Collection of Data Variation Using a High-Throughput Image-Based Assay Platform Facilitates Data-Driven Understanding of TRPA1 Agonist Diversity

Yuko Terada 1,†, Kenjiro Tanaka 2,†, Minami Matsuyama 1, Masaya Fujitani 2, Masatoshi Shibuya 2, Yoshihiko Yamamoto 2, Ryuji Kato 2,3,‡ and Keisuke Ito 1,*

1 Department of Food and Nutritional Sciences, Graduate School of Integrated Pharmaceutical and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan; yukoterada@u-shizuoka-ken.ac.jp (Y.T.); south59moco@gmail.com (M.M.)
2 Department of Basic Medicinal Sciences, Graduate School of Pharmaceutical Sciences, Nagoya University, Tokai National Higher Education and Research System, Furocho, Chikusa-ku, Nagoya 464-8601, Japan; tanaka.kenjiro@g.mbox.nagoya-u.ac.jp (K.T.); masaya.fujitani.0917@gmail.com (M.F.); m-shibu@ps.nagoya-u.ac.jp (M.S.); yamamoto-yoshi@ps.nagoya-u.ac.jp (Y.Y.); kato-r@ps.nagoya-u.ac.jp (R.K.)
3 Institute of Nano-Life-Systems, Institutes of Innovation for Future Society, Nagoya University, Tokai National Higher Education and Research System, Furocho, Chikusa-ku, Nagoya 464-8601, Japan
* Correspondence: sukeito@u-shizuoka-ken.ac.jp; Tel.: +81-54-264-5544
† These authors contributed equally.
‡ Correspondence of in silico analysis section.

Abstract: Because transient receptor potential ankyrin 1 (TRPA1) is involved in various physiological functions, TRPA1-targeting drugs have been energetically developed. Although TRPA1 is considered a multimodal receptor, the structural diversity of TRPA1 agonists is not fully elucidated. We hypothesized that collecting a wider variation of TRPA1–compound interaction data would aid the understanding of its complex mechanism and aimed to challenge such data collection using an “image-based TRPA1 assay system combined with in silico chemical space clustering concept.” Our library was clustered with 27 physicochemical molecular descriptors in silico, and structurally diverse compounds from each cluster were selected for a detailed kinetic assay to investigate variations of agonist structural rules. Through two sets of assays evaluating various compounds in parallel with validating effects of the previously established structural rules, we discovered that different chemical groups contribute to agonist activity, indicating that there are multiple agonist design concepts. A novel core structure for a TRPA1 agonist has been also proposed. Our new approach, “collection of TRPA1 activity data on compounds with physicochemical diversity,” will not only facilitate the understanding of the structural diversity of TRPA1 agonists but also contribute to the development of a new type of TRPA1-targeting drug.

Keywords: TRPA1 agonist; image-based assay; Ca\(^{2+}\) imaging; chemical library; physicochemical descriptors; clustering; in silico analysis; data diversity

1. Introduction

Transient receptor potential ankyrin 1 (TRPA1) is a Ca\(^{2+}\)-permeable cation channel expressed in sensory neurons and non-neuronal cells of different tissues. TRPA1 has been linked to various physiological functions, including pain and pungency perception, energy metabolism, hormone secretion, and vasodilation [1,2]. Because TRPA1 is a prospective target for treating obesity, diabetes, and atherosclerosis, and others, drugs regulating TRPA1 activity have been energetically developed in pharmaceutical companies.

Various stimuli, such as chemicals, temperature, and pH, activate TRPA1. Several chemical agonists for TRPA1 have been reported, including allyl isothiocyanate (derived from mustard oil), cinnamaldehyde (derived from cinnamon), piperine (derived from...
pepper), and menthol (derived from mint) [1,3]. Since TRPA1 was proposed as a multimodal receptor, multiple interaction mechanisms and positions have been discovered; however, interaction rules have yet to be clarified.

To design an effective agonist for TRPA1 with complex interaction forms, it is crucial to broaden the variations of structure–activity relationships. However, because such exploration of novel structure–activity rules is commonly cost- and labor-intensive, an effective approach is in high demand. In this study, we hypothesized that collecting a wider variation of TRPA1–compound interaction data would aid the understanding of complex TRPA1 agonist interaction mechanisms. Thus, this study challenged the collection of novel TRPA1 agonist variations using an “image-based TRPA1 assay system combined with an in silico chemical space clustering concept” to enhance the design possibilities of TRPA1 agonists. TRPA1 was selected as a model receptor because TRPA1 is an important target of drug discovery with multiple interaction mechanisms. Our approach presents a cost- and labor-efficient concept to collect wider variations of agonist candidates within the limited assay number compared to the straightforward exhaustive library screening, since it can expand the candidate variation in prior in silico. Practically, rather than focusing on specific chemical groups that have been previously investigated as agonists, we converted the chemical structure into multiple molecular descriptors in silico and attempted to minimize the evaluation of structurally similar compounds by the clustering of chemical space before the actual assay. Using a library of 707 compounds linked to their detailed synthesis processes as a model case, we were challenged to effectively collect TRPA1 interaction data with physicochemical diversity to deepen the understanding of agonist design possibilities. Our 197-compound assay provides multiple TRPA1 interaction data with novel agonist structures for further investigation of TRPA1-targeting drugs. Because TRPA1 is involved in various physiological functions, such as energy metabolism, insulin secretion, and vasodilation, TRPA1-targeting drugs are expected to treat obesity, diabetes, atherosclerosis, and others [1,2].

2. Materials and Methods

2.1. Image-Based Human TRPA1 Assay System

A detailed experimental method was previously described [4]. In brief, human TRPA1 was stably expressed in T-Rex™-293 cells (Invitrogen, Carlsbad, CA, USA), and the channel activity was evaluated by Ca²⁺ imaging. The TRPA1-encoding sequence was subcloned into pcDNA4/TO (Invitrogen). The plasmid was transfected into the T-Rex™-293 cells by using Lipofectamine 2000 reagent (Invitrogen), and the transfected cells were selected using 500 µg/mL zeocin and 10 µg/mL blasticidin to obtain cells that stably express TRPA1. The stable cell line was maintained in Dulbecco’s modified Eagle medium—high glucose (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 unit/mL penicillin, 100 mg/mL streptomycin, and 250 ng/mL amphotericin (Nacalai Tesque, Kyoto, Japan), 500 µg/mL zeocin, and 10 µg/mL blasticidin. The cells were cultured at 37 °C in a 5% CO₂ humidified incubator and passaged twice in a week. The intracellular Ca²⁺ concentrations were measured using a FlexStation III microplate reader system (Molecular Devices, Sunnyvale, CA, USA) at 37 °C. The cells were seeded in 96-well plates 24 h before each assay, and 1 µg/mL tetracycline was added to the cells to induce TRPA1 expression. The cells were loaded with 3 µM of Fluo-8 AM (Invitrogen) for 1 h at 37 °C in a measuring buffer (pH 7.4) which contained 5.37 mM KCl, 0.44 mM KH₂PO₄, 137 mM NaCl, 0.34 mM Na₂HPO₄, 5.56 mM D-glucose, 20 mM HEPES, 1 mM CaCl₂, 0.1% bovine serum albumin, and 2.5 mM probenecid. The test compounds were dissolved in DMSO and added to the measuring buffer. All of the test compounds were administered at 1, 3, 5, 10, 30, 50, and 100 µM to the TRPA1-expressing cells, because the compounds did not show nonspecific effects on parent cells, T-Rex™-293 cells not expressing TRPA1, at the concentrations. TRPA1 activity was calculated using the following equation: TRPA1 activity = ΔF/F₀, where F₀ (baseline) is defined as the mean fluorescence value at 0–30 s before sample addition, and ΔF is calculated by subtracting F (signal intensity, the highest
fluorescence value at 30–150 s after sample administration) from F0. For obtaining EC50 values, curve fitting and parameter estimations were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). Each experiment was repeated at least three times, and each data point represents the mean ± SEM. The assay results were divided into four categories (P1, P2, N1, and N2) as follows; P1: high-activity agonists (EC50 was determined), P2: medium-activity agonists (EC50 was too high to calculate), N1: difficult to evaluate activity due to its low solubility, N2: no activity. Because the TRPA1-expressing cells were seeded on the bottom of 96-well plates, test compounds need to be dissolved in the measuring buffer to reach the cells. Derivatization is a possible approach to improve the solubility of N1 compounds.

2.2. Generating Physicochemical Descriptors

KNIME Analytics Platform (version 4.4) was used to construct molecular descriptors from SDF (Structure Data File) format data of each molecule using the RDKit and Chemistry Development Kit (CDK). A total of 55 descriptors were analyzed by their correlation coefficients, and highly correlated descriptors were removed. Finally, 27 different types of descriptors were selected to characterize the physicochemical properties of compounds (detailed information listed in Table S1). To test the average differences of major physicochemical descriptors between P1 and N2 compounds, Student’s t-test was applied.

2.3. Chemical Library (Library707) Information

The library consists of 707 chemical compounds, named Library707, which are listed in Table S2. Further structural and synthesis information is available upon reasonable request to the corresponding author [5–7].

2.4. Hierarchical Clustering and Compounds Grouping

All the molecular descriptors were clustered by hierarchical clustering. The correlation coefficient was used as a distance metric in the average linkage method. We performed data standardization and clustering using the R program (version 4.1.1). A threshold correlation coefficient of 0.7 was used to cut the tree diagram. Hence, 127 clusters were obtained (detailed information listed in Table S2).

3. Results and Discussion

3.1. Design of Efficient Assay Platform for Collecting TRPA1 Agonist Variations

Although TRPA1 agonists have been widely studied for finding several interaction rules, further understanding of TRPA1 agonist interaction facilitates better agonist design. Therefore, in this study, we hypothesized that collecting various TRPA1 agonists would provide novel insights for understanding the receptor–agonist interaction and the design of agonist structures. We combined the image-based TRPA1 assay system with the in silico chemical space clustering approach to perform such an assay to efficiently collect agonist variations.

Our image-based assay can analyze the kinetics of TRPA1 agonists by monitoring the intracellular Ca2+ concentration changes induced by TRPA1 activation using Ca2+-sensitive fluorescent dye, Fluo-8. This system enabled the collection of kinetics data from the 96-well plate platform of TRPA1 agonists within 30 min (assay scheme illustrated in Figure 1).

For our assay target, we set Library707 for our TRPA1 agonist data collection (Table S2). There are two main reasons why we set this library for our study: one, it consists of our original compounds, all of which have been chemically synthesized, so intermediates or compound variants could be easily synthesized for further structure–activity relation studies, and two, it provides the opportunity to explore novel structural rules for further TRPA1 agonist study since this library consists of compounds with rare structural characteristics, which are not frequently detected in natural compounds.
To maximize the collection of data variation from the assay of Library707, we challenged ourselves to avoid making assays that analyze structurally similar compounds. For this objective, Library707 was clustered into 127 independent clusters using 27 physicochemical molecular descriptors (Figures 2 and 3a). Physicochemically similar compounds can be grouped objectively into clusters using multiple molecular descriptors. To obtain more compound variants in an assay, a single compound was selected from each cluster, and 127 representative compounds were evaluated in the first assay (Table S2). The clustering revealed that Library707 did not exhibit a strong bias on certain structures, as the largest cluster had 47 compounds and the average member number was 5.6 (Table S3).

**Figure 1.** Schematic illustration of the image-based TRPA1 assay platform. Human TRPA1 was stably expressed in the T-Rex293 cells. Activation of TRPA1 was quantitatively evaluated by measuring the intracellular Ca\(^{2+}\) concentration changes. TRPA1 activity was calculated using the following equation: TRPA1 activity = \([F \text{ (maximum fluorescence intensity)} - F_0 \text{ (baseline fluorescence)}]/F_0\).

**Figure 2.** Schematic chart of our TRPA1 agonist data collection. Starting from Library707, first and second assays were conducted covering 197 compounds. To collect interaction data from diverse candidates, 707 compounds in the library were clustered using physicochemical molecular descriptors to select 127 representative compounds for the first assay. From the first assay results, several clusters were selected for the second assay by two concepts: the clusters with a high rate of positive compounds, and the clusters with compounds carrying chemical groups that are associated with the previously known agonist mechanism. P1, P2, N1, and N2 show the categories of assay results: P1: high-activity agonists, P2: medium-activity agonists, N1: difficult to evaluate activity due to its low solubility, N2: no activity.
ated with the previously known agonist mechanisms. P1, P2, N1, and N2 show the categories of assay results; P1: high-activity agonists, P2: medium-activity agonists, N1: difficult to evaluate activity due to its low solubility, N2: no activity.

Figure 3. Library707 profiled by 27 molecular descriptors. (a) Hierarchical clustering of Library707 by 27 molecular descriptors. Compounds are classified objectively by their complete physicochemical compound properties. (b,c) UMAP visualization of chemical data space of Library707 for the first assay (b) and the second assay (c). Each dot indicates a chemical compound labeled with assay results from the first assay. Categories of their assay results are indicated as follows: P1 (high activity), dark red; P2 (medium activity), light red; N1 (not measurable), light blue; N2 (no activity), dark blue.

3.2. The First Assay (127 Compounds) Results and Obtained TRPA1 Agonists

From the first assay, evaluating 127 physicochemically diverse compounds in Library707, we obtained 64 TRPA1 agonists (22 P1s and 42 P2s) (Table S4a). The 64 compounds induced concentration-dependent Ca\textsuperscript{2+} responses in TRPA1-expressing cells but not in the parent cells, indicating that they are TRPA1 agonists (Figure S1a,b). As shown in Figure 3b, a diverse space of Library707 compounds could be assayed. The UMAP plot indicates that there was no “clustered region of compounds,” which resulted in finding agonists. In other words, these data indicate that the TRPA1 agonist molecular rule is complex and has multiple agonist forms.

From the first assay, we discovered compounds bearing electron-withdrawing groups (clusters 20, 21, 32, 100, and 120), which had been suggested to be the agonist structural rule previously [8,9]. These findings indicate that our assay platform can effectively evaluate TRPA1 agonists with electron-withdrawing groups. However, interestingly, compound 45 (cluster 22) and compound 285 (cluster 85), compounds with similar electron-withdrawing groups, did not show any activity. These findings imply that the conventionally known “structural rule of having an electron-withdrawing group to be TRPA1 agonist” is not a
universal rule. Furthermore, we discovered that compound 73 (cluster 33) does not resemble the previously reported TRPA1 agonists, indicating that our variety assay succeeded in finding novel candidate structures to be a potential TRPA1 agonist.

3.3. The Second Assay (70 Compounds) Results and Obtained TRPA1 Agonists

By detailed interpretation of the first assay results, we decided to dig deeper into the clusters with two concepts: the first is to dig the P1 clusters (clusters 12, 33, 35, 36, 38, 48, and 98), and the second is to focus on some clusters, which contain the electron-withdrawing group (e.g., clusters 20, 21, 22, 34, and 100) and can facilitate the Michael addition reaction of cysteine residues in TRPA1, matching to the knowledge of the previously investigated TRPA1 agonists. Conclusively, we assayed 70 compounds, focused on certain clusters in the second assay (Table S2).

As a result of the TRPA1 assay, 63 compounds were identified as TRPA1 agonists: their responses in the TRPA1-expressing cells were significantly higher than those in the parent cells; in addition, their Ca$^{2+}$ responses in the TRPA1-expressing cells were concentration-dependent (Table S4b, Figure S1c,d). As shown in Figure 3c, the focused clusters, which had characteristic electron-withdrawing groups, were extremely limited in the chemical space in Library707. These data indicate that if all assays are based on such prior knowledge, it would be difficult to investigate the wide range of structures for novel findings.

By the agonist activities confirmed in compounds bearing electron-withdrawing groups (Table S4b), the second assay data showed that our image-based assay platform works effectively to investigate compounds that match the prior knowledge of TRPA1 agonists. Interestingly, it was shown that compounds 46 and 51 diminished their activity, even if they had an electron-withdrawing group in the structure. Since electrophilic moieties of TRPA1 agonists are suggested to form a covalent bond with cysteine residues of TRPA1 via a Michael addition [8,9], their reactivity can be modified by peripheral substituents. Such knowledge accumulation should be important for avoiding the design of inactive agonists.

3.4. Interpretation of Assay Results from Previous Agonist Structures

Compounds belonging to clusters 25, 33, 37, and 38 are characterized by their triple bond and ring structures composed of benzene and 5- and 6-membered rings (Figure 4a). Among the compounds, compound 52 (cluster 25, EC$_{50}$ 1.0 µM, top one activity in the second assay), compound 92 (cluster 39, EC$_{50}$ 6.4 µM, top three activity in the first assay), compound 94 (cluster 33, EC$_{50}$ 7.1 µM, top five activity in the second assay), and compound 89 (cluster 37, EC$_{50}$ 24.0 µM) exhibited significant activities. The EC$_{50}$ value of compound 52 (1 µM) was the lowest in this study and was comparable with allyl isothiocyanate (EC$_{50}$ 0.5 µM), a natural TRPA1 agonist with high activity [10]. Compounds 52, 92, 94, and 89 have similar structures (two benzene rings, one triple bond, and one oxygen bind to a tetra-substituted carbon), hereafter called “structure A” (Figure 4a). In the first and second assay, 20 compounds had the structure A, and eight of them (8/12, 67%) were classified into P1, four of them (4/12, 33%) were P2, and none of them were N2 (0/12, 0%). When structure A was modified to “one benzene ring”, one triple bond, and one oxygen binding to a tetra-substituted carbon, the compounds did not exhibit TRPA1 activities. For example, compound 79 (cluster 25) and compound 86 (cluster 31) did not show TRPA1 activity (Figure 4a). These results indicated that structure A is related to high TRPA1 activity. To the best of our knowledge, no report has reported TRPA1 activation by structure A and its similar structure. Therefore, structure A is a candidate structure rule for designing new types of TRPA1 agonists.
Figure 4. Chemical structures and EC_{50} values of library compounds and TRPA1 agonists. Compounds characterized by their triple bond and ring structures (a), α, β-unsaturated amide moiety (b), α, β-unsaturated ester moiety (c), and ester moiety (d). Chemical moieties that can contribute to TRPA1 activity of the compounds are highlighted in pink (b,c).
Compounds belonging to cluster 100 have an α, β-unsaturated amide as a common structure (Figure 4b). All 13 compounds belong to cluster-100-activated TRPA1; three of them were classified into P1 and ten of them were P2. Compound 579 (EC\textsubscript{50} 6.6 µM, top four active compounds in the second assay) was the strongest in the cluster. Sanshools (derived from Japanese pepper) and piperines (derived from pepper) are representatives of TRPA1 agonists with an α, β-unsaturated amide structure (Figure 4b) \cite{11,12}. Compound 579 (EC\textsubscript{50} 6.6 µM) exhibited five to ten times higher affinity to TRPA1 compared with hydroxy-α-sanshool (EC\textsubscript{50} 69 µM) and piperine (EC\textsubscript{50} 30 µM). Menozzi et al. investigated the contribution of an amide moiety and α, β-unsaturation to TRPA1 activity using sanshool analogs \cite{13}. While amide and carbonyl moieties contributed to TRPA1 activation induced by sanshools, the presence of α, β-unsaturation did not significantly modify the TRPA1 activity.

Among compounds belonging to clusters 21, 22, and 120, many of them, including compound 655 (cluster 120, EC\textsubscript{50} 5.0 µM, top1 activity in the first assay) and compound 81 (cluster 22, EC\textsubscript{50} 45 µM), exhibited TRPA1 activity (Figure 4c). They have an α, β-unsaturated ester in common. Several TRPA1 agonists with α, β-unsaturated carboxyl moieties, such as cinnamaldehyde and N-methyl maleimide, have been identified (Figure 4c). Cysteines located in the N-terminus of TRPA1 have been reported to attack α, β-unsaturated carboxylic moieties of agonists and form a covalent bond via a Michael addition \cite{8,9}. The TRPA1 activity of compound 655 (EC\textsubscript{50} 5.0 µM) was comparable with that of cinnamaldehyde (EC\textsubscript{50} 5–19 µM) \cite{9,14}. Since compounds 655 and 81 have an α, β-unsaturated carboxyl moiety, they would activate TRPA1 through the same mechanism with cinnamaldehyde. The substituent of β and γ positions of the five-membered rings would modify their reactivity and TRPA1 activity because covalent bonds can be formed between the β position of their five-membered rings and cysteine residues of TRPA1.

Compounds belonging to clusters 54, 57, 60, and 98 commonly have an ester moiety and double bonds unconjugated with ester (Figure 4d). Several compounds, including compound 522 (cluster 57, EC\textsubscript{50} 6.8 µM, top four active compounds in the first assay) and compound 525 (cluster 98, EC\textsubscript{50} 7.8 µM, top five active compounds in the first assay), activated TRPA1. Terpenes, such as geranyl propionate and neryl acetate, are TRPA1 agonists with an ester moiety and double bonds unconjugated with ester as with those compounds (Figure 4d) \cite{15}. Terpenes are representatives of non-electrophilic TRPA1 agonists, and some of them are suggested to bind to the transmembrane domain 5 of the channel \cite{16}. Because the compounds of clusters 54, 57, 60, and 98 lack electrophilic moieties, they could activate TRPA1 through the same mechanism with terpenes.

### 3.5. Interpretation of Assay Results from Physicochemical Descriptors

From our assay results, we found that TRPA1 agonists concentrated in some clusters categorized by physicochemical molecular descriptors. Clusters 100, 31, and 33 revealed that compounds categorized in P1 and P2 were concentrated (Figure 5). Interestingly, such clusters were not designed by reflecting prior knowledge of TRPA1 agonists; it showed high hit rates of finding agonist candidates.

In cluster 100, within 17 compound members, all 15 selected candidates exhibited TRPA1 agonist activity, implying that the physicochemical descriptor combination, which defines this cluster, can define some TRPA1 agonist physicochemical rules (Figure 5a). Practically, the cluster 100 members can be described from the cluster property, as compounds with high Manhold LogP and carry amides conjugated to a long Pi chain (Table S2).

In cluster 31, 8 of 12 compounds exhibited TRPA1 agonist activities. However, compared with cluster 100, the Manhold LogP value of this cluster member compound was low (Figure 5b). The average Manhold LogP value of 31 cluster member compounds was 2.59 (707 compounds average: 3.05, SD: 0.73). The physicochemical property of cluster 31 can be expressed as compounds carrying a high bond polarization ratio and triple bonds.
In cluster 33, all four compounds exhibited TRPA1 agonist activities (Figure 5c). In that they have triple bonds, the compounds in cluster 33 have a structure similar to cluster 31. All compounds in this cluster have two benzene rings and higher Manhold LogP than compounds in cluster 31 (average Manhold LogP in cluster 33: 3.33).

Our clustering approach led to finding several clusters of TRPA1 agonists categorized by the compounds’ total physicochemical profiles, indicating that physicochemical molecular descriptor combinations can define some of the TRPA1 agonists with quantitative data. Such data-driven structure design will help assess or further optimize the agonist candidate with an in silico approach. However, our assay data also indicated that the physicochemical molecular rule was complex. When we attempted to assess the discrimination performance using several major molecular descriptors considered in designing compounds for drugs, it was difficult to clearly explain the structural difference between P1 and N2, although there was a slight discrimination effect for some physicochemical properties, such as Manhold LogP, Largest Chain, and Bond Polarizabilities (Figure 6). Consequently, the results indicated that the structure rules of TRPA1 agonists are complex.

Figure 5. Representative clusters consist of a high rate of P1 (high activity) and P2 (medium activity). (a) Cluster 100, (b) cluster 31, and (c) cluster 33. Heatmap represents the cluster’s physicochemical profile described by 27 molecular descriptors. The list of molecular descriptors, which is the same as (a), is abbreviated in (b) and (c). The molecular structures of assayed compounds in each cluster are illustrated.
In this study, to further understand the structural diversity of TRPA1 agonists, we integrated the image-based TRPA1 assay system with the chemical space clustering concept. To maximize the collection of data variation from the assay, the library compounds were classified into 127 clusters using 27 physicochemical molecular descriptors and subjected to the TRPA1 assay. We successfully obtained TRPA1 activity data of 197 compounds with physicochemical diversity. Various types of compounds, including alkyne, amide, and ester, showed TRPA1 activity and indicated the physicochemical diversity of TRPA1 agonists. Furthermore, structure A was proposed as a novel core for a potent TRPA1 agonist. The concept presented in this study would aid the understanding of the structural diversity of agonists and the development of various agonists. Our approach can be applied to other receptors and enzymes to investigate interaction mechanisms with ligands and substrates.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12031622/s1, Table S1: List of molecular descriptors selected from the total descriptors; Table S2: List of Library707 compounds with their molecular descriptor profiles and selections for assays (indicated in the order of cluster number); Table S3: Profile of 127 cluster members; Table S4: Chemical structures and assayed results of 129 compounds in the first assay (a) and 70 compounds in the second assay (b); Figure S1: Concentration response-curves of P1 (high-activity) and P2 (medium-activity) compounds.
Author Contributions: Conceptualization, Y.T., K.T., M.F., M.S., Y.Y., R.K. and K.I.; investigation, Y.T., K.T., M.M., M.F., M.S., R.K. and K.I.; writing—original draft, Y.T., K.T. and R.K.; writing—review and editing, R.K., M.S. and K.I. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Japan Society for the Promotion of Science (KAKENHI grant JP 21H02144A0 and 21K19092 to K.I. and 19K15792 to Y.T.) and the Platform Project for Supporting Drug Discovery and Life Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research: BINDS) from the Japan Agency for Medical Research and Development (AMED) under grant number JP21am0101099 (Y.Y.).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available upon reasonable request to the corresponding author.

Conflicts of Interest: The authors declare no potential conflict of interests.

References

1. Mahajan, N.; Khare, P.; Kondepudi, K.K.; Bishnoi, M. TRPA1: Pharmacology, natural activators and role in obesity prevention. Eur. J. Pharmacol. 2021, 912, 174553. [CrossRef] [PubMed]

2. Wang, Z.; Ye, D.; Ye, J.; Wang, M.; Liu, J.; Jiang, H.; Xu, Y.; Zhang, J.; Chen, J.; Wan, J. The TRPA1 channel in the cardiovascular system: Promising features and challenges. Front. Pharmacol. 2019, 10, 1253. [CrossRef] [PubMed]

3. Talavera, K.; Startek, J.B.; Alvarez-Collazo, J.; Boonen, B.; Alpizar, Y.A.; Sanchez, A.; Naert, R.; Nilius, B. Mammalian transient receptor potential TRPA1 channels: From structure to disease. Phys. Rev. 2020, 100, 725–803. [CrossRef] [PubMed]

4. Terada, Y.; Horie, S.; Takayama, H.; Uchida, K.; Tominaga, M.; Watanabe, T. Activation and inhibition of thermosensitive TRP channels by voacangine, an alkaloid present in Voacanga africana, an African tree. J. Nat. Prod. 2014, 77, 285–297. [CrossRef] [PubMed]

5. Yamamoto, Y.; Mori, S.; Shibuya, M. A combined transition-metal-catalyzed and photopromoted process: Synthesis of 2,3-fused 4-phenylnaphthalen-1-yl carboxylates from 1,7-diaryl-1,6-diynes. Chem. Eur. J. 2015, 21, 9093–9100. [CrossRef] [PubMed]

6. Mori, S.; Shibuya, M.; Yamamoto, Y. Ruthenium-catalyzed hydrocarbamoylative cyclization of 1,6-diynes with formamides. Chem. Lett. 2016, 46, 207–210. [CrossRef]

7. Yamamoto, Y.; Shibano, S.; Kurohara, T.; Shibuya, M. Synthesis of β-allylbutenolides via one-pot copper-catalyzed hydroallylation/cyclization of γ-hydroxybutyrate derivatives. J. Org. Chem. 2014, 79, 4503–4511. [CrossRef] [PubMed]

8. Hinman, A.; Chuang, H.H.; Bautista, D.M.; Julius, D. TRP channel activation by reversible covalent modification. Proc. Natl. Acad. Sci. USA 2006, 103, 19564–19568. [CrossRef] [PubMed]

9. Macpherson, L.J.; Dubin, A.E.; Evans, M.J.; Marr, F.; Schultz, P.G.; Cravatt, B.F.; Patapoutian, A. Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. Nature 2007, 445, 541–545. [CrossRef] [PubMed]

10. Terada, Y.; Masuda, H.; Watanabe, T. Structure-activity relationship study on isothiocyanates: Comparison of TRPA1-activating ability between allyl isothiocyanate and specific flavor components of wasabi, horseradish, and white mustard. J. Nat. Prod. 2015, 78, 1937–1941. [CrossRef] [PubMed]

11. Riera, C.E.; Menozzi-Smarrito, C.; Affolter, M.; Milag, S.; Munari, C.; Robert, F.; Vogel, H.; Simon, S.A.; Le Coutre, J. Compounds from sichuan and melegueta peppers activate, covalently and non-covalently, TRPA1 and TRPV1 channels. Br. J. Pharmacol. 2009, 157, 1398–1409. [CrossRef] [PubMed]

12. Okumura, Y.; Narukawa, M.; Iwasaki, Y.; Ishikawa, A.; Matsuda, H.; Yoshikawa, M.; Watanabe, T. Activation of TRPV1 and TRPA1 by black pepper components. Biosci. Biotechnol. Biochem. 2010, 74, 1068–1072. [CrossRef] [PubMed]

13. Menozzi-Smarrito, C.; Riera, C.E.; Munari, C.; Le Coutre, J.; Robert, F. Synthesis and evaluation of new alkylamides derived from alpha-hydroxysanshool, the pungent molecule in szechuan pepper. J. Agric. Food Chem. 2009, 57, 1982–1989. [CrossRef] [PubMed]

14. Iwasaki, Y.; Tanabe, M.; Kayama, Y.; Abe, M.; Kashio, M.; Koizumi, K.; Okumura, Y.; Morimitsu, Y.; Tominaga, M.; Ozawa, Y.; et al. Miogadial and miogatrial with alpha, beta-unsaturated 1,4-dialdehyde moieties—Novel and potent TRPA1 agonists. Life Sci. 2009, 85, 60–69. [CrossRef] [PubMed]

15. Terada, Y.; Yamashita, R.; Ihara, N.; Yamazaki-Ito, T.; Takahashi, Y.; Masuda, H.; Sakuragawa, S.; Ito, S.; Ito, K.; Watanabe, T. Human TRPA1 activation by terpenes derived from the essential oil of daidai, Citrus aurantium L. var. daidai Makino. Biosci. Biotechnol. Biochem. 2019, 83, 1721–1728. [CrossRef] [PubMed]

16. Meents, J.E.; Ciotu, C.I.; Fischer, M. TRPA1: A molecular view. J. Neurophysiol. 2019, 121, 427–443. [CrossRef] [PubMed]