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Research Article

Before or after mild acute heat exposure – does it matter for the therapy?

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Abstract

Adaptation to elevated temperatures poses a challenge that involves large-scale metabolic changes. It also affects the pharmacokinetics and pharmacodynamics of exogenous substances. Our experiments clearly showed differences in paw pressure thresholds in rats intraperitoneally injected with TDIFELLK—a C-terminal fragment of the calcium-binding, spermatid-specific protein 1—before and after exposure to 38.5 ± 0.5°C, compared to administration at 22 ± 2°C. Similarly, the analgesic effect of the substance, following antagonism of key analgesic receptors—namely opioid, cannabinoid, and serotonergic—was differentially influenced by heat exposure at normal temperature. The administration of the substance before heat exposure exerted a stronger effect than administration after heat exposure.

Keywords

heat stress, paw pressure test, pharmacodynamics, pharmacokinetics, TDIFELLK

Introduction

The frequency and severity of extreme heat events are increasing worldwide (Mu et al. 2022). According to the World Meteorological Organization, unprecedentedly high temperatures were recorded in the summer of 2023 (Hanfei Wang et al. 2024). Subjecting a living organism—regardless of biological species—to conditions drastically different from the usual, i.e., stressogenic, leads to two main effects:

1. the activation of adaptation processes aimed at preserving the organism's viability, and
2. the initiation of compensatory mechanisms aimed at restoring homeostasis.

Many exogenous environmental factors of different natures—such as physical, chemical, and biological—can be stressful, and for humans, psycho-emotional factors also play an important role. In humans, the stress reaction includes activation of the hypothalamic-pituitary-adrenal axis, which affects the function of vital body systems (nervous, endocrine, cardiovascular, respiratory, and immune), with indirect effects on the digestive and reproductive systems (DeMorrow 2018; Micale and Drago 2018). The complex response required to cope with stress involves readjustment of the body's metabolism, which can have serious consequences for overall health. Prolonged exposure to high temperatures may trigger physiological mechanisms such as ischemia, inflammatory

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responses, and rhabdomyolysis; many vital organs, including the brain, heart, and liver, may be severely affected (Wang et al. 2024).

The problem of global warming is becoming increasingly relevant. It encompasses numerous aspects, affecting many facets of human life—from the environmental climate and its effects on agriculture, animal husbandry, and energy to the socio-economic consequences for humanity (Shahzad 2015) and, ultimately, human health. At the same time, exposure to high temperatures presents a constant risk in various industries, including agriculture, construction, and transportation, and may be considered a specific stressogenic factor in occupational medicine (Varghesea et al. 2019; Fatima et al. 2021). Various circumstances may necessitate the administration of medications under elevated temperature conditions—for therapeutic, prophylactic, or supplementary purposes. Given the metabolic changes that accompany the stress response, it is possible that the pharmacokinetics (PhK) and/or pharmacodynamics (PhD) of drugs may be altered, potentially compromising their efficacy and/or potentiating adverse side effects.

This research aims to demonstrate the altered effect of an exogenously introduced substance depending on whether it is administered under normal (22 ± 2°C) or elevated (38.5 ± 0.5°C) temperature conditions. We also hypothesized that the timing of administration matters: when administered before heat exposure, the substance's PhK and PhD would be influenced by the pathogenetic processes involved in the body's stress response. In contrast, administration after the establishment of allostasis following heat exposure would affect the PhK and PhD differently.

The substance chosen for introduction into the model animal group fulfilled two criteria: (1) it produced an effect that could be relatively easily quantified (analgesic), and (2) it resembled naturally occurring molecular sequences in the body, ensuring the existence of well-conditioned pathways for its metabolism. Additionally, the substance has been the subject of various studies due to its biologically significant properties. The protein was first described by Jiahao et al. (2000), and it was later discovered that the molecule binds calcium during maturation in the epididymis (Akihiro et al. 2009). This target molecule was subsequently named calcium-binding protein spermatid-specific 1 (CABS1). Further studies showed that CABS1 is also present in saliva and includes a C-terminal heptapeptide (TDIFELL) and an octapeptide (TDIFELLK), which is analogous to the TDIFEGG sequence in submandibular rat protein 1 (St. Laurent et al. 2015; Ritz et al. 2017). Diversity of biological activities has been attributed to this sequence, including inhibition of inflammatory responses (Dery et al. 2004; Lane et al. 2004; Mathison et al. 2010), effects on erectile function (User et al. 2003; Messaoudi et al. 2004; Tong et al. 2006), modulation of tissue mineral balance (Rougeot et al. 1997), and analgesic activity (Rougeot et al. 2003). The latter activity supported the selection of the more active octapeptide sequence, TDIFELLK, for this study.

Thecurrent study comprises four parts: 1) determining the effect of TDIFELLK under normal temperature conditions (21 ± 3°C); 2) assessing potential changes in its analgesic effect under heat exposure (38.5 ± 0.5°C); 3) evaluating whether administration before or after heat exposure influences the analgesic effect differently; and finally 4) examining whether interaction with analgesia-related mediator systems—opioid, cannabinoid, and serotonergic (Kirkpatrick et al. 2016)—is affected by heat exposure. The study was based on the working hypothesis that heat exposure alters the effect of TDIFELLK compared to administration at normal temperature. Furthermore, we expected variations in its effect depending on the mediator systems involved and the timing of administration relative to the heat exposure.

Materials and methods

Animals

The experiments were carried out on male Wistar rats (180–200 g) obtained from the vivarium of the Medical University – Sofia. The animals were group-housed in polypropylene cages (40 × 60 × 20 cm) in a temperature-controlled colony room maintained at 21 ± 3°C under a 12 h light:12 h dark cycle, with lights on at 08:00 a.m. Water and standard rat chow were provided *ad libitum*. The experiments were conducted between 09:00 a.m. and 12:00 a.m.

After one week of acclimatization, rats were randomly assigned to different groups (6–8 rats per group): a control group and several experimental groups, as detailed in Table 1.

All procedures were approved by the Animal Care and Use Committee of the Medical University of Sofia. Permission was also granted by the Bulgarian Food Safety Agency (BAFS) (No. 353/14.06.23).

Acute heat exposure (HE) protocol

Rats in the experimental groups were transported in their home cages—with food, water, and bedding—into a temperature-controlled chamber and exposed to 38.5 ± 0.5°C for 1 h. Rats in the control group remained at room temperature during this period.

Drugs and treatment

The CB1 agonist N-arachidonoyl-ethanolamine (AEA, 1 mg/kg BW), CB1 antagonist N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251, 1.25 mg/kg BW), naloxone (Nal, 1 mg/kg BW), and the 5HT1A antagonist NAN-190 hydrobromide (NAN, 1 mg/kg BW) were dissolved in vehicle (Solinas et al. 2006; da Veiga et al. 2008) and intraperitoneally administered in different combinations before or after heat exposure, as described in Table 1.

Table 1. Treatments of animals from the different experimental groups.

№	Group	Treatment
1	Control	This group was not exposed to heat and was treated with saline.
2	TDIFELLK	Animals in the group were injected intraperitoneally (i.p.) with TDIFELLK (2 mg/kg).
3	HE	Animals in the group were exposed to heat (38.5 ± 0.5°C) for 1 hour (1 h).
4	TDIFELLK+HE	Animals in the group were injected i.p. with TDIFELLK (2 mg/kg), after which they were exposed to 1 h HE.
5	HE+TDIFELLK	Animals in the group were exposed to 1 h HE, after which they were injected i.p. with TDIFELLK (2 mg/kg).
6	Nal+TDIFELLK	Animals in the group were injected i.p. with the combination of naloxone (Nal, opioid receptor antagonist; 1 mg/kg) and TDIFELLK (2 mg/kg).
7	Nal+TDIFELLK+HE	Animals in the group were injected i.p. with the combination Nal (1 mg/kg) + TDIFELLK (2 mg/kg), after which they were exposed to 1 h HE.
8	HE+Nal+TDIFELLK	Animals in the group were exposed to 1 h HE, after which they were injected i.p. with the combination Nal (1 mg/kg) + TDIFELLK (2 mg/kg).
9	AM+TDIFELLK	Animals in the group were injected i.p. with the combination AM251 (AM, cannabinoid receptor type 1 antagonist; 1.25 mg/kg) + TDIFELLK (2 mg/kg).
10	AM+TDIFELLK+HE	Animals in the group were injected i.p. with the combination AM (1.25 mg/kg) + TDIFELLK (2 mg/kg), after which they were exposed to 1 h HE.
11	HE+AM+TDIFELLK	Animals in the group were exposed to 1 h HE, after which they were injected i.p. with the combination AM (1.25 мг/кг) + TDIFELLK (2 mg/kg).
12	NAN+TDIFELLK	Animals in the group were injected i.p. with the combination NAN-190 (NAN, 5HT1A-receptor antagonist; 1 mg/kg) + TDIFELLK (2 mg/kg).
13	NAN+TDIFELLK+HE	Animals in the group were injected i.p. with the combination NAN (1 mg/kg) + TDIFELLK (2 mg/kg), after which they were exposed to 1 h HE.
14	HE+NAN+TDIFELLK	Animals in the group were exposed to 1 h HE, after which they were injected i.p. with the combination NAN (1 mg/kg) + TDIFELLK (2 mg/kg).

Peptide moiety synthesis

The targeted molecule TDIFELLK, a C-terminal moiety of CABS1, was synthesized using solid-phase peptide synthesis via the Fmoc/Ot-Bu approach. Briefly, the peptide was synthesized on 2-chlorotrityl chloride resin using diisopropylcarbodiimide (Iris Biotech, Wunsiedel, Germany) as a coupling reagent by sequentially linking protected amino acids from the C- to N-terminus. The final peptide was deprotected and cleaved from the resin by treatment with a 50% TFA / 50% dH_2O:TIS (97.5:2.5 equiv.) mixture for 4 h. The raw TFA solution was precipitated using cold diethyl ether. TDIFELLK was obtained with a 63% yield; m.p. 213–215°C, $[\alpha]D^{20} = -15$ (c = 1, DMSO). Chromatographic purity of the peptide was 96%.

Pharmacokinetic study on the hydrolytic stability of TDIFELLK

To study the hydrolytic stability of TDIFELLK, three model systems simulating human stomach, blood plasma, and small intestine conditions were prepared following the 20th edition of the European Pharmacopoeia, with enzymatic modifications to mimic peptide hydrolysis.

Preparation of a model system with pH 2.0:

The system consists of two solutions

- Solution A preparation: In a volumetric flask, 119.0 mL of 0.1 mol/L HCl and 6.57 g of KCl in CO_2-free water were combined. The final solution was completed to 1000.0 mL with distilled water (dH_2O).
- Solution B preparation: A volumetric flask containing 5 mg of pepsin was filled to the brim with solution A. Consequently, the final model solution had a pH of 2.0 and a pepsin concentration of 0.5 mg/mL.

Preparation of a model system with pH 7.4:

In order to achieve a 0.1 mg/mL final trypsin concentration in the model system with pH 7.4, 0.1 g trypsin, 2.38 g Na_2HPO_4, 0.19 g KH_2PO_4, and 8.0 g NaCl were dissolved to a total of 1000.0 mL volume in dH_2O. One milliliter of blood plasma (ACCUCLOTM Reference Plasma, Normal, Sigma Diagnostics) was recovered using 15 mL of the pH 7.4 buffer that was obtained.

Preparation of a model system with pH 9.0:

In total, 420.0 mL of a solution containing 0.1 mol/L NaOH in dH_2O was combined with 1000 mL of a solution containing 6.18 g H_3BO_3 in 0.1 mol/L KCl in distilled water. To reach the final concentration of 0.1 mg/mL of trypsin, an additional 0.1 mg of trypsin was dissolved in the pH 9.0 solution and added to 10 mL in a volumetric flask.

Reversed-phase high-performance liquid chromatography on a Lichrospher RP-8 non-endcapped column with a pore size of 5 μm, i.d. 4.6 mm, and 150 mm length (Alltech, Lexington, KY, USA) and a Perkin-Elmer series 200 HPLC (Waltham, MA, USA) was used to monitor the pharmacokinetics expressed as hydrolytic stability of the targeted molecule. The detection was realized at 254 nm with a Perkin-Elmer series 200 detector (Waltham, MA, USA). The other parameters of the chromatographic system were as follows:

20 µL injection volume, temperature 37°C in order to mimic a human organism, and 0.70 mL/min flow rate. The gradient of the mobile phase was set at 0.0 min at 20% B, 10 min at 100% B, 10 to 13 min at 100% B, 13 to 14 min at 20% B, and 16.5 min at 20% B with mobile phases. A: Acetonitrile/HPLC water/TFA - 5:95:0.1 and B: Acetonitrile/HPLC water/TFA - 95:5:0.1.

Paw-pressure test (Randall-Selitto test)

Mechanical nociceptive thresholds were measured using an analgesimeter (Ugo Basile). Pressure was applied to the hind paw, and the weight (in arbitrary units, AU) required to elicit a nociceptive response (squeak or struggle) was recorded as the paw pressure threshold (PPT). A cut-off value of 500 g (25 AU) was used to prevent tissue damage (Randall and Selitto 1957).

Data analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test. Values are presented as mean ± SEM. A p-value < 0.05 was considered statistically significant.

Results and discussion

After administration at normal temperature, TDIFELLK (TDIFELLK group) showed an analgesic effect, with PPTs at 10 min exceeding those in rats exposed only to heat (HE group). The HE group exhibited high PPTs throughout the observation period, while PPTs in the TDIFELLK group declined, though analgesia persisted until 50 min (Fig. 1). Administration of TDIFELLK before heat exposure (TDIFELLK+HE group) reduced PPTs at 10 min compared to the TDIFELLK and HE groups. In contrast, TDIFELLK administration after heat exposure (HE+TDIFELLK group) had the opposite effect. At 20 and 30 min, PPTs in the TDIFELLK+HE and HE+TDIFELLK groups exceeded those of the TDIFELLK group, and the analgesic effect in these two groups was maintained until the end of the observation period (Fig. 1).

Opioid receptor antagonization at normal temperature prevented the onset of analgesia as early as the 10th min, with a trend towards hyperalgesia at the 20th min—this suggests that the analgesic effect of TDIFELLK is highly dependent on opioid receptors at normal temperature. Heat exposure reversed this relationship: administration of naloxone + TDIFELLK before HE showed analgesia that at the 10th min was superior to that in the TDIFELLK and HE groups. The administration of the naloxone + TDIFELLK combination after HE showed an analgesic effect curve similar to that at normal temperature, indicating that opioid receptors are more important for the analgesic effect after heat exposure than before it (Fig. 2A).

Differences were also observed in the interaction with cannabinoid receptors—their involvement in TDIFELLK's analgesic effect likely occurs at a later stage; therefore, at the 10th min, analgesia was observed in the animals from all three groups (AM+TDIFELLK, AM+TDIFELLK+HE, and HE+AM+TDIFELLK). Differences in PP thresholds, however, were noted later: decreasing PP thresholds in the AM+TDIFELLK group reached control values at the 30th min, then increased again, describing a U-shaped curve of the analgesic effect. The introduction of the AM+TDIFELLK combination before HE showed a sharp decrease in PP thresholds at the 20th min, and after the 30th min, they remained comparable to the control values. In the HE+AM+TDIFELLK group, PP thresholds decreased slowly at the 20th and 30th min, reaching control values at the 40th min. The administration of AM+TDIFELLK in combination with HE (whether before or after) did not show the U-shaped curve of the analgesic effect described with the exogenous administration of AM+TDIFELLK at normal temperature (Fig. 2B).

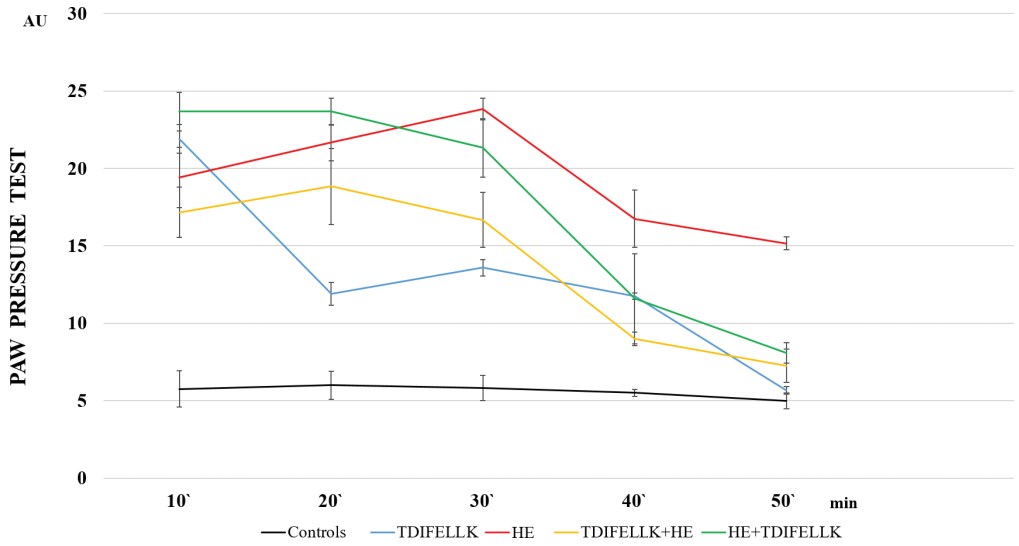

Figure 1. Effects of TDIFELLK administration without and with (before/after) 1 h of heat exposure (HE) estimated by the PP test in rats. Mean values ± S.E.M. are presented in arbitrary units (AU) at the 10th, 20th, 30th, 40th, and 50th min of the experiment.

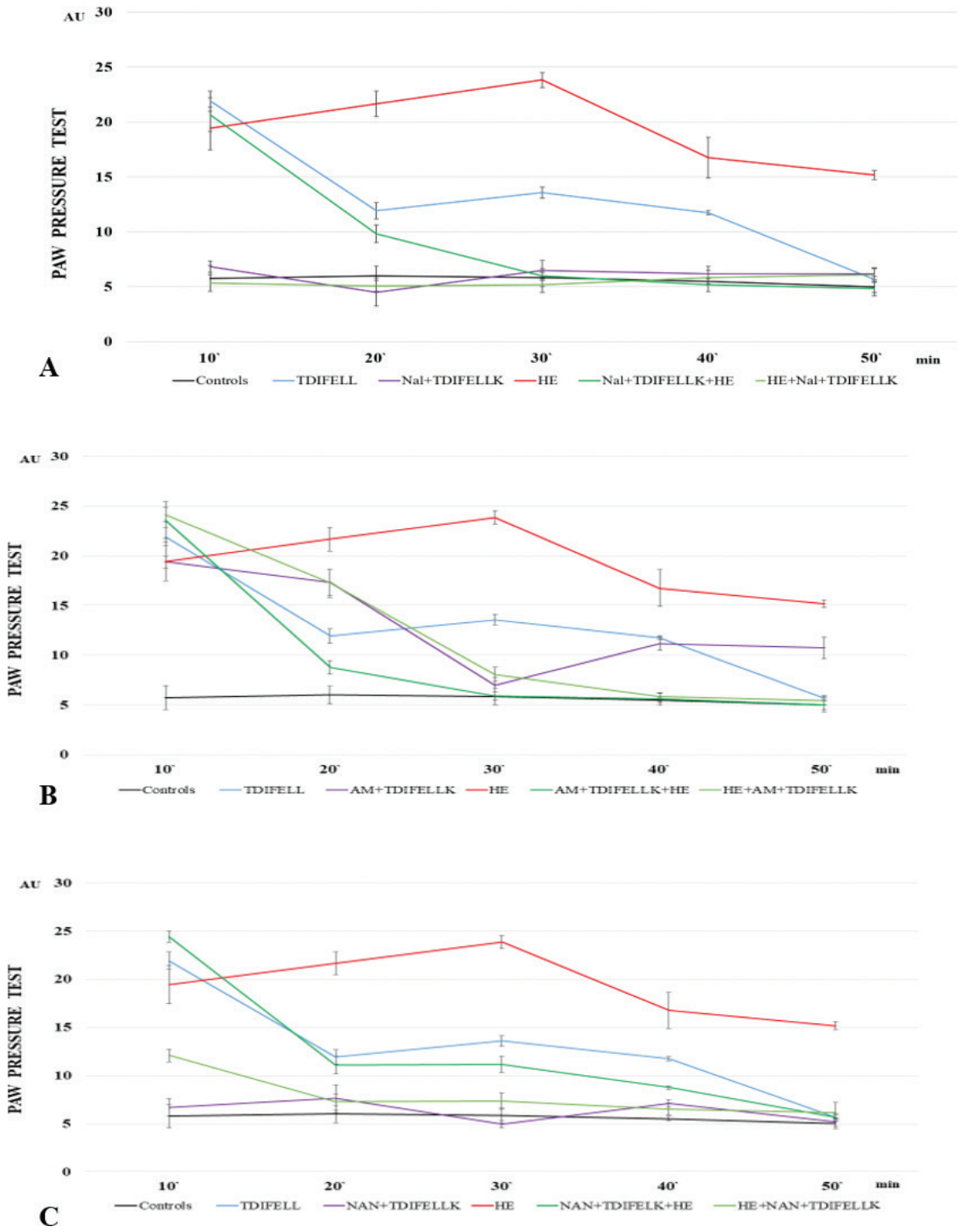

Figure 2. Effects of TDIFELLK administration without and with (before/after) 1 h of heat exposure (HE), estimated by the paw-pressure (PP) test in rats. Mean values ± S.E.M. are presented in arbitrary units (AU) at the 10th, 20th, 30th, 40th, and 50th minutes of the experiment.

Antagonism of serotonergic receptors with NAN-190 at normal temperature led to PP thresholds comparable to control values at the 10th and 50th minutes, while at the 20th and 40th minutes, a weak analgesic effect was reported, with a tendency towards hyperalgesia at the 30th minute. The introduction of the combination NAN+TDIFELLK under heat exposure conditions significantly changed the effect curve described above. At the 10th minute, PP thresholds in the NAN+TDIFELLK+HE group exceeded those of the TDIFELLK and HE groups; although followed by a significant decrease in the analgesic effect, the effect was maintained up to the 40th minute. Administration of NAN+TDIFELLK after HE also showed an analgesic effect, which, although more modest than that in the NAN+TDIFELLK+HE group, persisted until the end of the follow-up period (Fig. 2C). The observed differences in the effect of TDIFELLK administered alone at normal or higher than usual temperatures are likely due to changes in the

pharmacokinetics and/or pharmacodynamics of the substance. The effect of heat stress on the body has been of interest for a long time—one of the first known data points is from 1768 (Lind 1768). Studies performed later have primarily focused on occupational medicine (Buskirk 2003; Garrett et al. 2014; Nybo et al. 2014) or sports/military activity (Lorenzo et al. 1985; Buchheit et al. 2011; Racinais et al. 2014; Racinais et al. 2015). When taking into account adaptation changes, a number of additional factors should be considered: exogenous (temperature, humidity, solar radiation), endogenous (metabolic heat production, depending on specific physical work rate), and, last but not least, clothing (e.g., heavy clothing that impedes heat loss) (Périard et al. 2015; Périard et al. 2021). Acute heat exposure impairs aerobic exercise capacity via thermoregulatory-mediated cardiovascular adjustments, hyperthermia-induced skeletal muscle metabolism alterations, and central nervous system perturbations (Nybo et al. 2014). Immediately after exposure to higher than usual temperatures, an increase in core temperature is observed, along with accelerated cardiovascular and respiratory activity and cutaneous vasodilation, which stimulates sweating (Périard et al. 2016, 2021). These changes affect the percutaneous and intradermal administration of drugs. For example, more profuse sweating can cause dehydration with corresponding changes in blood flow, affecting drug distribution in different parts of the body. Similar changes in circulation can delay the substances reaching target organs and binding to receptors. In this regard, a delay in the onset of naloxone's antagonizing effect was observed when administered immediately before heat exposure, which may be the result of later receptor binding compared to administration at normal temperature. The delay could result from the aforementioned hemodynamic changes during the stress adaptation. An alternative explanation could be the different receptor expression (due to the generalized stress adaptation and its effect on body metabolism). Similarly, interaction with serotonergic receptors was probably delayed due to the overall effect of stress response circumstances on metabolism.

It is noteworthy that the effect of Naloxone+TDIF-ELLK, administered after heat exposure, does not differ from that at normal temperature. This finding could be attributed to the normalization of exchange processes when the animals are removed from the heat chamber, allowing the exogenously introduced substances to be metabolized under circumstances and pathways close to usual ones.

In contrast to skin vasodilatation, vasoconstriction is observed in the gastrointestinal tract, along with compromised tight junctions (Tellez et al. 2017), changes in the gut microbiota (Cao et al. 2021), and diarrhea, which can affect the oral intake of drugs (Elnesr and Abdel-Azim 2023).

In addition to pharmacokinetics, pharmacodynamics can also be affected by the general impact of heat adaptation on the body (Cramer et al. 2022).

The effect of high temperature itself may also be important—e.g., some degree of denaturation and conformational changes of receptors can be expected due to their protein nature. Some degree of partial denaturation or conformational shifts could alter receptors' ligand-binding affinity, ion channels' gating, or signaling efficiency (Masson et al. 2022). Heat is known to increase membrane fluidity, which could impact the mobility and clustering of membrane-bound receptors. As a result, rearrangements or disruption of receptor-ligand interactions and/or signal transduction are possible (Kurita et al. 2020).

Differences in the dependence of individual mediators on temperature changes are possible. For example, serotonin has high lipid membrane affinity, which is why a receptor-independent pathway is also possible for it (Dey et al. 2021). This highlights the importance of membrane fluidity.

At the same time, heat shock proteins released during temperature stress could have additional consequences, from refolding heat-damaged receptors to proteasomal degradation of irreversibly damaged receptors, as well as modulation of receptor trafficking and/or ligand binding (Hu et al. 2022).

Although the proposed study focuses more specifically on opioid, cannabinoid, and serotonergic receptors, it is important to remember that analgesia is a complex result of the activation of various receptors. Transient Receptor Potential channels are also known to be involved in antinociception and are characterized by direct activation by heat, which may also have influenced the documented results (Uchida 2024).

Changes in receptor expression, affinity, and reuptake, as well as the degradation of exogenous substances by the body's enzyme systems, should also be taken into consideration. Drugs' effects may also be influenced by differences in excretion rates (Vanakoski and Seppälä 1998).

A limitation of the publication could be the fact that the experiments were conducted between 9:00 a.m. and 12:00 a.m., considering that rats are nocturnal animals. It is known that chronobiology affects pain sensitivity through various mechanisms. For example, there are differences in receptor expression and activity, mediator production and deactivation, and overall body metabolism (Bumgarner et al. 2021; Guan et al. 2022; Yamakawa et al. 2024).

Pharmacokinetic studies express hydrolytic stability in three model systems that simulate different parts of the human body and show complete stability at three different pHs: 2.0, 7.4, and 9.0 for 24 h at 37°C (Fig. 3).

Conclusion

Environmental temperature plays an important role in regulating animal physiology and behavior. The presented data on altered organism-drug interactions under temperature stress conditions demonstrate that mild acute heat stress acts as a metabolic stressor, altering organismal metabolism (e.g., thermogenesis and energy metabolism) and affecting the pharmacokinetics (PhK) and pharmacodynamics (PhD) of administered substances. Consequently, this may influence both the therapeutic efficacy and side effects of drugs. The timing of drug administration

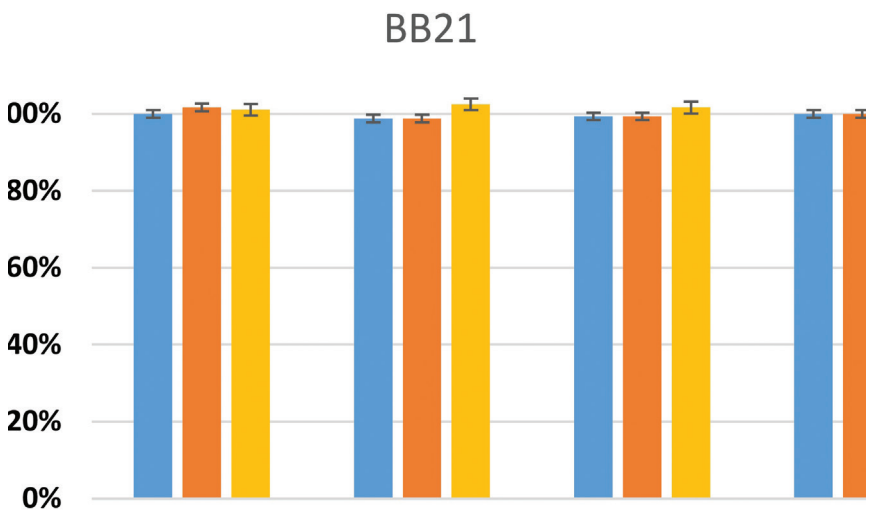

Figure 3. Kinetics of hydrolytic stability in three model systems of target molecule TDIFELLK.

appears to be crucial: application before heat exposure is of greater significance, as the metabolism of the exogenous substance is influenced by the pathogenetic mechanisms of the stress response. Investigating organism-drug interactions under heat stress is essential for optimizing the molecular design of newly synthesized compounds with potential therapeutic applications. It is also necessary to study changes in individual mediator systems during heat exposure, as they play a key role in determining the final pharmacological effect. This is particularly important for drugs targeting systems that are intensively involved in stress adaptation, including the cardiovascular, respiratory, nervous, and immune systems.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that no experiments on humans or human tissues were performed for the present study.

The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

Experiments on animals: All procedures have been approved by the Animal Care and Use Committee of the Medical University of Sofia, and a permission from BAFS has also been issued (№353/14.06.23).

The authors declared that no commercially available immortalised human and animal cell lines were used in the present study.

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Author contributions

Conceptualization, H.N. and D.D.; methodology, H.N., B.B., and R.T.; validation, B.B. and Y.S.; formal analysis, A.P., Y.S., and R.T.; data curation, D.D. and H.N.; writing—original draft preparation, H.N. and D.D.; writing—review and editing, R.T., D.D., and A.P.; visualization, A.P. and B.B.; supervision, D.D. and Y.S.; project administration, H.N. All authors have read and agreed to the published version of the manuscript.

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Data availability

All of the data that support the findings of this study are available in the main text.

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