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Topical Photodynamic Therapy Mediated with Hematoporphyrin-HCl using Glycerin as Penetrating Enhancer: In vitro and in vivo studies

Maha Fadel and Abdullah Ibrahim El-Kholy*

Nano-Pharmaceutical Lab., National Institute of Laser Enhanced Sciences (NILES), Cairo University, Egypt

Abstract

Purpose: In this study, the efficacy of a gel formulation containing Hp-HCl (a photosensitizer) dissolved in glycerin was evaluated for photodynamic treatment (PDT) of tumors.
Methods: The release kinetics of water-dissolved Hp-HCl and glycerin-dissolved Hp-HCl from gel formulae were studied in vitro, where the latter showed faster drug release. The growth rates of tumor size in animals, from different treatment groups, were measured.
Results: The group treated with the glycerin-dissolved Hp-HCl showed more significant decrease in tumor rate (smaller size) in comparison to the water-dissolved formula, after 35 post-implantation days. Histopathological examination of tumor sections revealed that glycerin-dissolved Hp-HCl effectively distributed the drug throughout the tumor, resulting in almost complete disappearance of tumor cells. Overall, the dissolution of Hp-HCl in glycerin not only increased drug release from the gel formulation but also improved its distribution and therapeutic efficiency. These findings were supported by histopathological results
Conclusion: Suggesting that the topical use of Hp-HCl dissolved in glycerin holds promise for improved PDT.

Keywords— Photodynamic therapy; Tumor; Topical; Glycerin-Hp-HCl

I. Introduction

Photodynamic therapy (PDT) is a promising approach in cancer treatment, utilizing the interaction between a photosensitizer, light, and molecular oxygen to selectively destroy malignant cells [1-4]. Over years, various photosensitizers have been investigated for their potential in PDT, and hematoporphyrin dihydrochloride (Hp-HCl) has emerged as one of the most widely used agents. Hematoporphyrin dihydrochloride, a derivative of hematoporphyrin, possesses excellent photochemical properties and has demonstrated effective tumor destruction in numerous preclinical and clinical studies. However, despite being significantly effective, there are ongoing efforts to optimize the delivery and efficacy of Hp-HCl-based PDT [5-7]. In recent years, there have been significant advances in the field of topical PDT, which offers several advantages over traditional systemic administration [8-11]. Topical PDT involves the application of a photosensitizer directly onto the target tissue, followed by localized light activation. This approach allows precise targeting of the photosensitizer to the lesion along with minimizing systemic side effects [3, 5, 12]. Among the various photosensitizers investigated for topical PDT, Hp-HCl has shown promising results due to its ability to penetrate the skin and selectively accumulate in cancer cells [13, 14]. This study aims to furtherly explore the potential of topical photodynamic therapy using Hp-HCl gel for the treatment of cancer, to investigate the in vitro and in vivo effects of Hp-HCl gel in various cancer cell lines and animal models and to focus on its efficacy, selectivity, and safety. Additionally, the optimal parameters for light activation, such as light dose and wavelength are also investigated, to maximize the therapeutic outcome. By optimizing the delivery and efficacy of Hp-HCl-based PDT, we aim to enhance treatment outcomes, minimize side effects, and improve the overall quality of life for cancer patients.

II. Materials and Instruments

Buffer used is N-[(2-hydroxy) piperazine-N-(2-ethanosulphonic acid)] (HEPES) and Hematoporphyrin dihydrochloride (Sigma, St. Louis, M.O, USA), Propyl paraben, Methyl paraben, and Carbopol 974 (Goodrich, USA), Dialysis sacks of cellulose tubing 35 mm (Sigma, St. Louis, M.O, USA), Tri-ethanolamine (Aldrich, USA), pH meter type 320 (Corning, USA), UV-Visible spectrophotometer (UV-2601, Rayleigh, Beijing, China), Diode laser of wave length 650 nm continuous wave, and maximum output power 500 mW (R 650-500, Intelite, USA) and light microscope (Biomed, Germany) with attached video camera (Panasonic, Japan).

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Corresponding author: Abdullah Ibrahim El-Kholy Email: abdullahelkhouli@gmail.com

III. METHODOLOGY

A. Preparation of standard curve of Hp-HCl

A stock solution of Hp-HCl with a concentration of 100 µM was prepared. An amount of 3.4 mg of Hp-HCl was dissolved in HEPES buffered saline using a magnetic stirrer for 4 hours and covered with aluminum foil. The pH of the solution was adjusted to 5.5 and a 50 ml volumetric flask was used to dilute the solution to the mark. Two milliliters of the solution were scanned for maximum absorption wavelength using spectrophotometer at 380 nm. Ten serial diluted samples were prepared from the stock solution, and their optical densities were measured at 380 nm. Measurements were done in triplets and linearity was achieved in concentrations from 0 to 0.02 mg/ml.

B. Preparation of Hp-HCl gel formulae

Two gel formulae (40 mg Hp-HCl) were prepared from Propyl paraben, Methyl paraben, and Carbopol 974 and tri-ethanolamine in the following concentrations: Carbopol 974 2%, propyl paraben 0.15%, methyl paraben 0.2% and tri-ethanolamine 10 drops.

1) Formula I (water-soluble Hp-HCl)

A stock solution of Hp-HCl with a concentration of 6×10^{-4} M was prepared by dissolving 40 mg Hp-HCl in 100 ml distilled water and stirring for 4 hours. The solution was filtered through a 0.2 µm pore diameter sterile filter. The stock solution was warmed below 37ºC, methyl and propyl paraben were added, and then Carbopol 974 was added after complete dissolution with continuous gentle stirring. Finally, triethanolamine was added drop by drop until complete gelling. The gel was adjusted to have a total of 40 mg Hp-HCl content.

2) Formula II (glycerin-soluble Hp-HCl):

Hp-HCl stock solution was prepared by dissolving 40 mg Hp-HCl in 50 ml glycerin and stirring for two hours. Distilled water was added in a volume ratio of 1:1 (v/v) glycerin/water and left under stirring for another 2 hours and the final concentration of the solution was 6×10^{-4} M. Then, the solution was filtered through a 0.2 µm pore diameter sterile filter. The prepared stock solution was then used for gel preparation following the procedure described in Formula I.

C. Release kinetics study

Three samples of 1±0.025 g of the prepared gel from each of Formula I and Formula II were packed in dialysis sacks. Each sack was placed in 50 ml HEPES buffered saline of pH 5.5 in a beaker with continuous stirring at 100 rpm and 37ºC. Samples of 1 ml were taken from the saline at different time points up to 3 hours and measured at 380 nm using spectrophotometer. The amount of drug released was estimated using the established calibration curve.

D. Release kinetics modeling

The released data were fitted to different equations to study the mechanism of drug release. The equations used were:

Zero-order release kinetics:

$$Q = Q_0 - k_0 t \quad (1)$$

First-order release kinetics:

$$\ln Q = \ln Q_0 - k_1 t \quad (2)$$

and Higuchi's diffusion kinetics:

$$Q = k_2 t_{1/2} \quad (3)$$

where: Q_0 is the original concentration of the drug per unit volume, Q is the amount of drug released per unit volume, t is the time of drug release, k_0, k_1, and k_2 are the release rate constants for zero-order, first-order and diffusion kinetics, respectively. Then, drug half-lives ($T_{1/2}$) in the prepared formulations were estimated from the release data [15-17].

E. In vivo study

1) Animals photodynamic experiment

Forty female albino mice were divided into four groups: Group A (healthy control), Group B (tumor control), Group C (PDT treatment with Hp-HCl gel formula I), and Group D (PDT treatment with Hp-HCl gel formula II). Tumor cells (Erlich tumor cell line) were injected subcutaneously into Groups B, C, and D to induce tumor growth. Groups C and D received photodynamic treatment (PDT) using Hp-HCl gel formula I and II, respectively. The gel was applied for three hours, followed by irradiation with 60 J/cm^2 Diode laser at wave length of 650 nm continuous wave. The spot size of the laser beam was 1cm^2. The energy deposited in the sample was calculated through the relation [14, 18]:

$$\text{Energy (joules)} = \text{Power (watts)} \times \text{Time (seconds)}/ \text{Surface area (cm}^2\text{)} \quad (4)$$

Treatment was conducted three times weekly for two weeks, and the tumor size was measured weekly for 35 days. At the end of the study, mice were sacrificed, tumors were excised, and histopathological examination was performed.

2) Histopathological examination

Mice were sacrificed 35 days post-tumor implantation, with overdose of general anaesthesia and autopsies were performed. Tumors were excised and pathologically examined using the light microscope and photographed with the attached video camera.

F. Statistical Analysis

The paired student's t-test was used to determine the statistical significance of the differences between drug release data for the two formulations as well as the tumor growth between the different groups. All p values were two tailed, and differences were considered significant when the p value was less than or equal to 0.05. Data were interpreted as means ± SDs.

IV. RESULTS AND DISCUSSION

A. In vitro studies

A reliable standard calibration curve of Hp-HCl in HEPS buffered saline (pH 5.5) was established and the resulted equation was utilized to colorimetrically measure the concentration of Hp-HCl in HEPES buffered saline by correlating the absorbance intensity of the molecule at 380 nm to its concentration according to Beer Lambert's law [19]. The drug release data was analysed using zero-order, first-order and Higuchi's diffusion control models. **Figure 1** (a) represents a nonlinear relationship between the drug released/cm^2 for both formulae I and II as a function of time in hours which indicate that the release is not a zero order. It is clear from the figure that the drug released from formula II was significantly ($P\leq 0.05$) higher than formula I at all

time intervals. **Figure 1** (b) represents the relation between ln drug released (lnQ) from gel as a function of time for the first order release kinetics. It is clear from the figure that there is a nonlinear relationship between lnQ and time for both formulae I and II when compared with the results in **figure 1** (c). **Figure 1** (c) shows the variation of %Q as a function of √t for both formulae. The release kinetics for both formulae I and II were compared. Formula II showed significantly higher drug release compared to formula I at all time intervals. Higuchi's diffusion control model provided the best fit for the drug release kinetics of both formulae. Formula II exhibited a higher diffusion rate compared to formula I, (31.1 ± 0.04) (mean ± SDs) and shorter half-life ($T_{1/2}$ = 0.9 ± 0.01 h), which can be attributed to the properties of glycerine in enhancing penetration.

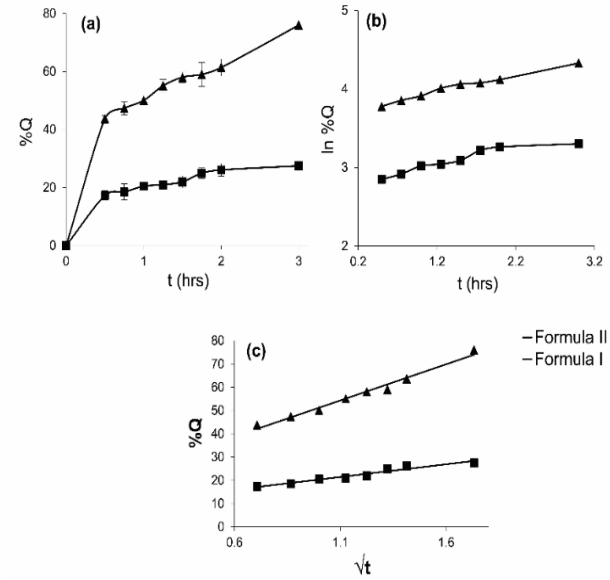

Figure 1*:* In vitro release kinetics for the two gel formulations I and II: (a) The relation between % drug released as a function of time in hours for zero order kinetics, (b) The relation between ln% drug released as a function of time in hours for first order release kinetics and (c) The relation between %drug release as a function of root time for Higuchi's diffusion.

B. In vivo studies

1) Tumor Growth

Figure 2 shows the growth rate of tumor size in animals from groups B, C, and D. Group D, which received treatment with formula II (Hp-HCl dissolved in glycerine), had significantly smaller tumor size compared to group C (Hp-HCl dissolved in formula I) after 35 days post-implantation. This indicates that the use of formula II resulted in better tumor growth inhibition.

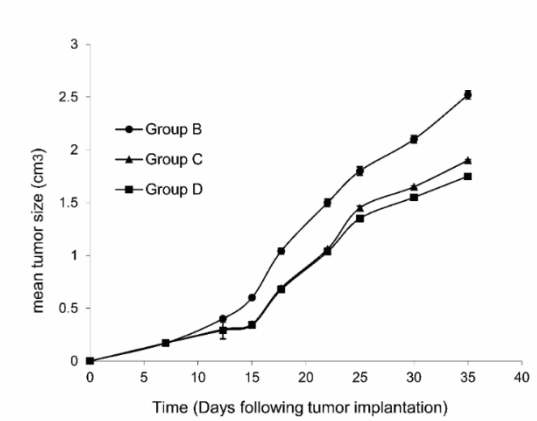

Figure 2: Average tumor size for the three groups untreated (**B**), group treated with gel formula I (**C**), and group was treated with gel formula II (**D**).

2) Histopathological Examination

Histopathological sections of tumors from groups B, C, and D are shown in **figure 3** (b, c, and d, respectively), while **figure 3** (a) a represents normal tissue. Tumors in group B (tumor control) exhibited characteristics such as large hyperchromatic and pleomorphic nuclei with scant cytoplasm and mitotic figures. In group C, there was vanishing of tumor cells at the periphery of the tumor, while the centre still showed tumor cells with hyperchromatic and pleomorphic nuclei, indicating limited drug penetration. In group D, there was almost complete disappearance of tumor cells, with only a few ghost cells remaining, indicating effective drug distribution throughout the tumor.

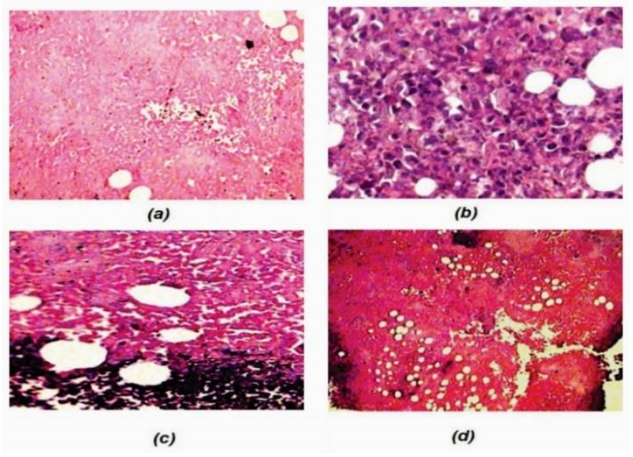

Figure 3: The histological sections of the different groups, (a) Normal tissue of group **A**, (b) Tumor tissues of group **B** with large hyperchromatic and pleomorphic nuclei, (c) Treated tumor tissues with gel formula I of group **C**; vanishing of tumor cells at the periphery of the tumor, and (d) The treated tumor cells with gel formula II of group **D**; almost complete vanishing of tumor cells with few ghost cells within the remaining tissues. 100X magnification.

C. Effect of Hp-HCl Dissolved in Glycerine

The improved in vivo results observed in group D (formula II) compared to group C (formula I) were attributed to the photochemical effect of Hp-HCl in the presence of glycerine. The 650 nm diode laser used in the study had no significant effect on tumor size or histopathology. Glycerine in formula II enhanced the diffusion process through biological tissue and cellular membranes. The polar nature

of glycerine facilitated H-bonding with Hp-HCl, increasing the number of di-cationic species that produce free radicals and undergo photo degradation. The high viscosity of glycerine prevented molecular rotation and aggregation of Hp-HCl, improving its photosensitization and therapeutic properties. The specific results of the histopathological examination of the tumor tissue indicated several noteworthy findings. The examination revealed a larger number of necrotic and ghost cells within the tumor tissue of the group treated with formulation II compared to the other groups. This observation suggests that the photodynamic treatment using Hp-HCl gel formulation II resulted in increased tumor cell death. Furthermore, the histopathological examination demonstrated that the effect of Hp-HCl was distributed throughout all parts of the tumor in the group treated with formulation II. This suggests that the use of glycerin as a solvent for Hp-HCl improved the penetration and distribution of the drug within the tumor tissue, potentially contributing to its enhanced efficiency in photodynamic therapy. Overall, these histopathological findings support the conclusion that the application of topical photodynamic therapy using Hp-HCl gel formulation II led to a greater number of necrotic and ghost cells within the tumor tissue and improved distribution of the photosensitizer, highlighting its potential as an effective treatment modality for Ehrlich tumors.

The improved distribution of the photosensitizer in photodynamic therapy can have significant implications for the treatment's effectiveness and overall outcomes. There are some potential implications such as enhanced tumor cell destruction due to that the photosensitizer is well-distributed throughout the tumor tissue, it increases the likelihood of more cancer cells being exposed to the therapeutic effects of photodynamic therapy. This can lead to a more comprehensive and efficient destruction of tumor cells, potentially improving treatment efficacy [18]. Furthermore, there is an increased treatment response due to the improved distribution of the photosensitizer which allows more uniform and thorough exposure of tumor cells to the activated photosensitizer and subsequent reactive oxygen species (ROS) production. This can enhance the treatment response and increase the likelihood of a favorable therapeutic outcome. The better coverage of
tumor heterogeneity was achieved as tumors often exhibit heterogeneity, with different regions having varying levels of oxygenation, vascularity, and cellular characteristics. By achieving an improved distribution of the photosensitizer, it becomes possible to target a larger portion of the tumor, including regions that may be more resistant to treatment. This can address the challenge of tumor heterogeneity and increase the chances of eradicating resistant or suboptimal regions. In addition, uneven distribution of the photosensitizer within the tumor can lead to untreated regions or "cold spots." These untreated areas may contribute to tumor recurrence or incomplete treatment response. By achieving a more uniform distribution, the likelihood of leaving untreated regions is reduced, minimizing potential treatment gaps and improving overall therapeutic coverage. Reduced potential for tumor regrowth and metastasis: Inadequate distribution of the photosensitizer may leave behind viable tumor cells, which can contribute to tumor regrowth or metastasis. Improved distribution increases the chances of effectively targeting and eliminating these cells, reducing the risk of tumor recurrence or the spread of cancer to other parts of the body. When Hp-HCl is dissolved in glycerin, several factors contribute to the enhanced photochemical effect such as the increased Di-cationic Species like Glycerin, being a polar solvent, forms hydrogen bonding with Hp-HCl. This interaction increases the number of di-cationic species of Hp-HCl. Di-cationic species are highly reactive and have a higher quantum yield for ROS generation. As a result, the presence of glycerin promotes the production of more free radicals, enhancing the photochemical effect of Hp-HCl. Furthermore, the enhanced solubility and diffusion by using glycerin which acts as a solubilizing agent, improving the solubility of Hp-HCl in the gel formulation. This increased solubility facilitates better diffusion of Hp-HCl through biological tissues and cellular membranes. Consequently, the drug can reach a larger area within the tumor, increasing its exposure to the activated light and enhancing the therapeutic effect [20]. The viscosity and molecular aggregation were affected in presence of glycerin which has a high viscosity, it prevents the molecular rotation and aggregation of Hp-HCl. Molecular rotation and aggregation can hinder the effective interaction of the photosensitizer with light and reduce its efficacy. By maintaining a higher viscosity, glycerin promotes the optimal orientation and dispersion of Hp-HCl molecules, maximizing their photosensitization capabilities [21]. Overall, the presence of glycerin in the gel formulation of Hp-HCl provides multiple benefits for the photochemical effect of the photosensitizer. It increases the production of reactive species, enhances solubility and diffusion, and improves the orientation and dispersion of Hp-HCl molecules. These factors collectively contribute to the improved therapeutic efficiency of Hp-HCl when dissolved in glycerin.

V. CONCLUSION

The dissolution of Hp-HCl in glycerine not only increased the drug release rate from the gel formula but also improved its distribution through animal tissues and enhanced therapeutic efficiency. These findings were supported by histopathological results. Overall, the topical use of Hp-HCl dissolved in glycerine showed significant improvement in its efficacy as a photosensitizer. It's important to note that while the information provided describes the experimental procedures, results, and conclusions, it lacks specific numerical data, such as the exact values of tumor sizes or statistical significance levels. This study outlines the potential of topical Hp-HCl-based PDT as an effective and minimally invasive treatment modality for various types of cancers. The findings of this study may have important clinical implications, as they can contribute to the development of more targeted and personalized approaches in cancer therapy. In summary, the improved distribution of the photosensitizer in photodynamic therapy can lead to enhanced tumor cell destruction, increased treatment response, better coverage of tumor heterogeneity, minimized treatment gaps, and reduced potential for tumor regrowth and metastasis. These implications highlight the importance of optimizing the

distribution of the photosensitizer to maximize the therapeutic benefits of photodynamic therapy.

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