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Regorafenib and reactive metabolite of sunitinib activate inflammasomes: Implications for multi tyrosine kinase inhibitor-liduced immune related adverse events

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Multi-targeted tyrosine kinase inhibitors have been developed for the treatment of various cancers, but they are associated with a significant incidence of idiosyncratic drug reactions (IDRs). There is compelling evidence that most IDRs are immune mediated. Activation of inflammasomes is often one of the early steps in the initiation of an immune response. This activation could involve the pharmacological effect of the drug, or it could involve the release of damage associated molecular patters (DAMPs) caused by a reactive metabolite. We tested whether sunitinib, regorafenib, lenvatinib and cabozantinib can directly activate inflammasomes in differentiated THP-1 cells. We found that regorafenib activated the inflammasome of differentiated THP-1 cells directly. We also found that the supernatant from the incubation of sunitinib with FLC-4 cells, which have a high capacity to metabolize drugs, led to activate the inflammasome of differentiated THP-1 cells. In the supernatant of FLC-4 cells with sunitinib, the heat shock protein (HSP) 90 was significantly increased. Sunitinib is known to be oxidized to generate a reactive, potentially toxic quinone imine. These results support the hypothesis that the reactive metabolite of sunitinib can cause the release of DAMPs from hepatocytes, which leads to activation of inflammasomes. Inflammasome activation may be an important step in the activation of the immune system by regorafenib and sunitinib, which in some patients, can cause IDRs.

1. Introduction

Excessive activity of tyrosine kinase induces phosphorylation of the receptor and activates downstream signaling pathways, which leads to uncontrolled cellular proliferation and survival, and often drives the initiation and progression of malignancy. Thus, many protein kinase inhibitors have been developed for the treatment of various cancers and used clinically. Tyrosine kinase inhibitors (TKIs) represent an important class of anti-cancer drugs, and their targets include vascular endothelial growth factor receptor (VEGF), platelet-derived growth factor receptor (PDGFR), stem cell factor (c-KIT), Flt3, and fibroblast growth factor receptor (FGFR). As tumor progression usually involves the action of multiple kinases rather than just one, multi-targeted TKIs are effective for cancer therapy. However, severe adverse events associated with targeted therapy by multi-targeted TKIs have also been reported. They include hypertension, venous thromboembolism (VTE), pulmonary embolism, interstitial pneumonia, cardiomyopathy, QT prolongation (Zhang et al. 2018). TKIs can also cause idiosyncratic drug reactions (IDRs), especially liver injury. There is compelling evidence that most IDRs are immune mediated. One step in the initiation of an immune response can be inflammasome activation. Our previous study revealed that TKIs of VEGF receptors activate inflammasomes, which might be involved in the mechanism of the IDRs caused by these drugs (Imano et al. 2021). The IDRs caused by multi-targeted TKIs may involve the same mechanism. However, this hypothesis has not been tested, and details of the mechanism have not been elucidated.

Several mechanisms have been proposed for how reactive metabolites can induce an immune response leading to an idiosyncratic drug reaction, but the dominant complementary hypotheses are the hapten and danger hypotheses (Cho and Uetrecht 2017). When reactive metabolites are produced, they can generate drug-modified peptides which are presented in the context of human leukocyte antigen molecules (hapten hypothesis). In general, a strong immune response is not induced by foreign proteins without a second signal, which is mediated by costimulatory molecules on antigen presenting cells (APCs) is required (Matzinger 1994). Reactive metabolites can cause cell damage, which leads to the release of damage-associated molecular patterns (DAMPs). DAMPs act through receptors such as the receptor for advanced glycation end products (RAGE) and toll-like receptors (TLR), which can lead to activation of inflammasomes, and this may be a common mechanism by which DAMPs activate APCs (Bettigole and Glimcher 2015).

In this study, we tested the ability of sunitinib, regorafenib, lenvatinib, and cabozantinib to activate inflammasomes in THP-1 cells, which are a human monocyte cell line. In addition, we incubated these drugs with functional liver cell (FLC)-4 cells, a human hepatocyte cell line with high drug metabolizing capacity and tested the ability of the supernatant from these cells to activate inflammasomes in THP-1 cells.

2. Investigations and results

2.1. IL-1β production and caspase-1 activity in THP-1 cells incubated with sunitinib, regorafenib, lenvatinib and cabozantinib

Incubation of THP-1 cells with regorafenib led to an increase in the production of IL-1β or caspase-1 activity (Fig. 1). However, incubation of THP-1 cells with sunitinib, lenvatinib, or cabozantinib did not increase the production of IL-1β or caspase-1 activity. The increase of the production of IL-1β or caspase-1 activity by regorafenib (30 μM) was significantly inhibited by adding the caspase-1 inhibitor YVAD to the culture medium of THP-1 cells (Fig. 1).

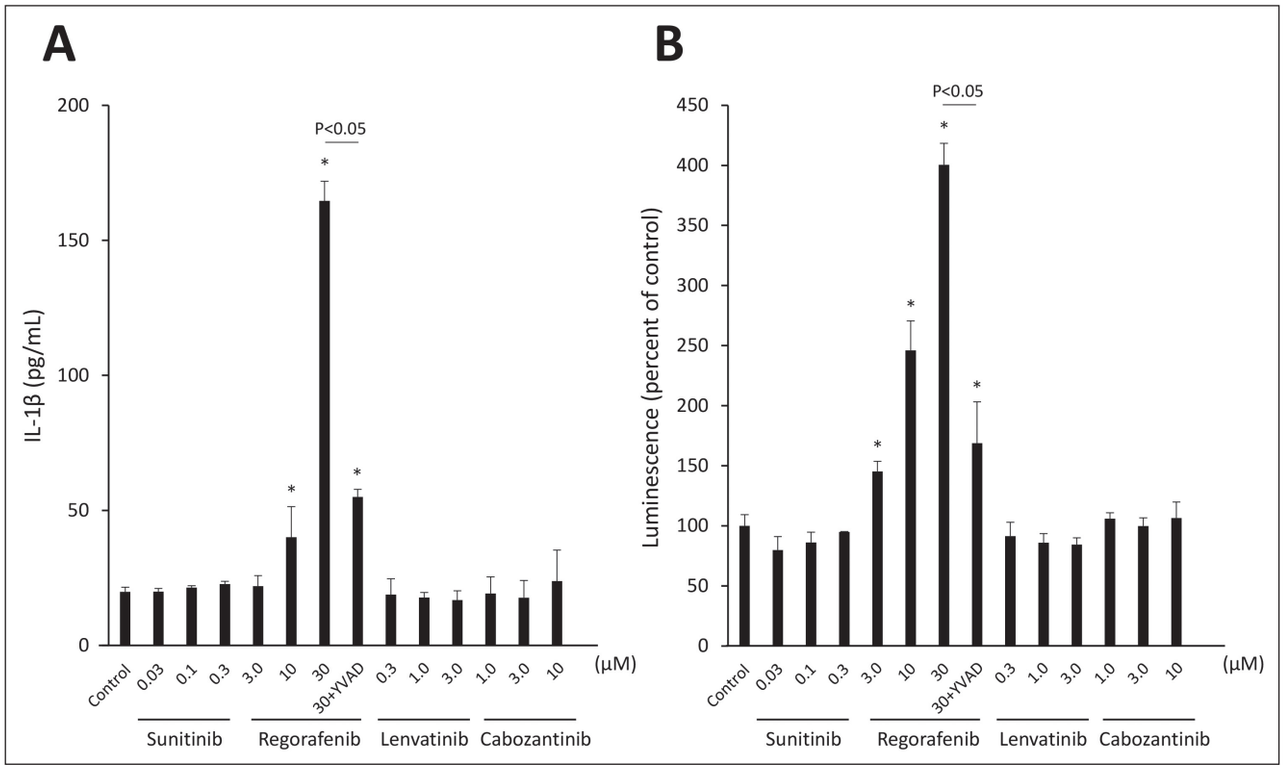

Fig. 1: Levels of IL-1β and caspase-1 activity in THP-1-derived macrophages in response to 24 h of treatment with sunitinib, regorafenib, lenvatinib and cabozantinib with or without a caspase-1 inhibitor, Ac-YVAD-CHO (YVAD). (A) Incubation of THP-1 cells with regorafenib led to release of IL-1ß. (B) Incubation of THP-1 cells with regorafenib also increased caspase-1 activity. Statistical significance was determined using the Tukey multiple comparison tests, where *, $p < 0.05$, vs. control (n=3).

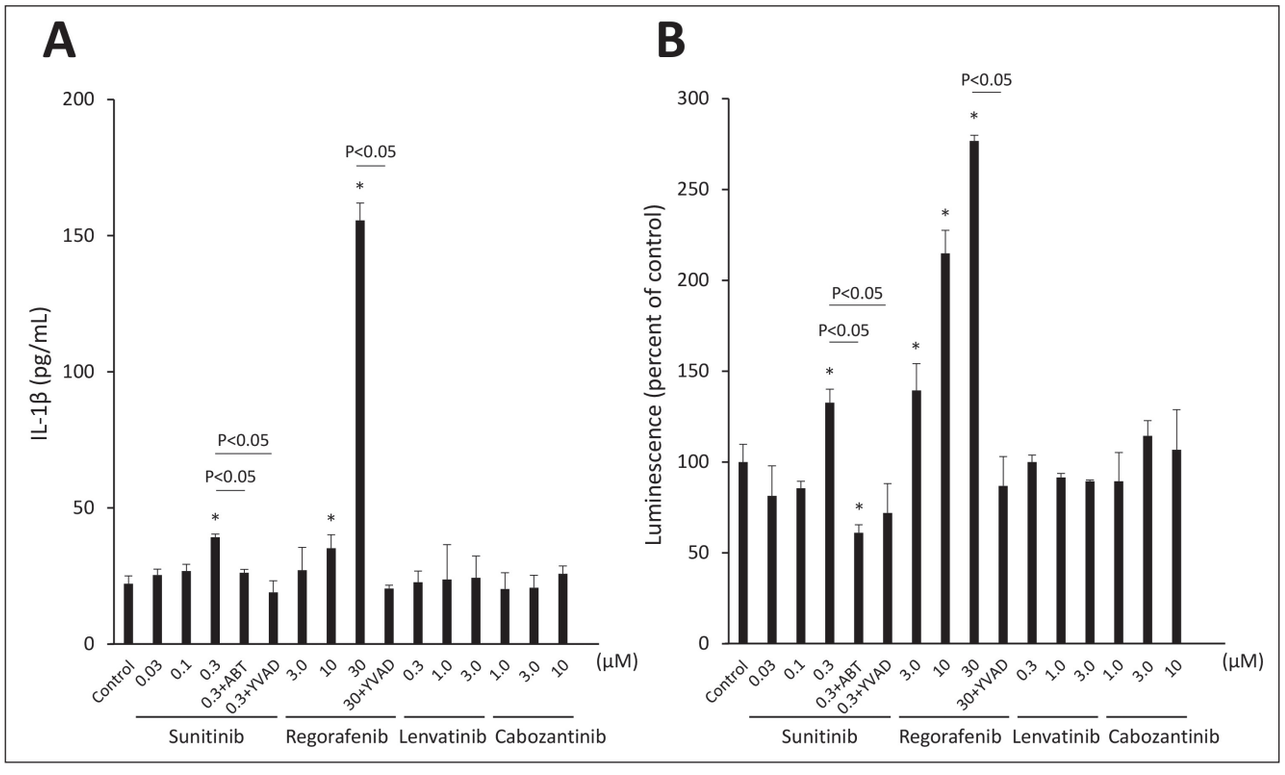

Fig. 2: IL-1b and caspase-1 activity of THP-1-derived macrophages in response to the supernatant from FLC-4 cells incubated with sunitinib, regorafenib, lenvatinib or cabozantinib with or without YVAD. (A) Incubation of THP-1 cells with the supernatant from FLC-4 cells incubated with sunitinib and regorafenib led to an increase in IL-1b. In the case of sunitinib, this was significantly inhibited by ABT added to the FLC-4 incubation. (B) Incubation of THP-1 cells with the supernatant from FLC-4 cells incubated with sunitinib and regorafenib led to an increase in caspase-1 activity. Again, this was significantly inhibited by ABT added to the FLC-4 incubation. Statistical significance was determined using the Tukey multiple comparison tests, where *, $p < 0.05$, vs. control (n=3).

2.2. IL-1β production or caspase-1 activity in THP-1 cells incubated with the supernatant from an incubation of FLC-4 cells with sunitinib, regorafenib, lenvatinib, or cabozantinib

Incubation of THP-1 cells with the supernatant from an incubation of FLC-4 cells with sunitinib or regorafenib led to an increase in IL-1ß production or caspase-1 activity (Fig. 2). However, incubation of THP-1 cells with the supernatant from an incubation of FLC-4 cells with lenvatinib or cabozantinib did not increase IL-1ß production or caspase-1 activity. The increase of the IL-1ß production or caspase-1 activity by sunitinib (0.3 µM) was significantly inhibited by adding the cytochrome P450 inhibitor ABT to the FLC-4 cell culture or by addition of the caspase-1 inhibitor YVAD to THP-1 cells (Fig. 2). In addition, the increase of the IL-1ß production or caspase-1 activity by regorafenib (30 µM) was significantly inhibited by addition of the caspase-1 inhibitor YVAD to THP-1 cells (Fig. 2).

2.3. Candidate DAMPs in the supernatants from FLC-4 cells incubated with sunitinib

In this study, we sought candidate DAMPs in the supernatants from FLC-4 cells incubated with sunitinib. As seen in Fig. 3A, there was no increase in high mobility group box 1 (HMGB1), heat shock protein (HSP) 32, HSP40, HSP60, HSP70, S100 calcium-binding protein (S100) A8, or S100A9 proteins. However, HSP90 was significantly increased in the supernatants from FLC-4 cells incubated with sunitinib, and it was decreased by adding ABT to the FLC-4 cell culture (Fig. 3).

3. Discussion

In this study, we revealed that regorafenib itself and the reactive metabolite of sunitinib activated the inflammasomes of differentiated THP-1 cells. Lenvatinib and cabozantinib did not activate inflammasomes of differentiated THP-1 cells within their therapeutic ranges.

In our previous studies, we demonstrated that several drugs that cause idiosyncratic drug reactions can lead to inflammasome activation (Mak et al. 2018). In the case of amodiaquine, myeloperoxidase in THP-1 cells oxidized amodiaquine, which led to activation of inflammasomes (Kato and Uetrecht 2017). While drugs such as amiodarone, carbamazepine or gefitinib that required bioactivation by cytochromes P450 did not directly activate THP-1 inflammasomes, but the supernatant from an incubation of these drugs with FLC-4 cells did activate THP-1 inflammasomes (Kato et al. 2019, 2020, 2021).

Although immortalized cell lines often lack the functions of drug metabolism including the major P450s, the FLC-4 cells have significant drug-metabolizing capacity (Kato et al. 2014), and they were used in this study to produce reactive metabolites of sunitinib. Presumably, this FLC-4 cell line oxidized amiodarone, carbamazepine, or gefitinib to their respective reactive metabolites leading to the release of DAMPs, which were responsible for activation of the THP-1 cells (Kato et al. 2019, 2020, 2021). Activation of the inflammasome caused by DAMPs may be a common factor in the mechanism of idiosyncratic drug reactions.

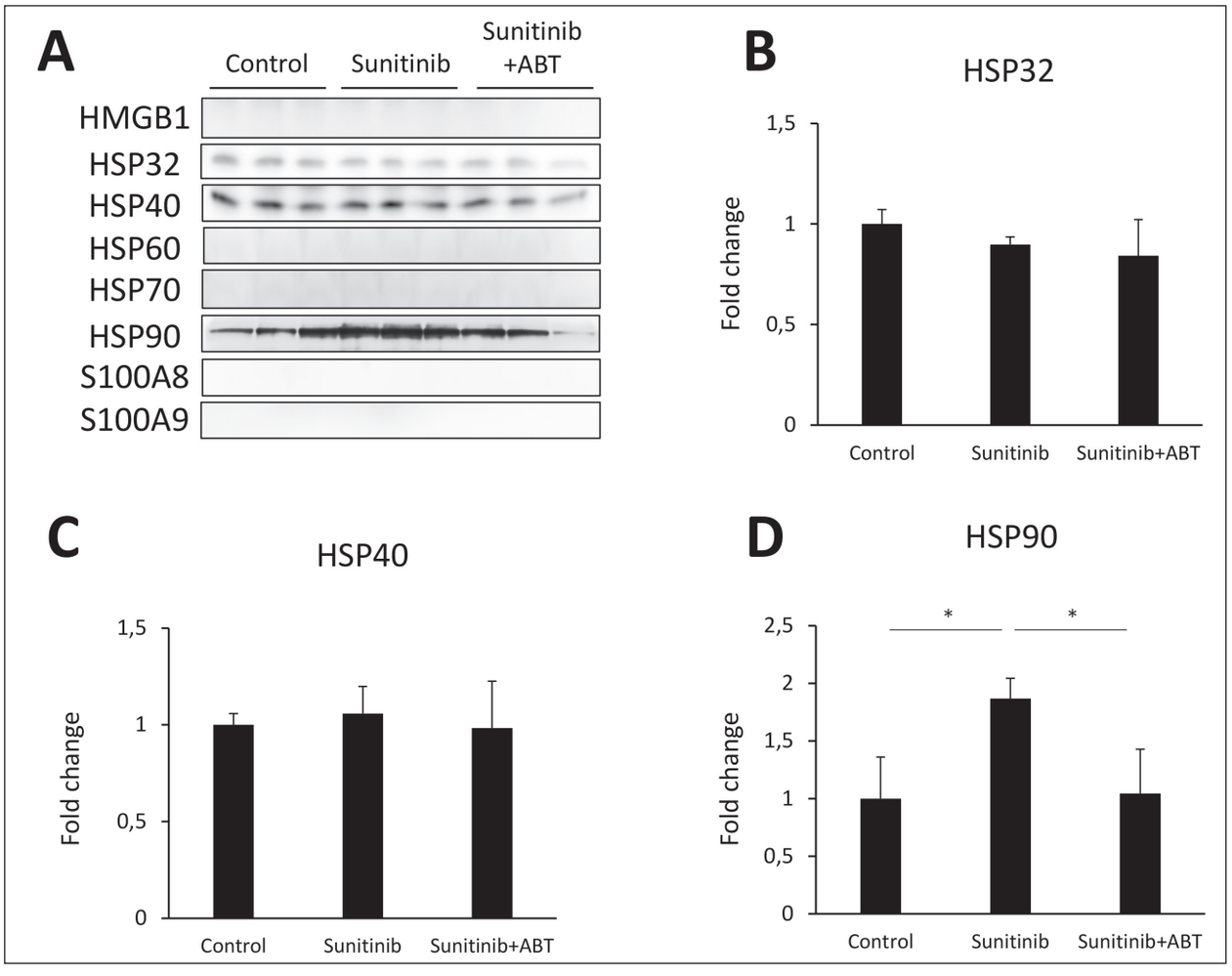

Fig. 3: Heat shock protein (HSP) 90 was released from hepatocytes as a danger associated molecular patterns (DAMP). (A) Western blot analysis of proteins [high mobility group box 1 (HMGB1), HSP32, HSP40, HSP60, HSP70, HSP90, S100 calcium-binding protein (S100) A8 and S100A9] that were released from hepatocytes incubated for 7 days with sunitinib (0.3 µM), with or without, a cytochromes P450 inhibitor (1-aminobenzotriazole, ABT), and their quantitative analysis of HSP32 (B), HSP40 (C) and HSP90 (D). Statistical significance was determined using the Tukey multiple comparison tests, where *, $p < 0.05$, n=3.

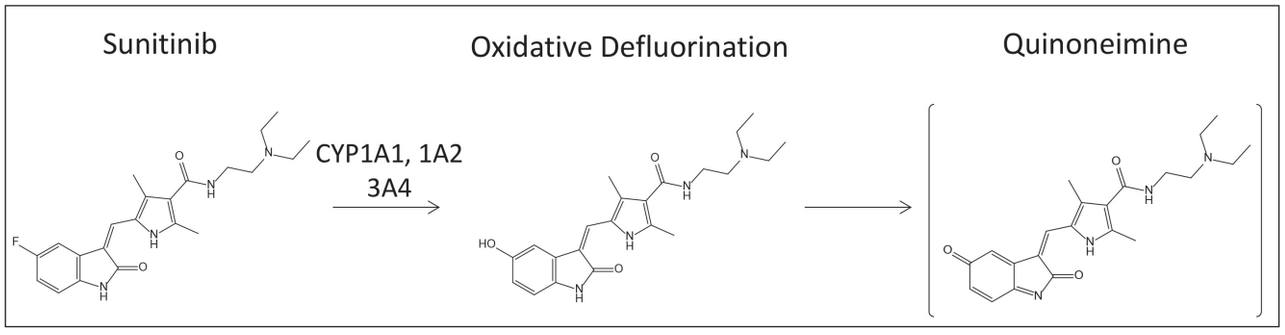

Fig. 4: Proposed bioactivation pathway of sunitinib that leads to the formation of a quinone reactive intermediate.

Sunitinib is oxidized to generate a reactive, potentially toxic quinoneimine (Amaya et al. 2018; Fig. 4). The quinone imine is generated by the oxidation of cytochrome P450 (CYP) in FLC-4 cells, and DAMPs were released from FLC-4 cells, which activates the inflammasome of THP-1 cells. Activation of inflammasomes was observed when regorafenib was incubated with differentiated THP-1 cells. The structure of regorafenib is almost the same as that of sorafenib. The difference between regorafenib and sorafenib is the addition of a fluorine atom to the central phenyl ring of sorafenib (Fondevila et al. 2019). Although there is no report that regorafenib is metabolized to generate a reactive metabolite, it could form an isocyanate like the reactive metabolite of ritonavir (Lin et al. 2013). In previous studies, we found that reactive metabolite caused by myeloperoxidase activates inflammasome of APCs (Kato and Uetrecht 2017), which may be the same mechanism that regorafenib activates inflammasome.

In this study, lenvatinib and cabozantinib did not activate inflammasomes of differentiated THP-1 cells. The inhibition of VEGF receptor leads to cause adverse events, which might include IDRs (Garcia et al. 2014). Lenvatinib and cabozantinib inhibits various kinds of tyrosine kinase including VEGF receptor and it is thus IDRs may be caused by their pharmacological reaction.

As mentioned, reactive metabolites of sunitinib can activate the inflammasome by inducing the release of DAMPs. HSP90 was significantly increased in the culture supernatant of FLC-4 cells treated with sunitinib. The cytochrome P450 inhibitor, ABT, prevented this increase, which strongly suggests that metabolites of sunitinib were responsible for release of HSP90. HSP90 is reported to be a DAMP, which can activate inflammasomes (Mayor et al. 2007; Kato et al. 2020).

In conclusion, our results support the hypothesis that activation of inflammasomes contribute to the idiosyncratic reactions associated with sunitinib and regorafenib. This method may provide a method to study the mechanism of idiosyncratic drug reactions and even predict which drug candidates are likely to cause such adverse reactions.

4. Experimental

4.1. Reagents

Sunitinib was purchased from MedChem Express (NJ, USA) and regorafenib monohydrate, lenvatinib mesylate and cabozantinib malate were purchased from Selleck Chemicals (TX, USA). 1-Aminobenzotriazole (ABT) and acetyl-tyrosyl-valyl-alanyl-aspartyl-chloromethylketone (YVAD) were obtained from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan) and Promega Corporation (WI, USA). Other reagents and solvents were commercially available extra-pure grade chemicals.

4.2. Cell cultures

Dulbecco's Modified Eagle Medium (100 µL, DMEM, 4.5 g glucose/L, Nacalai Tesque, Inc., Kyoto, Japan) containing 10% fetal bovine serum (BioSource International Inc., CA, USA) and a FLC-4 (JCRB0435, Health Science Research Resources Bank, Osaka, Japan) cell suspension (100 µL, 1.5×10^4 cells/mL) were applied to a Prime Surface 96U plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) for organoid culture. The cells in the 96U plate were cultured at 37 °C with 5% CO_2 in the presence of drugs and/or inhibitor for 7 days. THP-1 cells (JCRB0112, Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan) were differentiated in DMEM containing 10% fetal bovine serum (BioSource International Inc.) with phorbol 12-myristate 13-acetate (50 ng/mL, Merck KGaA, Darmstadt, Germany) for three days in a 24-well multiplate (4×10^5 cells/well). On the fourth day, each well was washed with Ca^{2+} and Mg^{2+} free phosphate-buffered saline, 500 µL of DMEM was added to each well, and the cells were incubated at 37 °C with 5% CO_2 for 24 h. After the aspiration of the medium, culture medium including drugs or from the FLC-4 cells that had been incubated with drugs for 7 days with/without inhibitors were added and incubated at 37 °C with 5% CO_2 for 24 h.

The drug concentrations were within their therapeutic concentrations (sunitinib, 0.03-0.3 µM; regorafenib, 3-30 µM; lenvatinib 0.3-3 µM; cabozantinib, 1-10 µM) (Speed et al. 2012; Mross et al. 2012; Boss et al. 2012; Nokihara et al. 2019). ABT (1 mM) was used to inhibit cytochromes P450 (Sharma et al. 2013; Parrish et al. 2016), and YVAD (1 µM) was used to inhibit caspase-1 activity.

4.3. Western blotting

Supernatants from FLC-4 cells were collected, and 20 µL was loaded onto a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The resolved proteins were then electro-transferred onto a polyvinylidene difluoride membrane (0.45 µm, Merck KGaA, Darmstadt, Germany). The primary antibodies, HSP32 rabbit polyclonal antibody (10701-1-AP, Proteintech Group, Inc., IL, USA), HSP40 rabbit polyclonal antibody (13174-1-AP, Proteintech Group, Inc.), HSP60 rabbit polyclonal antibody (15282-1-AP, Proteintech Group, Inc.), HSP70 rabbit polyclonal antibody (10995-1-AP, Proteintech Group, Inc.), HSP90 rabbit polyclonal antibody (13171-1-AP, Proteintech Group, Inc.), HMGB1 rabbit polyclonal antibody (10829-1-AP, Proteintech Group), S100A8 rabbit polyclonal antibody (GTX54721, GeneTex, Inc., CA, USA) and S100A9 rabbit polyclonal antibody (GTX129575, GeneTex, Inc.) were used in this study. They were detected by goat anti-rabbit IgG-peroxidase (SouthernBiotech, AL, USA) and bound peroxidase was visualized by using Luminata™ Classico Western HRP Substrates (Merck KGaA).

4.4. IL-1β concentration in culture medium

The culture medium of differentiated THP-1 cells was collected and stored at -80 ºC until analysis. IL-1β was measured in each culture medium sample using an ELISA kit (BioLegend, Inc., CA, USA).

4.5. Caspase-1 activity of differentiated THP-1 cells

Differentiated THP-1 cells were cultured with drugs or hepatocyte culture medium treated with drugs for 24 h in a 24-well plate (4×10^5 cells/well). Caspase-Glo® 1 Inflammasome Assay (Promega Corporation) was used to measure caspase-1 activity. Caspase-Glo reagent was added to each well and then incubated for 1 h at room temperature. The luminescence was measured with a plate reader.

4.6. Data analysis

Results are expressed as means±SD. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to assess for statistical significance ($p < 0.05$).

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Conflict of interest: None declared.

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