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Anticancer Properties for 4,4′-Dihydroxybenzophenone-2,4-dinitrophenylhydrazone (A-007)/3,7-Diaminophenothiazin-5-ium Double Salts

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Abstract—4,4′-Dihydroxybenzophenone-2,4-dinitrophenylhydrazone (A-007) formed stable double salts with phenothiazin-5-ium salts (**2a**–**d**), which have improved in vitro anticancer activities, as compared to A-007 alone. The stable salt between methylene blue (**2a**) and A-007 allowed the latter to diffuse into the dermis layers of skin. It is anticipated that these new salts will allow A-007 to penetrate into the deep lymphatic/vascular channels of the dermis, which contain metastatic cancer cells, and improve in vivo anticancer activities.

4,4′-Dihydroxybenzophenone-2,4-dinitrophenylhydrazone (A-007), **1**, has anticancer activities in vitro and in vivo when applied topically to metastatic cancer spread to the skin.[1–4] Metastatic cancer to the skin does not respond well to systemic therapy.[3] This communication describes the formation of stable double salts between A-007 and sulfur containing π-electron delocalized cationic (EDC) salts that, (1) improved A-007's anticancer activities, and (2) increased A-007's penetration into the dermis (Fig. 1).

Methylene blue (**2a**) and other phenothiazin-5-ium salts (**2b**–**d**) exist as π-electron deficient species due to sulfur's ability to utilize 3*d* orbitals (a characteristic of 3rd periodic level elements).[5–7] The orbital dynamics of the phenothiazin-5-ium salts have been utilized to produce stable salts with A-007 (Table 1).[8] Some chemistry and biological properties of these double salts are reported in this communication.

A-007 (63 mmol) in 10 mL of ethanol (need to heat to dissolve) was added, with stirring, to 63 mmol of **2a**–**e**, each dissolved in 10–15 mL of ethanol, and stirred at 75 °C for an additional 15–30 min. The solutions were allowed to stand at refrigerator temperatures overnight. The resulting precipitates (A-007/**2a**–**d**) were recrystallized from ethanol with 73–100% yield.[9] See Table 1 for structure compositions. All products analyzed correctly as a double salt (A-007/**2a**–**d**).

Fresh human cancers obtained at surgery, were minced and suspended in 16 mL of RPMI-1640 containing 10% FBS, 10 mg/mL neomycin, 5 mg/mL streptomycin and 5000 U/mL penicillin. The Cytostat® assay was used to determine in vitro anticancer activity for the double salts.[3] A-007 and salts were dissolved in DMSO (5 mg/mL) and used as a stock solution. Doxorubicin (DOX) (Table 1) and the respective base dyes were used as controls. The cultures were exposed to varying

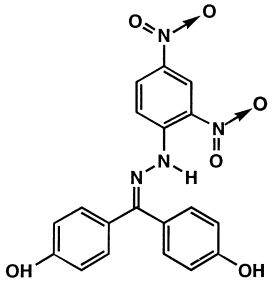

Figure 1. A-007.

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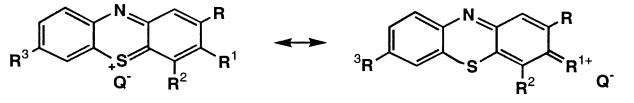
concentrations of A-007 salts for 72 h, then assayed for cytotoxicity.[3]

In triplicate, dermatomed (0.3–0.4 mm) skin samples from hairless rats were cut into multiple sections large enough to fit on 0.9 cm^2 Franz diffusion chambers. The dermal chambers consisted of 5% BSA in Hepes buffered Hank's balanced salt solution open to the ambient laboratory environment. The Franz diffusion chambers were placed in a diffusion apparatus and the dermal receptor solution stirred at 600 rpm and 32 °C.[10]

The test formulations were applied to triplicate skin sections as a 0.25% propylene glycol gel.[10] At timed intervals, the receptor solution was removed and stored at −70 °C until assayed for A-007. Standard H&E histological examinations were conducted on all treated and control skin specimens upon terminating each study. Quantitation of A-007 was determined by reverse-phase HPLC.[11]

A-007 formed double salts with 3,7-bis(dimethylamino)-phenothiazin-5-ium chloride (methylene blue or MEB, **2a**), 3-amino-7-dimethylamino-2-methylphenothiazin-5-ium chloride (toluidine blue or TB, **2b**), 3,7-bis(dimethylamino)-4-nitrophenothiazin-5-ium chloride (methylene green or MEG, **2c**), and 3,7-bis(diamino)-phenothiazin-5-ium acetate (Thionin or TN, **2d**), while phenothiazine (PT, **2e**) did not react with A-007. The latter observation may be associated with **2e** being unsubstituted (i.e., no –NH, etc.) and unable to stabilize through an EDC resonance, similar to what can exist for **2a–d.**

Table 1. Phenothiazin-5-ium salts



Dyes	R	R^1	R^2	R^3
2a Methylene blue (MEB)	H	$N(CH_3)_2$	H	$N(CH_3)_2$
2b Toluidine blue (TB)	CH_3	NH_2	H	$N(CH_3)_2$
2c Methylene green (MEG)	H	$N(CH_3)_2$	H	$N(CH_3)_2$
2d Thionin (TN)	H	NH_2	H	NH_2
2e Phenothiazine (PT)	H	H	H	H

$Q^- = Cl^-$ or acetate.

Anticancer activities for the A-007 double salts (**2a–d**) in a human cancer tissue explant culture assays are summarized in Table 2. The anticancer activities for the salts are significantly improved when compared to A-007 alone. The activities noted for breast, melanoma and lung are very encouraging. The base dyes (**2a–d**), alone, were not active ($IC_{50} > 30$ mcg/mL); NT = not tested. A-007/MEB and A-007 were tested against normal human peripheral blood dendritic cells and the IC_{50} were calculated to be > 10 mcg/mL.[12]

MEB improved A-007's penetration into tissue and the latter's ability to diffuse through the epidermis and enter channel networks draining the dermis (Table 2). This data supports the concept that π-electron deficient cations (EDC) may be able to deliver A-007 to lymphatic channels/spaces in tissues and thus increase anticancer activities. Histological examinations of the treated skin preparations from Table 3 revealed that A-007/MEB improved A-007's penetration into the dermis, when compared to A-007 alone, which only penetrated the epidermis. A-007 is deep red in color and stains tissues yellow; this property renders it easy to trace.

The above salts do not form acceptable crystals for X-ray crystallography analysis. In an attempt to appreciate the interactions between A-007 and the dyes, ^{15}N-A-007 was synthesized from $(^{15}N)_2$-2,4-DNPH and 4,4-dihydroxybenzophenone.[12] ^{15}N NMR studies failed to reveal an interaction between MEB and A-007's –NH moieties (**2a**). Comparative ^{13}C NMR spectroscopy did not demonstrate any π–π stacking for the **2a**/A-007 salt. No shifts in hydrogen were seen with comparative ^{1}H NMR spectroscopy for **2a**. In contrast, for the **2d** salt, a broad singlet at 3.6 ppm was observed with ^{1}H NMR spectroscopy while the phenolic protons of A-007 were absent; this could represent interactions between NH– and HO–. The latter shifts observed with **2d** are probably a result of the reduced bulkiness at the 3- and 7-

Table 2. Comparative IC_{50} sensitivities for primary human cancer culture

Tissue	No.	A-007 (mcg/mL)	A-007/MEB (mcg/mL)	A-007/MEG (mcg/mL)	A-007/TB (mcg/mL)	A-007/TN (mcg/mL)	DOX (mcg/mL)
Breast cancer	4	3≥10	0.3–2.5	NT	0.9–1.5	0.9	0.3–0.9
Melanoma	6	7≥10	0.5–1	0.03–0.4	0.7–4.0	NT	2≥5
Ovarian cancer	3	9	2	NT	NT	0.2	NT
Lung cancer (NSCLC)	4	4	1–2	0.75–1	3	>10	>30

Table 3. Percutaneous absorption in the Franz cell (A-007 vs A-007/MEB diffusion into rat skin)

Test material	Franz chamber (No.)	0.25% Gel applied (mg)	Actual A-007 applied (mg)	A-007 in receptor fluid (ng/mL)
A-007	12	17–56	0.04–0.14	None detected
A-007/MEB	3	14.6	0.05	9

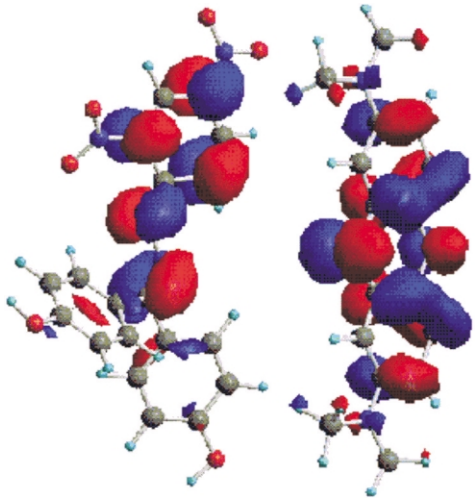


Figure 2. An AM1 generated HOMO–LUMO orbital presentation for A-007 (left) and methylene blue (right).

positions (primary amines) and the close proximity that could occur between A-007's –HOs and **2d**'s –NHs. Based on elemental analysis, IR stretching and NMR singlet shifts noted, the stability of the salts are possibly a result of interactions between the cationic sulfur and the anionic –NH of A-007, as well as the –OHs in A-007 and the 3- and 7-NHs groups in the phenothiazin-5-ium salts. An AM1 semi-empirical method generated a HOMO–LUMO orbital presentation for the A-007/MEB salt that revealed complimentary electronic derivatives that allow a stable configuration to exist (Fig. 2). At least one of A-007's aryl–OHs is required for salt formation.[12]

The described A-007 salts are stable, however, in the presence of tissue highly charged biopolymers and so on, the former are capable of dissociating, releasing A-007 (Table 2). Thus we have successfully generated products that both protect A-007 from non-selective tissue binding, as well as enhance its diffusion into avascular dermal structures that can harbor cancer cells.

Acknowledgements

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