Chem Biol Drug Des 2011

Research Letter

doi: 10.1111/j.1747-0285.2011.01105.x

Ethosuximide and Phenobarbital Promote Wound Healing via Enhancing Collagenization

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The fact that ethosuximide (ETO), phenobarbital (PHO), and barbituric acid (BARB) share structural and pharmacophoric homologies with phenytoin and allantoin, both known to have significant wound-healing properties, prompted us to evaluate them as wound-healing agents. Accordingly, ETO-, PHO-, and BARB-containing ointments were applied onto full-thickness excision and incision wounds created on the dorso-lumbar region of experimental rats. ETO-and PHO-treated incision wounds illustrated significant enhancement in breaking strengths (1380 ± 61 and 1240 ± 42 g, respectively) compared to vehicle controls (1070 ± 18 g) and BARB (1080 ± 45 g). Moreover, biochemical analyses revealed significant increase in hydroxyproline contents in ETO- and PHO-treated wounds compared to vehicle controls. Histological evaluation revealed that both ETO and PHO promoted collagen synthesis and deposition. This is the first time to describe the significant wound-healing merits of ETO and PHO as potential clinical agents for treatment of chronic wounds.

Key words: collagenization, ethosuximide, excision wound, hydroxyproline, incision wound, phenobarbital, tensile strength

Received 20 November 2009, revised 5 July 2010 and accepted for publication 13 February 2011

Wound healing starts immediately after injury and proceeds via complicated but well-organized sequence of interactions among various types of tissues and cells. Skin wound healing is composed of inflammatory, proliferative, and maturation phases, respectively. In the inflammatory phase, the recruitment of leukocytes such as neutrophils and macrophages into the wound site is the hallmark. In the proliferative phase, the migration and proliferation of keratinocytes, fibroblasts, and endothelial cells result in re-epithelialization and tissue granulation. In the maturation phase, collagen fibers are cross-linked and strengthened, while excess collagen in the wound site is degraded by several proteolytic enzymes leading to the completion of tissue repair (1–3). Hard-to-heal wounds constitute very common medical problem and present a staggering economic burden (4–6).

Phenytoin and allantoin (Figure 1) have been reported to promote wound healing (7–10). The fact that both compounds share a hydantoin core suggests that this scaffold is the pharmacophoric motif responsible for their wound-healing qualities (Figures 1 and 2).

Because hydantoin group exhibits alternating hydrogen bond acceptor/donor features, we were prompted to evaluate the wound-healing potential of compounds illustrating similar alternating pharmacophoric patterns. We decided to select phenobarbital (PHO), ethosuximide (ETO), and barbituric acid (BARB) (Figure 1) because of their established safety profiles (11,12). In fact, PHO and ETO are among the oldest and most widely used anticonvulsants worldwide (13,14), while BARB is the synthetic precursor of all barbiturate anticonvulsants. Cleary from Figure 3, ETO misses a hydrogen bond donor. Nevertheless, it shares, together with PHO and BARB, the planarity and the remaining alternating hydrogen bond donor/acceptor patterns of the hydantoin scaffold (Figures 2

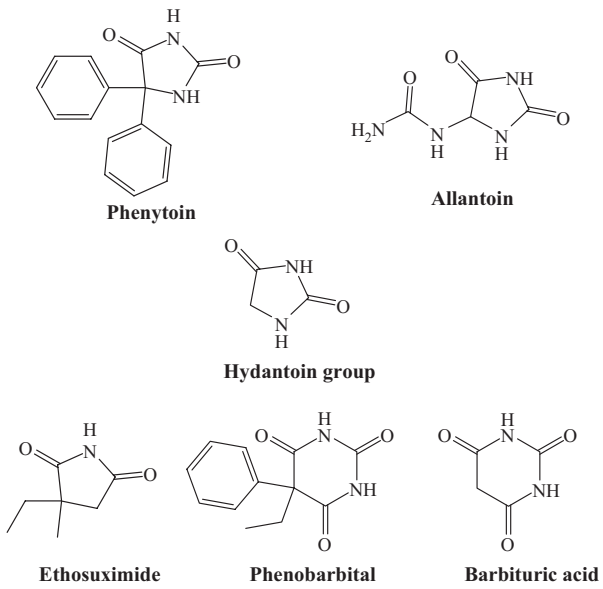

Figure 1: Chemical structures of phenytoin, allantoin, ethosuximide, phenobarbital, and the hydantoin scaffold.

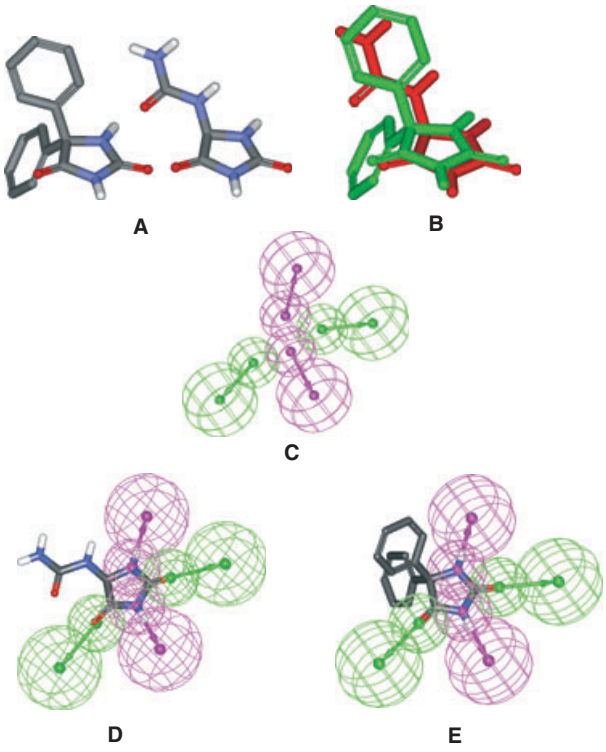

Figure 2: (A) The three-dimensional structures of phenytoin and allantoin, (B) alignment of the two structures (phenytoin in green, allantoin in red), (C) their common pharmacophoric pattern (hydrogen bond donors in red, hydrogen bond acceptors in green), (D) and (E) fitting allantoin and phenytoin, respectively, against their common pharmacophoric pattern.

and 3). Up to our best knowledge, PHO, ETO, and BARB have never been studied before as topical treatments for wounds.

In this study, we evaluated the healing potential of ETO and PHO on full-thickness excision and incision wounds created on the dorso-lumbar region of *Rattus norvegicus* UJ-1 rats. This was performed by assessing the rate of wound contraction, collagenization, as reflected by hydroxyproline content of the wound, healed wound tensile strength, and histology of the healing wound (1–4). BARB was excluded from excision wound experiments based on the lack of any potential in the incision wound model. The novelty of this work resides in discovering new healing promoters and specifying the pharmacophoric moieties responsible for wound-healing activity.

Materials and Methods

Molecular manipulation and visualization

Chemical structure drawing was performed employing ChemDraw Ultra 6.0 (Cambridge Soft Corp., Cambridge, MA, USA, http://www.cambridgesoft.com) installed on a Pentium 4 PC. Manual molecular modeling and manipulations were performed using Discovery Studio (Version 2.1; Accelrys Inc., San Diego, CA, USA, http://www.accelrys.com) installed on a Pentium 4 PC.

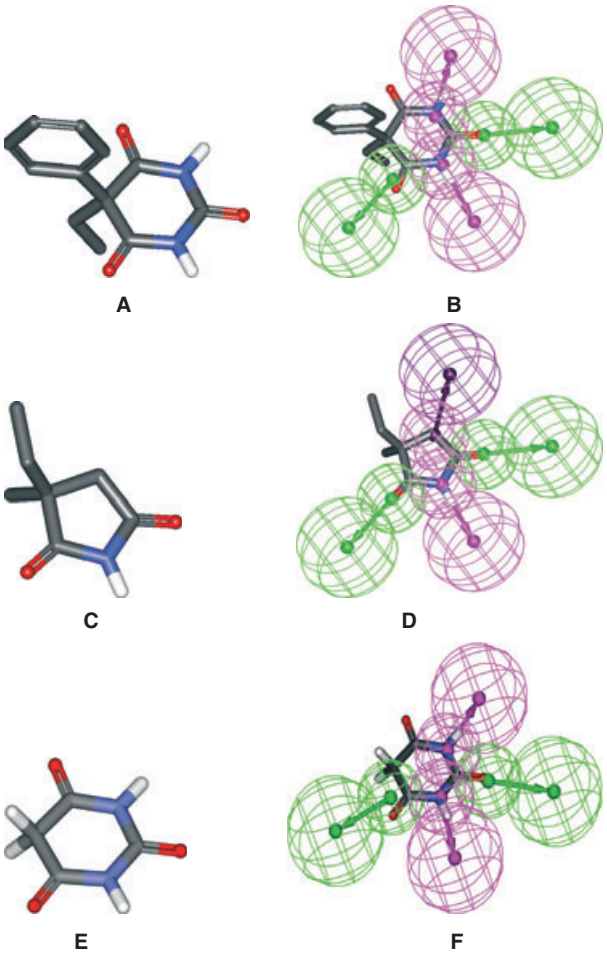

Figure 3: (A), (C) and (E) The three-dimensional structures of PHO, ETO and BARB, respectively, (B), (D) and (F) fitting PHO, ETO and BARB, respectively, against the pharmacophoric features deduced from phenytoin and allantoin (hydrogen-bond donors in red, hydrogen-bond acceptors in green, as in Figure 2).

Preparation and application of ointments

Drug-containing ointments were prepared by compounding ETO (10% w/w), PHO (1% w/w), or BARB (10% w/w) with soft paraffin. The resulting ointments were applied once daily as a thin film of *ca.* 100 mg per rat or *ca.* 150 mg per rat onto the incision or excision wounds, respectively, over 6 days after injury. The concentrations and applied amounts were chosen such as to deliver no more than 10% of the reported oral LD50 values (11,12).

Animals

The experiments were conducted employing albino rats (*Rattus norvegicus* UJ-1) weighing 140–180 g. A total of 114 rats were used, 24 for incision and 90 for excision wounds. Incision wounds were induced in male rats bred in the Animal House at the Applied Sciences University, while excision wounds were induced in rats of both sexes bred in the Department of Biological Sciences at the University of Jordan. Rats were bred in con-

ventional conditions of temperature and humidity and were provided with feed and water *ad libitum* during the course of the experiments. The procedures involving animals and their care conformed to the international guidelines, Principles of Laboratory Animals Care (24).

Incision wound model

Animals were anesthetized with intraperitoneal injection of 3.5% chloralhydrate (0.35 mg⁄g body weight). The dorsal backs of the rats were shaved and disinfected with ethanol. Then, 3-cm linear incisions (vertically from head to tail direction) were made to the depth of the subcutaneous tissue of dorso-lumbar region using a blade. The two edges of the wound were then kept close together with a single suture in the middle of the wound using a surgical thread. The rats were distributed randomly to treatment and control groups, and each rat was placed in a separate cage. The sutures were removed on the 8th day postwounding. On day 10 after incision, the tensile strength of healing incision wounds was determined for treated and control groups (six rats each). In brief, the rats were killed, and rectangular sections (a length of 3.0 cm and a width of 4.0 cm each) of the skin, including the healing incision wounds, were excised. The tensile strengths of these sections were measured using a tensiometer designed according to the method of Vaisberg *et al.* (15). In brief, one edge of the rectangle parallel to the wound was fixed, while applying incremental loads to the other edge (separated from the first edge by 2.0 cm). The tensile force was applied in such a way to be perpendicular to the wound. The tensile strength was then taken to be the load in grams required to disrupt the wound and was determined for four experimental groups: ETO-, PHO-, BARB-, and vehicle-treated incision wounds.

Excision wound model

Rats were anesthetized and shaved in the same way as for incision wounds. Then, a full-thickness 4-cm^2 rectangular excision wound was created in the dorso-lumbar region of each rat to the depth of the subcutaneous tissue. The wounds were left undressed, and no local or systemic antimicrobial agent was used. The rats were distributed randomly to treatment and control groups, and each rat was placed in a separate cage. For each of the ETO-, PHO- and vehicle-treated groups, 30 rats with excision wounds were used, where six rats from each group were killed at 4-day intervals up to 20 days of wounding to determine wound contraction rate, hydroxyproline content, and histological improvement. Barbituric acid was ruled out based on the results of the incision wound experiments and so was not involved in any further investigation.

Determination of wound contraction rate

Excision wound margins were traced on transparent paper on day zero and on days 4, 8, 12, 16, and 20 postwounding, and the wound areas were then measured using a caliper. The wound contraction rate was then calculated as percentile reduction in wound area compared to day zero, as in the following formula:

$$\text{Rate of wound contraction} = \frac{\text{Area at day 0} - \text{Unhealed wound area}}{\text{Area at day 0}} \times 100\%$$

Determination of hydroxyproline content in excision wounds

The wet granulation tissues taken on days 4, 8, 12, 16, and 20 postwounding were digested using 6 N HCl over 3 h at 130 °C, and the content of hydroxyproline in the hydrolyzate was then determined using the method of Woessner (16).

Histological analysis

Three excision-wound model rats were treated with ETO, PHO, or vehicle for 6 days and killed on the 16th day postwounding. Subsequently, five-micrometer-thick sections were prepared from their granulation tissues and adjacent normal tissues for histological evaluation. The sections were stained with Masson's trichrome for the assessment of collagen content and maturation.

Statistical analysis

One-way ANOVA analysis was applied to test the significance of differences between the results of ETO-treated, PHO-treated (and BARB-treated in tensile strength experiment), and soft paraffin-treated (negative control) groups. Dunnett's test was used to determine which means differ significantly from negative control group. The difference was considered significant at the conventional level of significance ($p < 0.05$).

Results

Table 1 compares the breaking strength of the four experimental groups on the 10th day postwounding. ETO and PHO significantly improved tensile strength of incision wound compared with vehicle-treated rats. BARB did not show such an improvement and was, therefore, excluded from further investigations. We believe the ionization of BARB ($pK_a = 3.9$) under physiological pH (17) renders the compound too hydrophilic to penetrate cellular membranes to promote wound healing.

Table 1: Breaking strength of experimental groups on the 10th day postwounding

Groups	Breaking strength (g)
Phenobarbital ointment	1240 ± 42*
Ethosuximde ointment	1380 ± 61*
Barbituric acid ointment	1080 ± 45
Vehicle	1070 ± 18

Values are mean ± SE of six animals in each group on the 10th day postwounding.
*$p < 0.05$ as compared to the vehicle group.

Table 2: Rate of contraction of excision wound (%) of experimental groups on the indicated days

	Rates of wound contraction (%)				
Groups	Day 4	Day 8	Day 12	Day 16	Day 20
Phenobarbital ointment	13.2 ± 1.3*	50.1 ± 3.6	85.6 ± 1.9	92.5 ± 1.9	96.6 ± 1.1
Ethosuximide ointment	10.9 ± 2.0	53.6 ± 2.8	84.9 ± 0.6	91.7 ± 1.5	95.6 ± 0.7
Vehicle	7.30 ± 2.1	48.3 ± 6.4	85.0 ± 2.0	91.9 ± 0.94	96.3 ± 0.7

Values are mean ± SEM (n = 6).
*$p < 0.05$ as compared to the vehicle group.

As for excision wounds, neither ETO nor PHO affected wound contraction rate (Table 2). However, both treatments improved hydroxyproline content in all experimental days (Figure 4), albeit only ETO yielded statistically significant improvements. Furthermore, both ETO and PHO increased collagenization of healing excision wounds. In Figure 5, we compare Masson's trichrome-stained sample sections of both treatments and vehicle at day 16 postwounding. Not only was the intensity of collagen fibers increased compared with the vehicle-treated wounds, but the arrangement was obviously more organized into bundles as well. Collagenization in different slides was scored from 1 to 4 by a blinded observer and average score for each treatment calculated. The average scores (± standard deviation) for ETO, PHO, and vehicle on the indicated day were 2.42 (±0.58), 2.62 (±0.53), and 1.88 (±0.53), respectively, suggesting ETO and PHO significantly promoted collagenization compared to vehicle control, as measured by Dunnett's test.

Discussion

The strength of healing skin incisions in the first several weeks postwounding depends upon the amount of new collagen present in the wound and the degree of its cross-linking (18–22). Breaking collagen liberates free hydroxyproline and its peptides. Measurement of wound hydroxyproline, therefore, has been used as an index of collagen turnover and wound healing (16,18–21). According to the results of hydroxyproline content and histological assessment, the increase in breaking strength of ETO- and PHO-treated incision wounds is mainly because of the increase in collagen concentration and stabilization of its fibers. Therefore, the results suggest that treatment with ETO or PHO ointments have beneficial influence on incision wound-healing process.

On the other hand, our results showed that excision wound contraction rates for ETO and PHO were not significantly different from vehicle-treated controls. Although it is known that wound contrac-

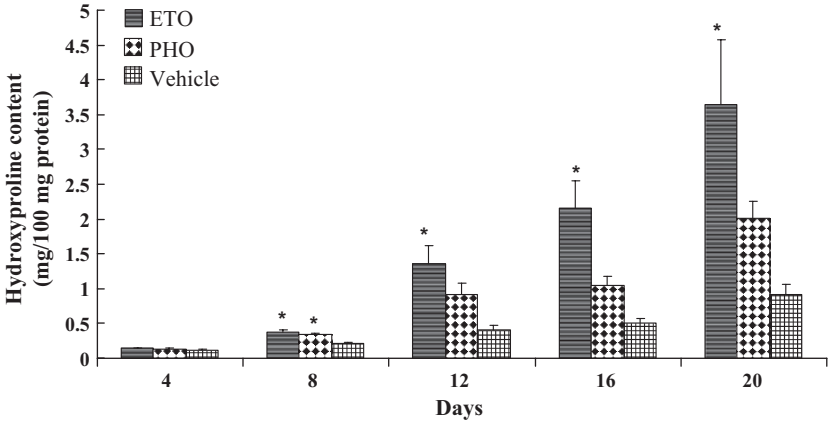

Figure 4: Hydroxyproline content of ETO-, PHO-, and vehicle-treated groups on the specified days postwounding. Values are mean ± SEM (n = 6). *$p < 0.05$ as compared to the vehicle group.

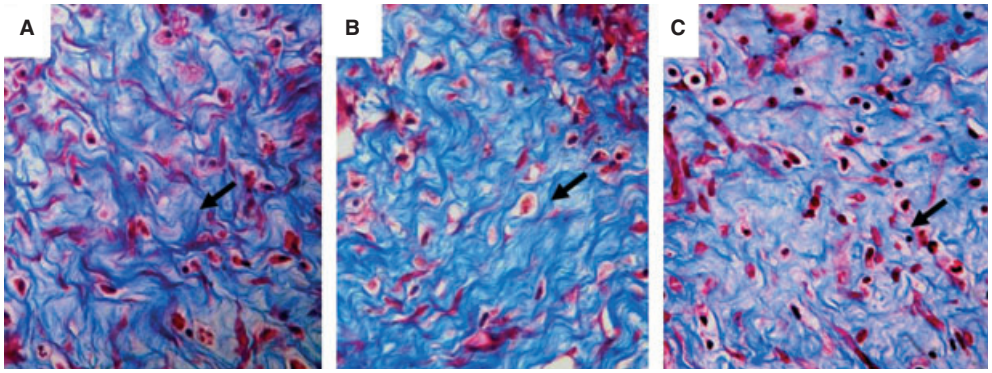

Figure 5: Photomicrographs of sections stained with Masson's trichrome of the wounds taken 16 days postwounding. (A) Ethosuximide-treated wound, (B) Phenobarbital-treated wound, (C) Vehicle-treated wound. Arrows point at sample collagen fibers. (Magnification: 900×).

tion begins almost concurrently with collagen synthesis (18), it is also known that gross wound contraction does not seem to depend on collagen synthesis (20). In fact, the rate of contraction might even be inversely proportional to lattice collagen concentration (22).

Qualitative histological assessment suggested noticeable decrease in the number of fibroblast cells in PHO- and ETO-treated wounds on the 12th and 16th days compared with vehicle group. Because greater number of fibroblasts in the granulation tissue is indicative of immature healing process (22), one can assume that ETO and PHO sped up the initial events in wound healing, thus advancing the time frame for fibroblastic proliferation, collagen formation, and collagen maturation.

To further establish the wound-healing potential of the two compounds, we compared hydroxyproline content and tensile strength of ETO- and PHO-treated wounds with those previously published for phenytoin-treated wounds (7). Apparently, there was no statistical difference in these criteria, suggesting comparable wound-healing potential for the three compounds.

Chemical and three-dimensional similarities between phenytoin, ETO, and PHO suggest common prohealing mechanism, probably by accelerating the autocrine and paracrine activity of growth factors within fibroblasts through upregulation of the related receptors (23). However, further work is required to pinpoint the exact prohealing mechanism of ETO and PHO, particularly because phenytoin was reported to modify the expression of about 1500 different genes in fibroblasts (23).

Conclusion and future directions

This study demonstrates significant wound-healing efficacy for ETO and PHO that may be attributed to their ability to enhance collagen synthesis and deposition. In addition, the study highlights the structural modality that may be responsible for these activities. It is very difficult to comment on the exact locations and mechanisms of the prohealing actions of these drugs applied topically. Therefore, better understanding of ETO and PHO mechanisms of actions at the molecular level is required to elucidate exactly their effects on the wound-healing cascade of events. Such events can be probably studied by analyzing the *in vitro* effects of ETO and PHO on cultured fibroblasts.

Acknowledgments

This work was supported by a grant from the Deanships of Academic Research at the University of Jordan and Applied Sciences University, Jordan. The authors are thankful to Miss Haifa Hattab, Miss Amal Majdalawi, and Mr. Salem Shawabkeh for their technical help. The authors thank Hamdi-Mango Center for Scientific Research at the University of Jordan for their generous funds.

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