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ORIGINAL ARTICLE

Differential Immunomodulatory Activities of Schiff Base Complexes Depending on their Metal Conjugation

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Abstract— Immunomodulatory compounds have become crucial with advances in immunotherapy. Using our own immune system cells, we can direct the immune cell function and develop desired response against a certain threat. Immunotherapy applications have been suggested against tumors, autoimmune disorders, and infectious diseases. Vaccination can be considered as one of the best known example of immunotherapy. Infectious agent's signature molecular structures are introduced to the immune cells together with the adjuvants that further activate the immune cells to mount a proper immune response and memory. Immunotherapy and vaccine formulations are in constant need of a library of immunomodulatory reagents that can be applied depending on the target. In order to expand the number of immunomodulatory reagents that can find medicinal applications, our group has been testing unique chemical structures on the immune system cells, especially macrophages. Schiff base complexes are known for their anti-inflammatory and antimicrobial activities. In this study, we used previously characterized Schiff base complexes with different metal conjugations. These molecules had differential immunostimulatory and immunomodulatory potentials on macrophages *in vitro* depending on the type of the conjugated metal. After light exposure, these complexes changed their characteristics and became powerful anti-inflammatory complexes. Due to their possible antimicrobial potentials, we also tested their activities against gram negative and gram positive bacteria. All of the complexes exerted antimicrobial activities which were not light responsive. Here, we present Schiff base complexes with differential immunostimulatory and immunomodulatory activities that can also efficiently eliminate gram positive and gram negative bacteria. Upon photo activation, they block the

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production of inflammatory TNFα cytokine. Therefore, together with the light, they can be used to treat bacterial infections associated with damaging inflammation.

KEY WORDS: immunomodulation; macrophages; PDT; antimicrobial activity; inflammation.

INTRODUCTION

Our immune system has all the necessary tools to deal with different types of infections and tumor cells, as well as injuries, including those of hard tissues such as the bone [1–15]. In recent years, the medicinal approaches changed their direction towards exploiting the immune system cells to cure disease conditions [1–15]. Immunotherapy of cancer increased its pace to such an extent that T cells are specifically engineered against tumor cells [1–15].

Other than introduction of cells from outside sources, the patient's immune system cells can also be directed towards desired immune response against the danger [1–15]. This approach is known as immunomodulation: regulation of the immune system cells' activities [1–24]. In order to achieve this, proper and fine control over the activation status and functional polarization of the immune system cells are crucial [1–24].

Adjuvants could be one example of how immunomodulation works for the patients as well as public health [1–24]. It is mostly a preventive approach but the aim is pushing the immune system cells for a strong and right type of an activation status against the target antigens of the infectious agents [1–24]. This strong activation later on turns into the formation of a strong immune cell memory that can be utilized in later encounters with the pathogen [1–24]. Adjuvants are stimulator or activator immunomodulatory reagents. They work by activating the toll-like receptors (TLRs) of the innate immune system cells, especially of macrophages [1–24].

Immunomodulatory reagents do not have to have a stimulant role; for example, in the case of autoimmune diseases, the field would prefer compounds that would suppress the immune cell activation [1–24]. This suppression should not be a complete shutdown as in the case of steroids [1–24]. Instead, it should be a fine tuning towards the generation of the right type of response that would start wound healing processes in the tissue [1–24].

In our studies, we have been taking advantage of a mammalian macrophage cell line to screen possible immunomodulatory potential of newly produced or unique and previously non-investigated chemicals [16–24]. Previous studies suggest that Schiff bases have anti-inflammatory activities [25–29]. Moreover, they have a photodynamic activation potential which can be altered by addition of different types of metals [25–29].

In this study, we focused on new Schiff base complexes with different metal conjugations that were characterized chemically in our previous publications [30–32]. Photodynamic activation will further enable control over their activities so that they can be turned on at a certain location of the tissue or body at a desired time [33–49]. Due to a well-known antimicrobial potential of Schiff base complexes, we also deciphered their antimicrobial activities on gram negative and gram positive bacteria as well [25–29]. Our results suggest differential dark *versus* photodynamic immunomodulatory activities of Schiff base complexes that depended on the type of metal it was conjugated with. These compounds had dark antimicrobial activities which did not alter by light exposure.

MATERIALS AND METHODS

Synthesis of Metal-Conjugated Schiff Bases

Synthesis of Schiff Base and Its Transition Metal Complexes

Schiff base (HL) was prepared by using 2,4-dimethoxy aniline with 5-chloro-2-hydroxybenzaldehyde in methanol. The Schiff base complexes with different transition metal ions $[Cu(L)_2]$**(1)**, $[Co(L)_2].CH_3OH$**(2)**,$[Ni(L)_2]$ **(3)** were produced according to protocols in our previous publications [32] (Fig. 1).

Determining the Immunostimulatory and Immunomodulatory Functions of Metal-Conjugated Schiff Bases. Mammalian macrophage cell line RAW 264.7 cells (ATCC) were grown in 37 °C 5% CO_2 incubator by using RPMI 1640 mixed with 10% fetal bovine serum and 1% antibiotics (streptomycin and penicillin) [16–24]. Immunostimulatory roles of the complexes were measured by using them in10 and 100 μg/mL concentrations without LPS. The incubations were

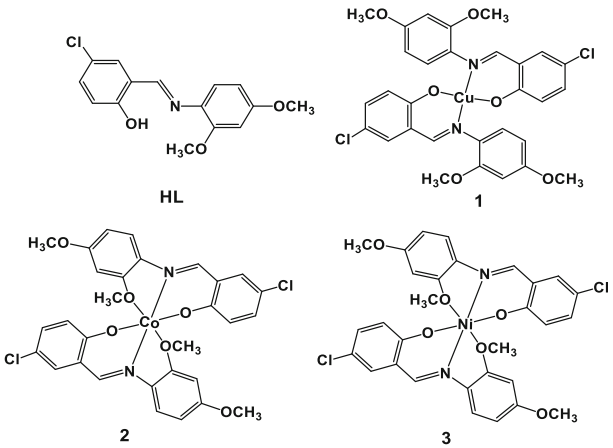

Fig. 1. Chemical structures of Schiff base and its transition metal complexes.

done for 24 h and 10^6 cells/well in 1 mL were put into each well of the 24 well plates. Immunomodulatory potentials of the compounds were tested by using the same experimental setup and putting 1 μg/mL LPS together with the complexes. Media in each well were collected and analyzed by TNFα ELISA kit (BD Biosciences) by following the manufacturer's guidance. Other than dark conditions, these experimental setups were also conducted with 5 min and 10 min of Xenon light treatment right before 24-h incubation period. Student *t* test was applied through Graphpad Prims Version 5 after obtaining statistically meaningful data by at least three biologically independent repeats of the experiments [16–24].

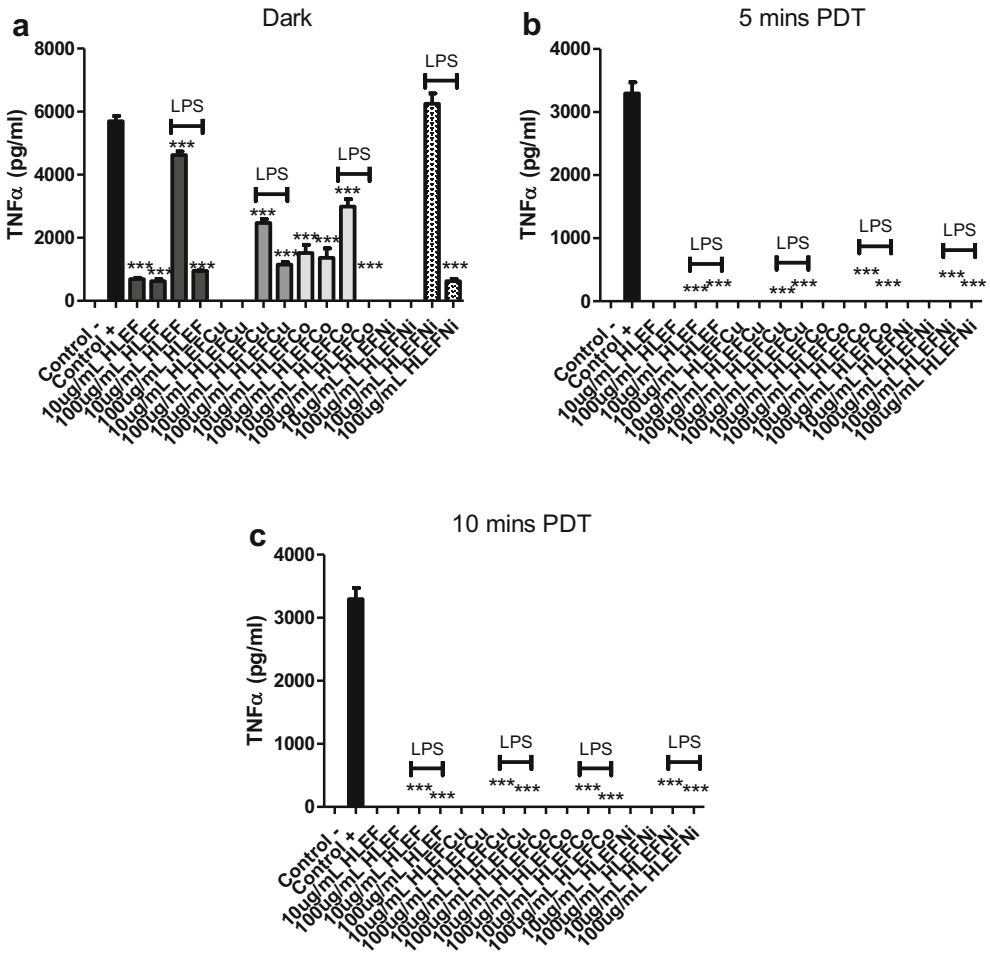

Fig. 2. TNFα ELISA results after 24-h incubation. The concentrations of complexes were 10 μg/mL and 100 μg/mL and they were applied with or without 1 μg/mL LPS. Other than dark conditions, these experimental setups were also conducted with 5 min and 10 min of Xenon light treatment right before 24-h incubation period. $*p < 0.001$, $**p < 0.0005$, $***p < 0.0001$, $N = 3$.

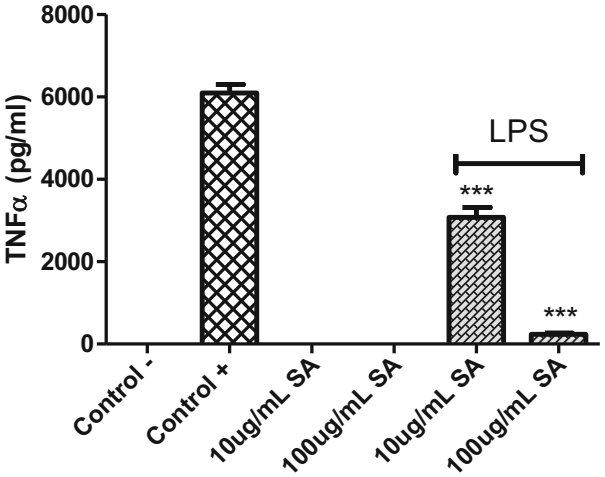

Fig. 3. TNFα ELISA results after 24-h incubation. The concentrations of salicylic acid were 10 μg/mL and 100 μg/mL and it was applied with or without 1 μg/mL LPS. Other than dark conditions, these experimental setups were also conducted with 5 min and 10 min of Xenon light treatment right before 24-h incubation period. *$p < 0.001$, **$p < 0.0005$, ***$p < 0.0001$, $N = 3$.

Determining the Antimicrobial Potential of Metal-Conjugated Schiff Bases

IC50 values of each compound on gram negative *Escherichia coli* (ATCC25922) and gram positive *Staphylococcus aureus* (ATCC29213) bacteria were determined by using microtiter broth dilution assay. In control groups, neomycin was used and its IC50 values were calculated for each bacterial strain. A total of 1, 10, 50, and 100 μg/mL concentrations of the complexes were tried to calculate the IC50 values for each on two bacterial strains. Bacteria were grown overnight on 37 °C shaker after picking specific colonies into Mueller Hinton Broth (MHB) media. By using sterile PBS, the bacterial concentrations were adjusted to 0.5 McFarland and 10 μL of bacterial samples were added into each well of sterile 96 well plates supplemented with 100 μL of fresh MHB in each well. After addition of the specified concentrations of the complexes, the plates were incubated on 37 °C shaker for 18 h and absorbance values were measured at 600 nm. In order to prevent the interference of internal absorbance values of complexes at 600 nm, the absorbances of our compounds were measured at 600 nm without having the bacteria in the wells of 96 well plates and from sample absorbances this value was subtracted. On top of the dark conditions, these experimental setups were also conducted with 5 min and 10 min of Xenon light treatment right before 24-h incubation period. Student *t* test was applied through Graphpad Prims Version 5 after obtaining statistically meaningful data by at least three biologically independent repeats of the experiments [16–24].

RESULTS

Depending on the Type of Metal in the Complex Schiff Base Complexes Had Differential Immunostimulatory Effects on the Inflammatory Activity of Macrophages

HL, **1**, **2**, and **3** were first incubated with the macrophages in the absence of LPS stimulant. In this way, their immunostimulatory activity was examined with their 10 μg/mL and 100 μg/mL concentrations by measuring TNFα production level. After 24 h of incubation compared with negative control wells, HL that does not contain any metal had immunostimulatory activity at both concentrations (Fig. 2). Moreover, addition of copper or nickel to the structure completely knocked out the immunostimulatory activity of HL structure (Fig. 2). Whereas, cobalt-conjugated HL still pertained its immunostimulatory effect at both concentrations and higher than that of HL (Fig. 2). This immunostimulatory activity was eradicated by light treatment. Even with 5 min of Xenon light treatment, the pro-inflammatory potential of HL and **2** was completely gone (Fig. 2). Longer (10 min) period of light exposure had similar effects as short period (5 min) light treatment (Fig. 2).

Cobalt and Copper Decreased the Dark Anti-inflammatory Activity of Schiff Base Complex

In order to test the effect of our compounds on already activated macrophages, they were used in 10 μg/mL and 100 μg/mL concentrations together with 1 μg/mL LPS on macrophages. After 24 h based on TNFα production levels, their immunomodulatory potential was assessed in dark conditions as well as after light exposure (Fig. 2). In dark conditions, HL had strong anti-inflammatory activity higher than that of salicylic acid control groups on the macrophages (Fig. 2). Addition of cobalt or copper did not alter the complex's anti-inflammatory character but increased its effectiveness (Fig. 2), whereas nickel decreased the anti-inflammatory effectiveness of HL (Fig. 2). **1** and **2** complexes had a stronger anti-inflammatory effect than HL on stimulated macrophages (Fig. 2). Nickel had a depreciating effect on the anti-

inflammatory potential of the complex (Fig. 2). Ten micrograms per milliliter of **3** did not affect the TNFα production levels by stimulated macrophages compared with positive controls (Fig. 2). The situation was different after light exposure; all of the complexes gained strong anti-inflammatory characteristics even at their 10 μg/mL concentrations (Fig. 2). They were stronger than salicylic acid in anti-inflammatory potential (Figs. 2 and 3). They completely knocked out TNFα production by stimulated macrophages compared with positive controls (Fig. 2). Even 5 min of light exposure was enough to observe the effect and 10 min of light exposure gave the same results (Fig. 2). We measured the cell viability after 24 h of incubation in the dark, and in the 5-min and 10-min light conditions, our complexes did not alter the cell viability of the macrophages (Fig. 4). Therefore, the observed effects were independent of the changes in the cell viability.

Schiff Base Complexes Had Strong Antimicrobial Activity Against Gram Negative and Gram Positive Bacteria in Dark Conditions and Photodynamic Activation Did Not Alter Their Capacity

Our compounds' IC50 values were determined separately against gram negative *E. coli* and gram positive *S. aureus* both in dark conditions as well as after 5 min

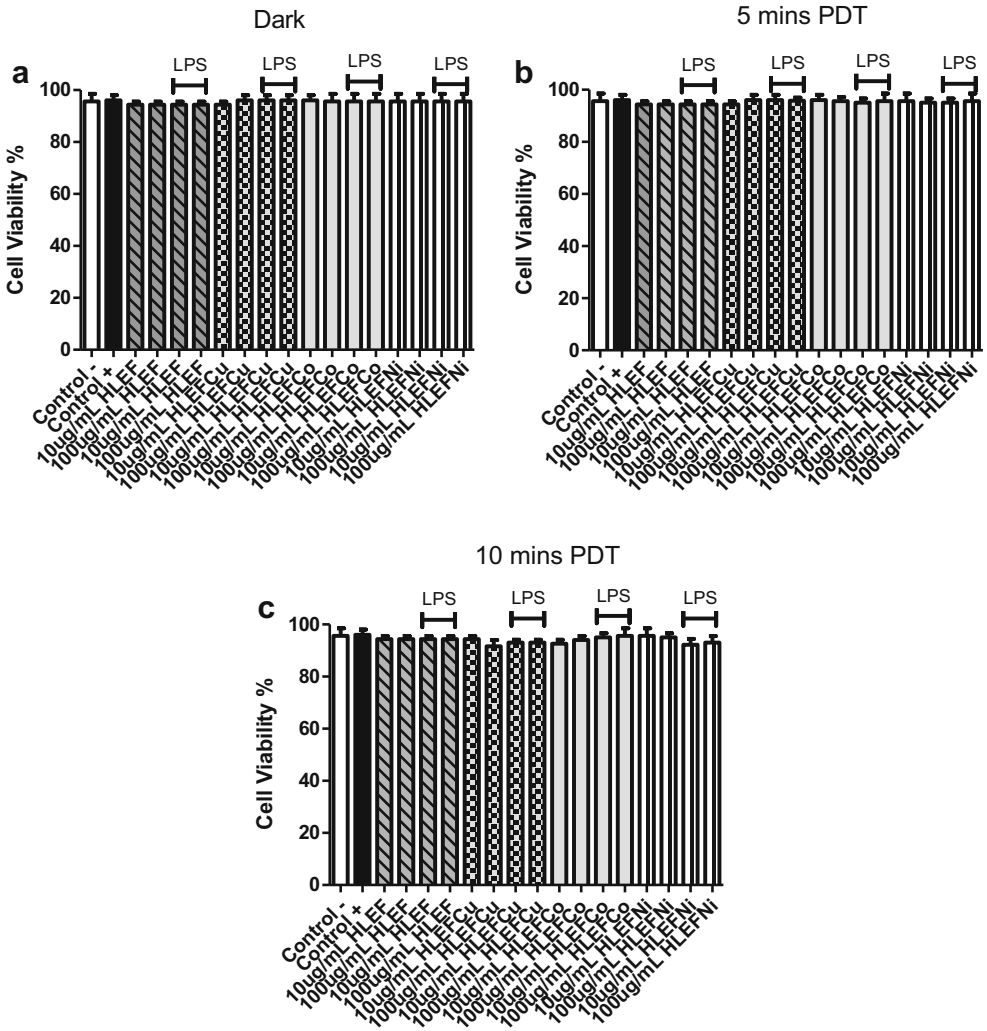

Fig. 4. Viable cells were determined by trypan blue after 24-h incubation. The concentrations of complexes were 10 μg/mL and 100 μg/mL and they were applied with or without 1 μg/mL LPS. Other than dark conditions, these experimental setups were also conducted with 5 min and 10 min of Xenon light treatment right before 24-h incubation period. *$p < 0.001$, **$p < 0.0005$, ***$p < 0.0001$, $N = 3$.

Table 1. IC50 Values for Each Schiff Base Complex Against *E. coli*

IC50 for *E. coli* (μg/mL)					
	HL/PDT	1/PDT	2/PDT	3/PDT	Neomycin
(Dark)	1.95	2.6	3.81	1.45	45
(5 min)	1.94	3.46	3.79	1.66	40
(10 min)	1.95	3.79	3.77	1.48	41

and 10 min of light exposures (Tables 1 and 2). Compared with neomycin, our compounds had substantially stronger antimicrobial activity against both bacterial strains (Tables 1 and 2). Light exposure did not alter their IC50 values; therefore, their antimicrobial activity was independent of photodynamic activation (Tables 1 and 2). Moreover, addition of metals to the complex structure slightly decreased their antimicrobial effect compared with HL, but this slight difference is negligible (Tables 1 and 2).

DISCUSSION

Schiff base complexes and their derivatives have been studied for their anti-tumor, antimicrobial, and anti-inflammatory potentials [25–29]. These active chemical structures also have photodynamic activation characteristics [25–32]. Photodynamic activation enables their controlled and localized activation which can decrease the required effective dose for the treatment as well as the side effects associated with uncontrolled activation and high dosage [25–49].

In this study, we focused on previously only chemically characterized but novel Schiff base complexes [30–32]. These complexes had different metal conjugations and we tested the effect of metals on the immunostimulatory and immunomodulatory as well as antimicrobial potentials of the complexes. Moreover, their activity changes after photodynamic activation was also measured.

Immunostimulatory compounds are important for the generation of new adjuvant candidates [16–24]. Adjuvants work by activation of TLR signaling pathways on macrophages [16–24]. In our experimental setup, mammalian macrophages were analyzed and their pro-inflammatory cytokine TNFα secretion levels were measured. TNFα cytokine is crucial for development of Th1-type immune responses that are responsible for the effective fight against intracellular pathogens as well as tumor cells [1–24]. Schiff base complex HL was the starting material and it lacked metal in its structure. HL was able to stimulate the macrophages to produce significant amounts of TNFα. Copper and nickel eradicated this property of HL structure since the complexes with copper and nickel did not lead to TNFα production by themselves. Whereas cobalt had a different effect, it actually increased the TNFα production even further than HL itself. All these effects were observed in dark. The situation completely changed after light exposure. The complexes lost their ability to activate the macrophages by themselves.

When immunomodulatory activity of these complexes was examined in the presence of LPS, HL had anti-inflammatory activity which got stronger with addition of either copper or cobalt to the structure, whereas **3** did not alter TNFα production compared with positive control in its low concentration. Higher concentrations of it were required to get a significant drop in the pro-inflammatory cytokine TNFα production levels compared with positive control. These results were obtained in dark conditions. After light exposure, all the complexes independent of which metal complex they had in their structures had very strong anti-inflammatory effect on activated macrophages compared with salicylic acid control groups. They got even stronger than salicylic acid after light exposure in terms of their anti-inflammatory capacity based on the level of decrease in TNFα secretion.

Table 2. IC50 Values for Each Schiff Base Complex Against *S. aureus*

IC50 for *S. aureus* (μg/mL)					
	HL/PDT	1/PDT	2/PDT	3/PDT	Neomycin
(Dark)	1.95	3.93	3.95	1.65	65
(5 min)	1.96	3.63	3.95	1.7	63
(10 min)	1.89	3.05	3.42	2.34	60

Using an anti-inflammatory molecule during a chronic inflammation or excessive inflammation could be beneficial; but if the reason of the inflammation is an infectious bacteria, then the reason of the infection should be eliminated as well [1–15, 50–57]. Therefore, we tested the antimicrobial activity of these complexes in the dark and after light exposure. All of the complexes had comparable antimicrobial activities against both gram negative and gram positive bacteria. This situation did not depend on photo activation since both in the dark and after light exposure complexes' IC50 values were similar and much lower than that of neomycin against both bacterial strains.

In conclusion, these novel Schiff base complexes had differential dark- and photo-activated effects on the macrophage inflammatory activity depending on the type of metal that it was conjugated with. After light exposure, these complexes had better anti-inflammatory activity than salicylic acid. Independent of photo activation, our compounds were strong antimicrobial agents against gram negative and gram positive bacteria. Their effectiveness was better than that of neomycins. These complexes have great potential to be used as immunomodulatory agents and their proper usage requires further characterization in terms of determining their molecular targets and pharmacokinetics.

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Authors' Contributions

Authors equally contributed to the manuscript and approved the final version of it.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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