.05$	$p > 0.05$
	Ald + Lova + LPS + GOH	Ald + Lova + LPS + ManA	Ald + Lova + LPS + GOH + ManA	P5	P6
IL-1β (pg/mL)	2811 ± 157.1	2673 ± 305.8	264.5 ± 3.94	$p > 0.05$	$p > 0.05$
IL-18 (pg/mL)	730.6 ± 17.77	611.6 ± 6.3	118.9 ± 13.88	$p < 0.01$	$p < 0.05$
TNF-α (pg/mL)	1841 ± 44.84	2329 ± 195.2	394.1 ± 81.49	$p < 0.01$	$p < 0.01$

Data were representative of three distinct experiments. Mean concentration and SE are reported.

P1, p value of the comparison between Ald + Lova + LPS + GOH + Tip respect and ALD + Lova + LPS + GOH; P2, p value of the comparison between Ald + Lova + LPS + GOH + Tip respect and ALD + Lova + LPS + Tip; P3, p value of the comparison between Ald + Lova + LPS + GOH + Lon respect and ALD + Lova + LPS + GOH; P4, p value of the comparison between Ald + Lova + LPS + GOH + Lon respect and ALD + Lova + LPS + Lon; P5, p value of the comparison between Ald + Lova + LPS + GOH + ManA respect and ALD + Lova + LPs + GOH; P6, p value of the comparison between Ald + Lova + LPS + GOH + ManA respect and ALD + Lova + LPS + ManA.

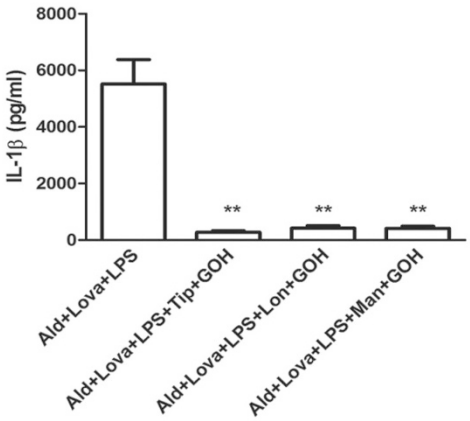

Figure 4. GOH enhances the anti-inflammatory effect of FTIs. Cells were incubated with 100 μM alendronate (Ald) and 20 μM lovastatin (Lova) (Ald + Lova) for 20 h, and then with 10 μg/mL LPS for supplementary 24 h. FTIs (10 μM ManA, 5 μM Tip, 5 μM Lon) and 100 μM GOH were added simultaneously with Ald and Lova. *Bars* represent the mean concentration (pg/mL) ± SE of three experiments. **$p < 0.01$ significantly different from cytokine concentration in Ald-Lova-LPS cells.

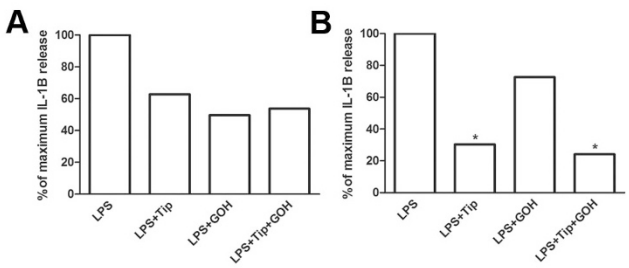

Figure 5. Tipifarnib, alone or in combination with GOH, is able to lower the LPS-induced IL-1β secretion in MKD monocytes. LPS-induced IL-1β secretion in monocytes from two MKD patients (P1-4A; P2-4B) incubated in the absence or presence of 10 μM GOH for 20 h, or 5 μM Tip for 1 h, or their combination. Cells were then treated 1 μg/mL LPS for supplementary 20 h. Absolute values (pg/mL) were obtained from one experiment performed in triplicate. *$p < 0.05$ significantly different from absolute value of cytokine concentration in LPS cells (100%).

and they were replicated with GOH in this study to compare the two different anti-inflammatory approaches.

It seems that the anti-inflammatory effect of Tip and Lon, as well as of GOH, was independent from the type of secreted cytokine, IL-1β, IL-18, or TNF, indicating a mechanism acting before the specific and different inflammatory signaling pathways. IL-1β and IL-18 were produced after the caspase-1 activation, an event specifically induced by the inhibition of mevalonate pathway as reported by Mandey *et al* (2). For what concern the inhibition on TNF secretion, no theory has been yet proposed, even if previous works showed that the block of mevalonate pathway induced TNF secretion from monocytes underling the causative link between cholesterol metabolism and TNF (23). We hypothesize that a small GTP protein or a prenilated protein could be involved in the signaling leading to TNF production, similarly to what happens with the activation of caspase-1 (2).

We first tried, unsuccessfully, to decrease the cytokines secretions to the basal condition with augmenting concentration of both FTIs and GOH, considering their similar anti-inflammatory effect (data not shown). We then tried with the combination of Tip or Lon and GOH: this significantly reduced the cytokines production (Fig. 4). Beyond expectation, FTIs and GOH showed a synergic effect, probably related to the bypassing of a compensative dose-related mechanism induced by each single compound, or alternative to the different mechanism of action of FTIs and GOH.

Our results suggest that the manipulation of the mevalonate pathway through the use of FTIs or GOH with the purpose to rebalance the intermediates levels acts above and in an aspe-

cific mechanism. Tip used in monocytes isolated from two MKD patients showed interesting results (Fig. 5) even if a certain interindividual variability and the limited number of patients did not allow us to definitively demonstrate the efficacy *ex vivo* of this compound.

The different entity of response between the two patients could be explained taking in account the interaction between genetic defect and compounds concentration. In MKD, residual MK activity varies from <0.5 to 7% depending on the type of *MVK* mutations (24). Considering that MK activity affects the levels of GGPP and the consequent shortage of geranylgeranylation, monocytes from subjects carrying different *MVK* mutations could need different amount of Tip to exhibit the same anti-inflammatory effect. A quantitative residual MK activity evaluation was not performed in our patients because it is quite expensive and not so informative for the clinical follow-up, but the possible residual MK activity could at least in part explain the different response to isoprenoids in the two patients carrying different mutations.

In conclusion, in agreement with previously reported data (2,4,6), our findings suggest once more that the recovery of GGPP flux through the geranylgeranyltransferase, in this case through the use of Tip and Lon, has an anti-inflammatory effect, both in pharmacologic or in genetic blockage of the mevalonate pathway. These molecules are nowadays used in clinical protocols as anticancer drugs, so when considering our findings and those already reported in the literature, we would like to propose the use of Tip and Lon as a novel therapeutical approach for the still orphan disease MKD.

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