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# Effect of essential oils, such as raspberry ketone and its derivatives, on antiandrogenic activity based on in vitro reporter gene assay

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ABSTRACT

The effect of essential oils, such as raspberry ketone, on androgen (AR) receptor was investigated using a MDA-kb2 human breast cancer cell line for predicting potential AR activity. Among them, eugenol had the highest AR antagonistic activity with its $IC_{50}$ value of 19 μM. Raspberry ketone, which has threefold higher anti-obese activity than that of capsaicin, also had AR antagonist activity with its $IC_{50}$ value of 252 μM. Based on these findings, a more precise CoMFA model was proposed as follows: $pIC_{50}$ [log (1/$IC_{50}$)] = 3.77 + [CoMFA field terms] ($n$ = 39, $s$ = 0.249, $r^2$ = 0.834, $s_{cv}$ = 0.507, $q^2$ = 0.311 (three components).


Quantitative structure–activity relationship (QSAR) models and qualitative SAR approaches have met with some measure of success in identifying and depicting the structural features that contribute to the ability of a chemical to interact with the androgen receptor.[1–5] In addition, our previous study developed a good Comparative Molecular Field Analysis (CoMFA) model[6] to analyze the structural requirements necessary to disrupt AR function and a significant CoMFA model for AR pure antagonist, suggesting that the steric and electrostatic properties were sufficient to describe the structural requirements for AR antagonist activity.[7] This study revealed that some alkyl phenols, such as *p*-*t*-octyl phenol, act as AR pure antagonists. Recently, some essential oils extracted from plants have been used as aromatherapy for their anti-obese activity or mitigation. For example, raspberry ketone, a major aromatic compound contained in red raspberries (*Rubus idaeus*), has 3 times higher anti-obese activity than that of capsaicin,[8] and increased skin elasticity and promoted hair growth by increasing dermal IGF-I production.[9] Eugenol is a very potent inhibitor of arachidonic acid (AA)-induced platelet aggregation and is 29-fold more potent than aspirin in inhibiting AA-induced human platelet aggregation.[10]

Since raspberry ketone is a derivative of alkyl phenols, we addressed the effect of raspberry ketone and its related essential oils with alkylphenol moiety on androgen-mediated transcription activity by using well-established MDA-kb2 human breast cancer cells stably expressing AR and the GR-responsive luciferase reporter gene, MMTV-luc.[7,11–13] This cell line elicits an increase in luciferase activity in the presence of the androgens. Briefly, the cells were treated with various concentrations of test chemicals from $10^{-8}$ to $10^{-3}$ M to determine the AR agonist and/or antagonist activity in the absence and presence of 0.2 nM dihydrotestosterone (DHT), respectively. Data are expressed as the induced luciferase activity (induced luc activity (%)) compared to that of 0.2 nM DHT in the absence or presence of 0.2 nM DHT, that is, induced luciferase activity (%) = [RLU (test chemical)−RLU (vehicle control)/RLU (0.2 nM DHT)−RLU (vehicle control)] × 100. When a test chemical has no activity, the obtained induced luc activity (%) is 0% because the value of RLU (test chemical)−RLU (vehicle control) is zero. When a test chemical has AR agonistic activity, its induced luc activity (%) increases as its concentration increases in the absence of DHT. When a test chemical has AR antagonist activity, its induced luc activity (%) decreases as its concentration increases in the presence of 0.2 nM DHT. Therefore, the value of $IC_{50}$ for AR antagonist activity is the concentration of the test chemical producing 50% inhibition of 0.2 nM DHT induced luc activity. Since microscope observation of the damage to treated cells correlated with the decreased induced luc activity (%) in the absence of DHT, induced luc activity less than 85% in the absence of DHT was assigned as cell toxicity and the corresponding concentrations were not used to calculate $IC_{50}$ in the presence of DHT. The data were analyzed by Student's *t*-distribution with Excel (Microsoft, USA) and $p$ values less than 0.05 were considered as significant. The measured activities are expressed as the means ± SD resulting

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from at least three separate experiments with quadruplicate wells for each treatment dose.

Among the tested essential oils (Fig. 1), the $IC_{50}$ value of raspberry ketone (**a**), eugenol (**b**), zingerone (**c**), and *p*-hydroxyphenylacetone (**d**) demonstrated a sufficient decrease of induced luc activity with the increase of their concentrations in the presence of 0.2 nM DHT, implying that they acted as AR antagonists with calculated values of $IC_{50}$ were 252 ± 26, 19 ± 7, 153 ± 8, and 420 ± 120 μM, respectively (Fig. 1). No tested compounds showed any increase of induced luc activities even at their highest treated concentration. Capsaicin, which has a similar chemical structure to raspberry ketone and anti-obese activity, showed no increase or decrease of induced luc activities at its highest treated concentration of $1 \times 10^{-3}$ M (data not shown).

Although eugenol itself did not have any induced luc activity in the absence of DHT, it had the highest $IC_{50}$ value among them, suggesting that it acts as a pure AR antagonist while it had 20-fold lower AR antagonistic activity than that of a well-known AR antagonist, flutamide ($IC_{50}$ = 1 μM).

Since we have already developed a CoMFA model to analyze the structural requirements for AR antagonists, their $IC_{50}$ values were predicted based on the previous equation.[7] To assumed the fully optimized conformation of the molecules to be the active conformation for binding, compounds (**a**–**d**) were constructed using the SYBYL ver. 7.3[14] standard values for bond lengths and angles. A systematic search in SYBYL was applied to all rotatable bonds. The low-energy conformer of each compound obtained by a systematic search was then optimized by the semiempirical PM3 method.[15] For the optimized coordinates of all compounds, atomic charges were calculated using MNDO.[16] Molecular electrostatic potentials of the molecules were computed from the MNDO atomic charges and used in CoMFA studies. The compounds were superimposed on the reference compound, testosterone, according to a similar rule to flutamide.[7] Atoms used for the superimposition of the compounds are indicated in Figure 2.

The observed $IC_{50}$ value of eugenol (**b**) was threefold higher than the predictive value obtained from our previous model; however, the observed activity of three other compounds (**a**, **c**, and **d**) was 10-, 3-, and 8-fold lower than the calculated values based on our previous model, respectively (data not shown). Since it was predicted to not be disadvantageous even if a longer methylene chain were substituted between the phenyl ring and the ketone group in our previous model, their estimated AR antagonistic activities were considered to be higher. We re-analyzed the antagonistic activity ($pIC_{50} = \log(1/IC_{50})$), including four compounds (**a**–**d**) by CoMFA, to obtain a more precise model as follows.

$$pIC_{50} = 3.74 + [\text{CoMFA field terms}] \quad (1)$$

$n = 39$, $s = 0.248$, $r^2 = 0.834$, $s_{cv} = 0.488$, $q^2 = 0.359$ (three components), relative contribution: steric 43.2%, electrostatic 56.8%, where

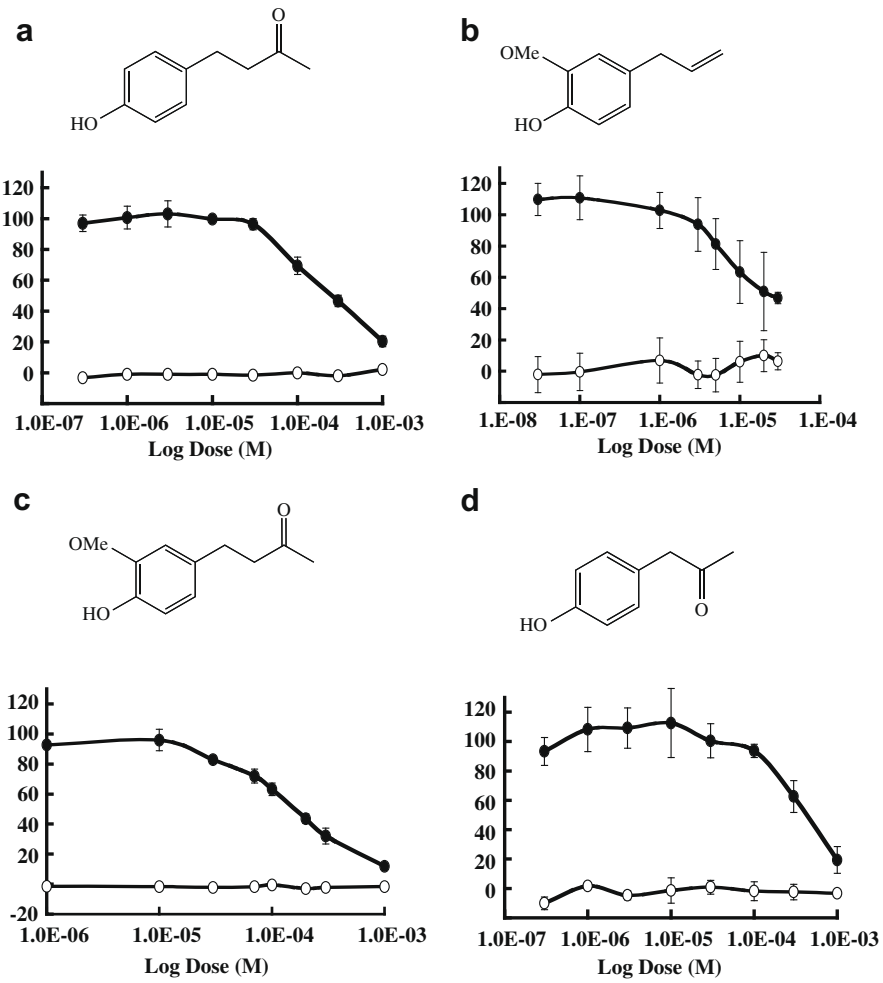


**Figure 1.** Effects of essential oils on AR in the absence (open circle) and presence (closed circle) of 0.2 nM DHT. (**a**) raspberry ketone, (**b**) eugenol, (**c**) zingerone, (**d**) *p*-hydroxyphenylacetone. Data are expressed as the mean of induced luciferase activity (%) of three independent experiments with quadruplicate wells, that is, induced luciferase activity (%) = [RLU (test chemical)−RLU (vehicle control)/RLU (0.2 nM DHT)−RLU (vehicle control)] × 100.

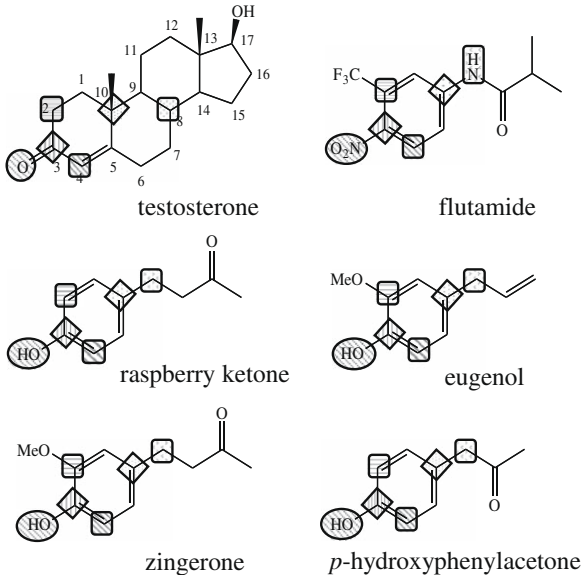


**Figure 2.** Atoms used for superimposition of compounds in this study. Pairs of atoms are indicated by the same symbols.

$n$ is the number of compounds, $r$ is the correlation coefficient, $q$ is the cross-validated (leave-one-out) correlation coefficient, $s$ is the standard error, and $s_{cv}$ is the cross-validated (leave-one-out) standard error.

The calculated $pIC_{50}$ values by Eq. (1) as well as the observed values are shown in Table 1. However, this CoMFA model predicted the AR antagonistic activity of eugenol (**b**), having the highest activity among four compounds, to be much lower than actual. Since the dataset contains diverse-structural compounds, only phenolic compounds (12 compounds including compounds (**a**–**d**) were analyzed by CoMFA and significant result was obtained (three components, $s = 0.101$, $r^2 = 0.988$, $s_{cv} = 0.763$, $q^2 = 0.310$). This equation could predict the activity of compounds (**a**–**d**), but the major steric and electrostatic potential contour maps drawn according to the equation were not consistent with those for all compounds (data not shown). Therefore, the conformation of the compounds (**a**–**d**) was re-considered based on the energy difference between the folded forms obtained from the systematic search and the extended forms in which the butenyl or acetylalkyl groups were planer for a phenyl ring. The energy difference between the extended forms and the most stable folded forms of compounds (**a**–**d**) were 0.86, 0.38, 0.81, 3.16 kcal/mol by the PM3 optimization and calculation. As the extended forms were only slightly unstable, we chose simple extended forms and analyzed AR antagonistic activity ($pIC_{50}$) including four compounds by CoMFA as follows;for all compounds:

$$pIC_{50}[\log(1/IC_{50})] = 3.77 + [\text{CoMFA field terms}] \quad (2)$$

**Table 1**
Refined data based on the newly evaluated CoMFA model

| No. | Compounds | $IC_{50}$ (μM) | $pIC_{50}$ (obsd) | $pIC_{50}$[a] (calcd) | $pIC_{50}$[b] (calcd) |
|---|---|---|---|---|---|
| **a** | Raspberry ketone | 252 | 3.60 | 3.84 | 3.78 |
| **b** | Eugenol | 19 | 4.72 | 4.06 | 4.42 |
| **c** | Zingerone | 153 | 3.82 | 3.82 | 3.89 |
| **d** | *p*-Hydroxyphenylacetone | 420 | 3.38 | 3.54 | 3.49 |

[a] Calculated by Eq. (1).
[b] Calculated by Eq. (2).

$n = 39$, $s = 0.249$, $r^2 = 0.834$, $s_{cv} = 0.507$, $q^2 = 0.311$ (three components), Relative contribution: steric 41.5%, electrostatic 58.5%.
for phenolic compounds:

$$pIC_{50}[\log(1/IC_{50})] = 3.77 + [\text{CoMFA field terms}] \quad (3)$$

$n = 12$, $s = 0.140$, $r^2 = 0.977$, $s_{cv} = 0.711$, $q^2 = 0.402$ (three components), Relative contribution: steric 53.0%, electrostatic 47.0%. The $IC_{50}$ values of compounds (**a**–**d**) calculated from Eq. (2) were shown in Table 1. Although $q^2$ of Eq. (2) was slightly lower than that of Eq. (1), Eq. (2) had the almost same statistical quality as Eq. (1) and better prediction for the activity. In addition, $q^2$ of Eq. (3) was higher compared to that of Eq. (1) for the folded forms. Figure 3 represented the overlay of eugenol (**b**) with the major steric and electrostatic potential contour maps drawn according to Eq. (2). A favorable methylene chain length was found between the phenyl ring and the ketone group. The newly predicted yellow areas indicated regions where submolecular bulk was unfavorable for activity at the D ring site of DHT (Fig. 3). The contour maps drawn according to Eq. (3) was consistent with those in Figure 3 (data not shown). The obtained result did not contradict the proposed hypothesis, the Near 10 Å Polar Interaction Rule.[17]

Although our previous study showed that substitution with a bulkier group, such as methylcarboxylate, at the *meta* position significantly decreased AR antagonist activity,[7] the fact that substitution of a methoxy group of (**c**) for a hydrogen atom of (**a**) at the *meta* position slightly increased AR antagonist potency clearly indicated that a *meta*-substituent, such as methyl or trifluoromethyl group, is one of the most effective substituents inducing interaction with the hydrophobic pocket surrounded by Gln711, Met745, and Leu707 in the AR-LBD. This plays a prominent role in increasing AR binding activity as an AR antagonist while the existence of a "meta" channel extending away from the steroidal A-ring region of the glucocorticoid receptor (GR) ligand binding domain enhances the efficacy of potent GR agonist activity. Therefore, this obvious difference between AR-LBD and GR-LBD at the 'meta' channel has very attractive potential for receptor-selective drug design of AR and GR agonists and/or antagonists, respectively, because the amino acid residues necessary for the hydrogen bond in their LBD are very similar between AR and GR.

The red areas in Figure 3 indicate regions where negative electrostatic interactions with the receptor binding site increase activity. Since the length and bulkiness of *p*-hydroxyphenylacetone (**d**) are similar to those of eugenol (**b**), and the carbonyl group of (**d**) was fitted in this red area, just like the double bond of (**b**), the AR antagonist activity of *p*-hydroxyphenylacetone (**d**) was predicted to be much higher than its actual value based on our previous model. However, its $IC_{50}$ value was 1.6- and 10-fold lower than that of raspberry ketone (**a**) and eugenol (**b**), respectively. That this negative electrostatic interaction site in AR-LBD is surrounded by Leu704, Gly708, Trp741, and Met895 means that this area becomes

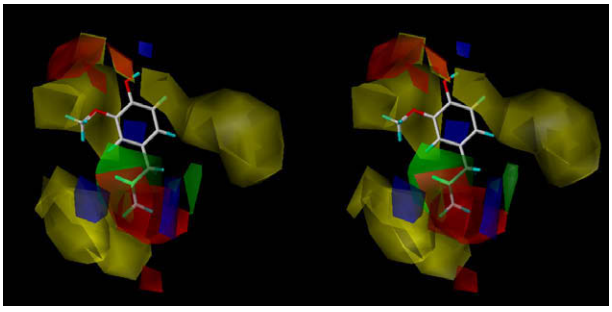

**Figure 3.** Stereoviews of contour diagrams of steric and electrostatic fields with eugenol according to Eq. (2) for AR pure antagonists.

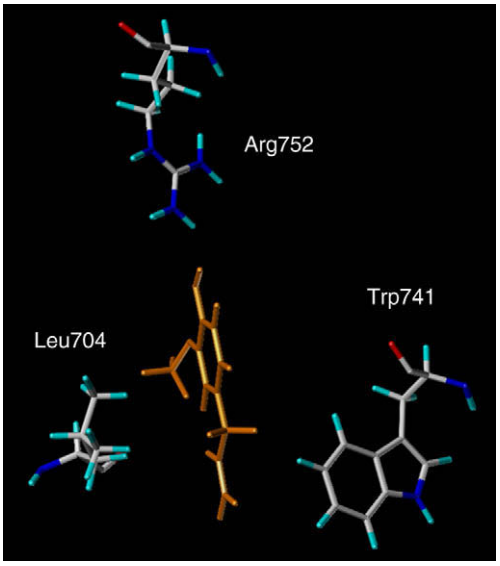


**Figure 4.** Interaction between eugenol and key amino acid residues (Trp741, Leu704, and Arg752) in AR ligand binding domain.

hydrophobic; therefore, carbonyl oxygen of (**d**) becomes rather disadvantageous because there are no electrophilic amino acid residues in this area.

On the other hand, π electrons of the double bond of (**b**) made a CH-π interaction with both Trp741 and Leu704, This means that the plane of the indole moiety of Trp741 faces the double bond of butenyl moiety of (**b**) vertically. Figure 4 shows the interaction between (**b**) and these two amino acid residues in AR-LBD when (**b**) was superimposed on R1881 of the crystal structure of the AR-R1881 complex (PDB:1XQ3), according to the similar rule to superposition on testosterone. The closest distances between carbon atoms of the double bond of (**b**) and hydrogen atoms of Trp741 and Leu704 were 3.625 and 3.603 Å, respectively. This new finding of CH-π interaction between eugenol and Trp741 interprets the relationship between its high AR antagonistic activity and completely plane conformation of eugenol.

Since eugenol with 2-methoxyestradiol (2-ME2) inhibited the growth of prostate cancer cells and induced apoptosis at lower concentrations than either single agent alone,[18] our present result provides strong evidence that the combination of eugenol with 2-ME2 may offer a new clinically relevant treatment procedure. Although raspberry ketone prevented high-fat-diet-induced elevations in body weight and the weights of the liver and visceral adipose tissues, its anti-obese activity was observed with 1% raspberry ketone treatment for 10 weeks.[8] Therefore, the blood concentration of raspberry ketone was calculated for the 10 g/kg (1%) dose taken to have anti-obese activity based on the reported bioavailability of *p-t*-octylphenol with male Wistar rats because raspberry ketone has a similar chemical structure to alkylphenol. After dosing with 200 mg/kg *p-t*-octylphenol, it was detected in the range of 80–100 ng/ml blood.[19] The blood level of raspberry ketone was calculated in the range of 4000–5000 ng/ml blood after dosing with 10 g/kg (1%). Its concentration in blood was 24–30 mM as its formula weight is 164.2 g. Although this assumption is estimated by the bioavailability of *p-t*-octylphenol, the calculated concentration is about 100-fold higher than that of its own AR antagonist activity, assuming an intake of 10 g/kg.

In summary, we have demonstrated that essential oils with alkylphenol moiety act as a novel AR modulator that interacts with AR receptor based on the Near 10 Å Polar Interaction Rule. This study provided a more comprehensive model based on the current data, with the newly predicted yellow areas in present study indicating regions where submolecular bulk is unfavorable for activity at the D ring site of DHT, and a *meta*-substituent, such as methyl or trifluoromethyl groups, is one of the best substituents to induce interaction with the hydrophobic pocket surrounded by Gln711, Met745, and Leu707 in AR-LBD. Moreover, the negative electrostatic interaction site in AR-LBD, surrounded by Leu704, Gly708, Trp741, and Met895, prefers a CH-π interaction with both Trp741 and Leu704 rather than electrophilic functional groups, such as carbonyl groups, in this area. This demonstrates the useful potential of designing novel and highly receptor selective nonsteroidal ligands.

Furthermore, although raspberry ketone and eugenol may have a significant impact on chemopreventive and therapeutic approaches for cancer, anti-obese activity and human platelet aggregation, our findings highlight the careful use of such essential oils with alkylphenol moiety as health care supplements in regulating physiological activities of AR in vivo.

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