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Light-controlled HDAC inhibitors: towards photopharmacological chemotherapy

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Abstract

Cancer treatment suffers from limitations that have a major impact on the patient's quality of life and survival. In case of chemotherapy, the systemic distribution of cytotoxic drugs reduces their efficacy and causes severe side effects due to non-selective toxicity. Photopharmacology allows a novel approach to address these problems, as it employs external, local activation of chemotherapeutic agents using light. We report here the development of photoswitchable Histone Deacetylase inhibitors as potential antitumor agents. Analogs of the clinically used chemotherapeutic agents Vorinostat, Panobinostat and Belinostat were designed with a photoswitchable azobenzene moiety incorporated into their structure. The most promising compound exhibits high inhibitory potency in the thermodynamically less stable *cis* form and a significantly lower activity for the *trans* form, both on HDACs activity and proliferation of HeLa cells. This approach offers a clear prospect towards local photo-activation of HDAC inhibition, to avoid severe side effects in chemotherapy.

Keywords

chemotherapy; photopharmacology; photoswitches; HDAC inhibitors; cytotoxicity

Introduction

Chemotherapy, besides radiotherapy and surgery, is one of the crucial elements of cancer treatment.[1] It relies on the systemic administration of cytotoxic antineoplastic agents, which are infamous for their severe side effects.[2] Efforts towards targeted delivery of chemotherapeutics are often insufficient and therefore the lack of drug selectivity remains a major problem in care for cancer patients.[3] Ineffective cancer treatment causes severe

emotional and societal burdens to the patient and his environment and has massive economic impact as well.[4] In case of cancer types that warrant localized treatment, high spatiotemporal control over the activity of the drug, i.e. the possibility to externally modulate the potency of the chemotherapeutic agent, would allow for avoiding the systemic side effects by minimizing the concentration of active compound outside the area of treatment, thereby tremendously increasing the quality of the patient's life.[5]

Photopharmacology[6,7] aims at using light as an external non-invasive control element to modulate drug activity (Figure 1). Light can be delivered with very high spatiotemporal precision, with a wide range of intensities and wavelengths[8,9] and very limited effects on the patient's body. From a medicinal chemistry perspective, photopharmaceuticals are developed by incorporation of photoresponsive molecular switches into existing drugs to enable alteration of their biological properties upon light irradiation.[8–10] Photoswitching is reversible by irradiation with light of different wavelength or occurs spontaneously in a thermal process.[11] Recent examples of bioactive compounds with photomodulated potency include photocontrolled ion channel blockers,[12] antibiotics[13] and enzyme inhibitors.[14] However, successful examples for switch-on chemotherapeutics are currently lacking.

For a possible therapeutic application, the potential photopharmaceuticals must fulfil a number of criteria. Firstly, they must show high potency, being at least comparable with the parent, clinically useful drug that inspired their design. Secondly, the difference in activity between the inactive and active state should be sufficiently large to allow for “off-on” switching of their activity under physiological conditions. Finally, the thermodynamically less stable form should show higher biological activity, preferably with a known mechanism of toxicity, to enable precise local activation as presented in Figure 1. Photopharmaceuticals have been described that meet some of these requirements, i.e. high potency,[15] >20× difference in activity[16] and enhanced potency for the unstable state.[13] However, fulfilling all these criteria in one molecule remains a major challenge and is crucial for the development of a clinically useful, photoactivated drug.

In order to demonstrate the viability of this novel approach with the ultimate goal of photoresponsive chemotherapy, we have chosen Histone Deacetylases (HDACs) as a pharmacological target. The function of HDACs is the deacetylation of ϵ -acetylated lysine residues on histone tails, which restores the positive charge of the histones and their electrostatic interactions with DNA, leading to condensed and transcriptionally silent chromatin structures.[17]

The HDAC family members are categorized in four classes (I–IV), based on their primary structure, size and sequence homology to the respective yeast enzymes.[18,19] The mechanism of deacetylase activity is zinc-dependent for classes I (HDACs1–3 and 8), II, subdivided to classes IIa (HDAC4, 5, 7 and 9) and IIb (HDAC6 and 10), and IV and NAD⁺-dependent for class III. As epigenetic regulators of both histone and non-histone proteins, [20] HDACs play a pivotal role in a vast array of biological processes, including DNA repair, cell differentiation, proliferation and apoptosis. As a result, alterations in expression or mutations of genes encoding for HDACs can lead to aberrant gene transcription,

disruption of cell homeostasis and, subsequently, to tumorigenesis.[21–23] Recent evidence demonstrates that individual HDACs are strongly associated with neurodegenerative[24–26] and inflammatory diseases,[27,28] tissue fibrosis and metabolic disorders.[21]

The link between abnormal HDAC activity and cancer initiation and progression is best shown in classes I, II and IV.[21] A large variety of natural and synthetic compounds has been reported as potential agents for cancer prevention or treatment of different stages of several tumor types. Depending on their structure, they can be categorized to hydroxamic acids, cyclic peptides, benzamides and short-chain fatty acids.[20,22] Up to date, there are 11 different HDACis (HDAC inhibitors) undergoing clinical trials as monotherapy or in combination with other antitumor approaches in cancer patients.[29] The most successful inhibitors until now proved to be the hydroxamic acid type of pan-HDACi from which three obtained FDA approval for clinical use: Vorinostat (SAHA), Panobinostat and Belinostat. [30] These compounds non-selectively inhibit classes I, II and IV HDACs with a nM scale potency[31] and are successfully applied as chemotherapeutic agents for the treatment of hematologic malignancies.[30,32–34] However, despite significant efforts, the specific HDACs that are responsible for the clinical effects of these successful inhibitors have not been elucidated as of yet.[31,34]

The crystal structure of HDLP (Human Deacetylase-Like Protein) with SAHA shows that SAHA binds inside the catalytic pocket by inserting the chain into the enzymatic channel (Figure 2).[35] The hydroxamic acid interacts with the zinc cation at the polar bottom part and also forms hydrogen bonds with catalytic residues. Moreover, the aliphatic chain makes Van der Waals interactions with residues at the hydrophobic part of the pocket, while the cap group serves in packing the inhibitor at the rim of the active site.

The insertion of SAHA into the channel of the deacetylase and the flexibility of the aliphatic chain suggest that the inhibitory activity may be controlled with changes in length, shape and substituents of the molecule. During the studies reported here, a patent was published that demonstrates an increased activity for the *cis* isomers of azobenzene-benzamide type of HDAC inhibitors.[36] Despite their potential clinical relevance, compounds of this class lack the high toxicity for cancer cells as generally reported for hydroxamic acid type of HDAC inhibitors. Therefore, we have chosen to employ the clinically approved inhibitors SAHA, Panobinostat and Belinostat as starting points for the design of photoswitchable HDACis, as potential photocontrolled chemotherapeutic agents for improved, safer cancer therapy with less severe side effects. We aimed at the design of a potent compound that would show high activity in the thermodynamically unstable *cis* state and very little cytotoxicity in the stable *trans* state.

We have chosen the azobenzene photoswitch as a photoresponsive element that, when incorporated into the structure of chemotherapeutic agents, should provide control over their activity with light. Azobenzene molecules can be switched, usually by using UV-irradiation, from a flat, *trans* isomer to the bent *cis* isomer (Figure 3a).[11] The latter, being thermodynamically less stable than the former, will switch back in time to the initial state (Figure 3a). This reverse process can also be achieved using visible light irradiation. Importantly, the two forms show major differences in their shape and polarity, and therefore

the light-induced isomerization will result in the switching of the properties of an azobenzene-modified drug, which may consequently change the drug's biological activity.

Azobenzene photochromes with different properties have been used in photopharmacological projects due to their easy preparation, efficient photoswitching and low rate of photobleaching.[8,9] Their electronic properties determine the half-lives of the *cis* isomers and their absorption maxima. Accordingly, azobenzenes with various substitution patterns were included in our newly designed inhibitors in order to optimize their photophysical properties and HDAC inhibition with respect to potency and selectivity. We also used a new class of tetra-*ortho*-substituted azobenzenes that can be photoisomerised using visible light (up to 650 nm), which allows for lower toxicity and deeper tissue-penetration.[37,38]

Results and Discussion

Two distinct approaches were taken in the molecular design of photoswitchable HDACi. In the first one (compounds **1-7**, Figure 3b), the photoswitches were introduced into the cap moiety of the SAHA molecule (Figure 2b). In order to systematically study the influence of the substitution pattern on the photochemical and pharmacological properties of the obtained photocontrolled chemotherapeutic agents, all possible attachments of azobenzene to SAHA (*para* (**1**), *meta* (**2**) and *ortho* (**3**)) were evaluated. Additionally, a *para*-MeO substituent was introduced to the terminal aromatic ring in compounds **4** and **5**, in an attempt to increase the *cis* content in the irradiated samples, as described for other azobenzenes.[39] The influence of the length of the aliphatic chain in the SAHA core was tested using compound **6**, a shortened homolog of compound **2**. Finally, we studied the visible light-switchable compound **7**, to evaluate the possibility of using higher and more biocompatible wavelengths of light for the photocontrol of bioactivity.

In the second approach (compounds **8-12**) the linker part of the inhibitor (Figure 2b) was modified, because it is more tightly embedded within the protein. We hypothesized that introducing a photoswitch into this part of the SAHA molecule would lead to increased photocontrol over binding, as one of the photoisomeric forms should fit the tunnel better than the other one. Compounds **8**, **9**, **10** and **11** were designed to test this hypothesis. In compound **9**, an additional benzyl moiety was introduced to mimic the cap in the SAHA molecule (Figure 2b). Homologous compounds **10** and **11** were prepared to test the influence of the inhibitor's flexibility and linker length in comparison to **8** and **9**. Finally, inspired by the structure of two known clinically approved inhibitors, Belinostat and Panobinostat,[40] we introduced a double bond in the linker part of compound **12**.

The biological activity assay (*vide infra*) that we performed was based on the addition of the respective compound as a stock solution in DMSO to a buffered enzyme solution and, after 1 h incubation at rt, measurement of the conversion of a pro-fluorogenic substrate.[41] Therefore two photochemical properties of the inhibitors are of importance: the content of *cis* isomer that can be obtained upon irradiation in DMSO, and the half-life of the thermodynamically unstable *cis* isomer under the assay conditions (Figures 3c and 3d, respectively). The latter value is also crucial for the photopharmacological application

(Figure 1), in which one would envision the use of *cis-trans* isomerisation for slow auto-inactivation of compounds that have been locally activated.

All of the compounds show a satisfactory photostationary state (PSS > 76%, defined as the content of *cis*-isomer, at equilibrium, under the $\lambda = 365$ nm light irradiation at ~2 mg/mL) in DMSO (Figure 3c). In the isomeric series **1-3**, the lowest PSS was obtained for the *meta*-substituted compound, and the highest (95%) for the *para*-isomer. Introduction of methoxy substituents in compounds **4** and **5** indeed allowed to increase the PSS with respect to parent structures **1** and **2** (from 95% (**1**) and 83% (**3**) to 97% (**4**) and 95% (**5**)). With compound **7** we were able to achieve 78% PSS using blue light (400 nm) and 61% PSS using green light (530 nm), which is in line with similar tetra-*ortho*-substituted systems,[37,38] and permits the switching of these compounds with visible light. For all the *para*-alkoxy-substituted compounds **8-12**, a high PSS was reached (>92%).

By changing the substitution pattern we were able to control the half-life of the inhibitors from minutes to hours, which is of key importance in designing potential chemotherapeutics that are meant, after photoactivation, to auto-inactivate in the patient's body (Figure 3d). Very high stability was observed for compound **7**, which is in line with earlier studies on tetra-*ortho*-substituted systems.[38]

Importantly, all of the studied compounds are stable towards reduction in cellular environment, as shown by repeating switching cycles[42–44] in buffer in the presence of 10 mM glutathione (GSH, Supporting information, Figure S5), the highest concentration of GSH found in cells.[45]

Compounds **1-12** were initially screened for their inhibitory potency of crude enzyme activity (Figure S11 and S12, Supporting Information) using a published protocol.[41] Nuclear extracts from HeLa cells were used as a source of class I HDACs.[46] Gratifyingly, for the *meta*-substituted compounds **2** and **5**, we observed even higher potency than for the original drug. A significant difference in activity between the two isomeric forms, which is crucial for photopharmacological applications, was apparent in the *meta*- and *ortho*-substituted compounds, with compound **3** showing the highest *trans:cis* activity ratio (approximately 3×). Compounds of the second design (**8-12**, Figure 3), turned out to be less potent than the ones from the first design. However, in all cases, a stronger HDAC inhibition was observed for the *cis* isomer, whose potency increased from μ M to nM scale with the introduction of a second carbon in the chain.

We tested selected compounds for inhibition of human recombinant class I HDACs (**1-3** and **8**) and HDAC6 (class IIb) (Table 1, Figure 4). The active form of compound **1** (*cis*, except for HDAC3) was less potent than SAHA, while compounds **2** and **3** showed a remarkable potency in their *trans* form, with an IC₅₀ value of 12 nM (15× more potent than SAHA) in HDAC2 for compound **2** and 7.7 nM in HDAC3 for compound **3**. The same profile was observed with the visible light-switchable compound **7** (up to 12× more potent than SAHA in HDAC2). The highest difference in IC₅₀ values between the two isomers was observed for compound **3** (10× for HDAC3), whereas the same ratio was moderate for compounds **1** and **2** and relatively good for compound **7** (almost 6× for HDAC3). Considerable inhibitory

potency was also observed for compound **6** (*trans* form), the shorter analog of **2**, but the *cis:trans* activity difference was significant only in case of HDAC3. Despite the promising results from the HeLa nuclear extracts (Figure S11), the potency of *trans*-**5** in HDACs1-3 was comparable to SAHA and the *cis:trans* activity ratio was rather small (Figure 4a).

In the second design, the most active form of the inhibitor is the less stable *cis* isomer, which is advantageous for photopharmacological applications (Figure 1). Compounds **8** and **12** proved to have lower potency than SAHA in HDACs1-3 (Table 1, Figure 4b). However, we were delighted to observe a large increase in the IC₅₀ ratio between the *trans* and *cis* isomers in case of HDAC2 (19 times for inhibitor **8** and nearly 40 times for **12**, Figure 5b). Importantly, both these compounds in the active (*cis*) state showed inhibition in the same concentration range as SAHA (IC₅₀ = 0.44 μM for **8** and 0.56 μM for **12** vs. 0.18 μM for SAHA).

Interestingly, opposite activity results, as compared to HDACs1-3, were obtained for HDAC8, since the potency of all the inhibitors (including SAHA) was dramatically reduced (Table 1, Figure 4a and b). This can be explained by structural and sequence alignments of class I HDACs, which reveal a particularly malleable active site for HDAC8.[47] *Trans*-**3** was found to be more active (IC₅₀ = 70 nM, 16× more potent than SAHA) and exhibited the biggest difference in potency (comparing with the *cis* form) for HDAC8 inhibition.

Moreover, we examined the activity of the selected compounds against HDAC6, an isoform with a critical role in tumorigenesis and cancer cell metastasis.[48] A considerable inhibitory potency was achieved in case of the *trans* isomer of **1** and **2**, with the *cis* form being comparable to the reference compound (Table 1, Figure 4a and b).

The experiments described above were aimed at selecting the optimal photocontrolled HDACi for selective chemotherapy. Compound **12** fulfills all the criteria that we describe in the introduction section, i.e. high potency of the *cis* state (HDAC2 IC₅₀ = 0.56 μM) with a very large difference from the *trans* state. Furthermore, we tested if its reversible photochromism (switching back and forth between the isomers) is reflected in reversible changes in activity. To that end, compound **12** was tested for HDAC1 inhibition using cycles of *trans-cis* isomerization of the same stock solution. The residual HDAC1 activity had a reversible profile and dropped from 90% (*trans*) to 50% (*cis*) after each isomerization (Figure 5c). This experiment excludes the possibility that the irradiation results in irreversible formation of compounds with altered inhibitory activity against HDACs.

Next, we evaluated the possibility to use light to change the activity of compound **12** *in situ*, during the HDAC2 enzymatic activity assay. After 30 min incubation with the more active *cis* form, white light irradiation was applied to the enzymatic reaction to induce the switching to the less active *trans* form. In line with our expectations, the rate of the product formation was considerably increased (Figure 5d) due to the poor inhibitory potency of *trans*-**12**. This experiment confirms that light can be used in a biological setting to change the activity of photoswitchable HDAC inhibitors presented here.

Finally, we elucidated the global cytotoxic activity of photoswitchable HDACi and the extent to which it can be photocontrolled. Chosen compounds were incubated, in both isomeric forms, with HeLa cells for 16 h at 37°C, followed by measurement of cell viability (Figure S33). In line with the results obtained for recombinant enzymes, the most promising results came from compound **12**, where the *cis* form was significantly more toxic to the HeLa cervical cancer cells than the *trans* (Figure 5e). At 100 μ M, nearly full selectivity is obtained: the *cis* isomer kills almost all the cells, whereas the *trans* leaves almost all the cells intact. This selectivity is remarkable, since the half-life of *cis*-**12** at 37°C is relatively short (67 min). Moreover, both isomers proved to result in increase in histone acetylation (mainly histone H4) at concentrations around 2 μ M (Figure 5f), which indicates inhibition of intracellular HDAC activity. Since **12** is the only inhibitor prone to undergo conjugate addition (Figure 3a), a possible attack from nucleophilic residues of cellular proteins may lead to irreversible inhibition. We observed, however, competitive inhibition (Lineweaver-Burk analysis, Figure S19), proving a non-covalent binding to HDACs.

Conclusions

In conclusion, we present here the development of potential chemotherapeutic agents whose activity on their molecular targets can be externally controlled using light. Around the azobenzene photoswitchable moiety, inhibitors were designed that are structurally related to the clinically approved HDAC inhibitors SAHA, Panobinostat and Belinostat. The inhibitors were optimized towards high potency and pronounced inhibitory activity difference between the more active, thermodynamically less stable *cis* isomer and the less active *trans* isomer.

Notably, introduction of the photoswitchable moiety did not compromise the HDAC inhibitory activity, as some of the compounds show potencies comparable, or even superior, to the original SAHA drug. By changing the substitution pattern of the azobenzene, we were able to strongly influence not only the potency for different HDACs, but also the growth of HeLa cervical cancer cells. Favorably, it also enabled the control of other important features of the photocontrolled inhibitor, such as photostationary state, λ_{max} and *cis* isomer half-life in aqueous medium.

The most exciting results were achieved with inhibitor **12**, which shows many characteristics of a privileged photocontrolled chemotherapeutic agent: high potency (in selected HDACs comparable to SAHA), very high difference in activity between the photoisomers on isolated enzymes (up to 39 $\times$ for HDAC2) and whole cells, stable photoswitching and stability against GSH reduction. Importantly, the photochemically accessible, less stable *cis* isomer of **12** is the one that shows much higher inhibitory potency, which is in line with the ultimate applications envisioned in Figure 1. In these applications, one can envision the use of visible light for inactivation of the drug outside the site of action. Alternatively, advantage can be taken of the relatively short half-life of this isomer in aqueous buffer (67 min), which would allow for relatively fast auto-inactivation of the drug outside the irradiated area.

Here we provide proof of concept that development of photoswitchable inhibitors for a range of HDACs, in particular HDAC2, is feasible and that this photoswitching is also reflected in cytotoxicity in HeLa cells. This creates a perspective towards several clinical applications.

The use of the photoreactive agents presented here, requiring UV irradiation, would currently be limited to topical or intraoperative chemotherapy, such as for example used in HIPEC procedure.[49] However, we have also shown that significant activity can be obtained with visible-light switchable compound **7**, and due to the low toxicity and higher tissue penetration of visible light, this offers a promising approach towards fully non-invasive photopharmacology. Employing such compounds[42,43,50] provides the prospects for future precision chemotherapy, combining molecular tracers for tumor imaging with chemotherapeutic agents that can be photoactivated with high spatio-temporal precision.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparison of the principles behind classic chemotherapy (a) and high-precision photopharmacological chemotherapy (b,c). The reversible photoswitching between the inactive (blue) and active (red) chemotherapeutic agent (b) allows for local activation of the drug and permits its use at elevated concentrations, without systemic side effects (c).

Figure 2.

Structural design of photoswitchable HDAC inhibitors. a) Crystal structure of HDLP from *Aquifex aeolicus* in complex with SAHA. The aliphatic chain is inserted into the enzymatic channel, where the hydroxamic group interacts with the zinc cation, leaving the cap at the rim of the catalytic pocket. The picture was adapted from the PDB file 1C3S.[35] b)

Molecular design of photoswitchable SAHA analogs, with the azobenzene moiety introduced into the cap (first design) or the linker region (second design).

Figure 3.

Structure and photochemical properties of compounds **1-12** (for synthetic procedures and analytical data, see Supporting Information). a) Reversible photochromism of an azobenzene molecule. b) Molecular structure of compounds **1-12**. c) The content (%) of the *cis* isomer at the photostationary state in DMSO, $\lambda_{\text{irrad}} = 365$ nm, determined by ^1H NMR spectroscopy. d) Half-life (in the dark, at room temperature) for the thermodynamically unstable *cis* isomer, determined by UV-vis spectroscopy at room temperature in the HDAC assay buffer with 1 vol% DMSO.

Figure 4.

Inhibition of human recombinant class I HDACs and HDAC6 (class IIb). Inhibitory potency of *trans* (black) and *cis* (red) form of selected compounds of the a) first and b) second design. The corresponding IC₅₀ ratios of the two isomers are also reported. The logIC₅₀ values are presented as mean values of three independent measurements with their respective standard deviations.

Figure 5.

Studies on the performance of compound **12**. The IC₅₀ curves in a) HDAC1 and b) HDAC2 recombinant enzymes of *trans* and *cis* form of the inhibitor. c) Inhibition of HDAC1 activity using 50 nM of inhibitor **12** (black) and reversible photochromism (blue) after 4 isomerization cycles. d) HeLa cell viability was measured after 16 h incubation with various concentrations of each isomeric form of the inhibitor. e) *In situ* photoisomerization of **12**, after 30 min incubation of the *cis* form (1 μM) with HDAC2, using white light. The formation of the product was monitored at the indicated times. f) On blot luminescence detection and Coomassie Blue staining of acetylated histones (4 μg) after 16 h incubation with 1 and 2 μM of *cis* and *trans*-**12** using the Anti-Acetyl lysine antibody. a-e) The data are presented as mean values of three independent measurements with their respective standard deviations.

Table 1
Inhibition of HDAC activity by photoswitchable SAHA, Panobinostat and Belinostat analogs.

	HDAC1 IC ₅₀ [μM]		HDAC2 IC ₅₀ [μM]		HDAC3 IC ₅₀ [μM]		HDAC6 IC ₅₀ [μM]		HDAC8 IC ₅₀ [μM]	
	<i>trans</i>	<i>cis</i>	<i>trans</i>	<i>cis</i>	<i>trans</i>	<i>cis</i>	<i>trans</i>	<i>cis</i>	<i>trans</i>	<i>cis</i>
1	0.081±0.012	0.045±0.008	2.185±0.327	0.401±0.133	0.144±0.052	0.335±0.103	0.010±0.002	0.048±0.014	1.609±0.423	0.777±0.160
2	0.014±0.006	0.035±0.023	0.012±0.002	0.056±0.012	0.019±0.009	0.022±0.007	0.013±0.003	0.047±0.013	0.125±0.025	0.087±0.016
3	0.013±0.003	0.057±0.011	0.043±0.010	0.185±0.042	0.008±0.002	0.073±0.020	0.335±0.105	0.210±0.080	0.070±0.022	0.320±0.101
5	0.040±0.010	0.047±0.011	0.113±0.020	0.098±0.014	0.030±0.011	0.067±0.023	0.394±0.083	0.151±0.027	0.843±0.204	0.445±0.107
6	0.025±0.011	0.024±0.006	0.034±0.004	0.045±0.012	0.011±0.003	0.096±0.042	0.043±0.011	0.082±0.033	0.247±0.057	0.099±0.027
7	0.008±0.002	0.050±0.011	0.015±0.003	0.091±0.018	0.025±0.006	0.061±0.011	0.132±0.041	0.198±0.065	0.122±0.036	0.095±0.021
8	0.855±0.196	0.137±0.029	8.506±2.331	0.444±0.119	0.428±0.086	0.094±0.033	0.353±0.088	0.190±0.063	0.830±0.149	1.313±0.403
12	0.658±0.141	0.080±0.020	21.65±8.095	0.555±0.121	0.320±0.115	0.071±0.013	0.114±0.024	0.110±0.039	0.237±0.047	0.462±0.137