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Survey and critical appraisal of pharmacological agents with potential thermo-modulatory properties in the context of artificially induced hypometabolism

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Survey and critical appraisal of pharmacological agents with potential thermo-modulatory properties in the context of artificially induced hypometabolism
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Dear Author,

After careful consideration, your ms can be eligible for publication in JCTR with major revision. In particular, both reviewers have expressed some concern on the choice to use only the degree of Tb cooling, without normalization for body mass or Ta. This issue needs to be addressed, either justifying the choice or producing at least one kind of normalization (body mass for instance).

Matteo Cerri M.D., Ph.D.

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40126 Bologna Italy

*****Reviewer comments*****

REVIEWER 1

This manuscript aims to identify a group of drugs with a high potential for induction of anapyrexia (downward shift of the thermoregulatory set point or thermoneutral zone, as stated by the authors). The authors collected their information from a series of reviews (Clark, W.G., Neurosci Biobehav Rev) to identify drugs that produce anapyrexia. To identify such substances, the authors tabulated the temperature body changes, compounds and species tested. This type of data organization allowed them to identify eight agents with a strong potential to lead to a large Tbody decrease.
Even if the manuscript were well written, and the idea for such a report was of interest, I have major concerns about the approach used for the identification of these agents. Moreover, the approach used by the authors does not give any information about the ability of the selected drugs to have an anapyretic effect (downward shift of the thermoregulatory set point), but instead, it simply gives a different graphical representation of the data from Clark, W.G. concerning the pharmacologically-evoked decreases in Tbody. Furthermore, the approach used by the authors is limited, and even less analytic than the compilation from Clark, W.G.

Major
As also mentioned by the authors, their analytical approach as strong limitations:
The number of animals in each report was not taken into account.
Tb was not corrected for the Ta
Differences in the dosage and administration route were not accounted for.
The manner in which experiments were performed was discounted.

On top of this, the authors didn't consider that the mass of the animal also contributes strongly to the thermal dispersion and the ability to lower the Tbody. In this regard, it is curious to notice that the group of drugs selected is, in fact, causing large Tbody changes in mice, but not in bigger species. Surely, if the authors normalize the changes in Tbody to the animal's mass, they will find a completely different result.
One of the drugs that the authors selected based on their calculation is acetaminophen. This is a COX1-2 inhibitor commonly used as an antipyretic. Even if cases of mild hypothermia have been reported, these occur only in patients in whom the thermoregulatory system is already activated, or functionally compromised, by bacterial and viral infections, and by clinical trauma, such as cancer or stroke [Samir S. A. et al., PNAS 2004]. Moreover this hypothermic effect has only been demonstrated in mice. Thus, it is unclear that this class of drugs would induce hypothermia in a normal subject. This is just one example of how important it is to consider the experimental basis used to obtain the data whose effects are then extrapolated in this review.
In the "limitation" section, the author reports that experiments with helium and DMSO were performed at an average Ta of 10.0 ± 13.3 °C and 12.5 ± 10.3 °C. These large standard errors for Ta emphasize the large Ta range to which different mice were exposed in these experiments. It is

not surprising that DMSO caused such a big change in Tbody in a small species like a mouse or rat when exposed to such a heavy cold thermal load.
None of the data reported provides any insight into potential changes in the thermoregulatory set point, indeed, if it was altered at all. The reported changes in Tbody could simply reflect a fall in Tbody that was due to inhibition of neurons in thermoregulatory centers in the brain or to effects on thermoeffector function. Indeed, many recent discoveries in the field of thermoregulation have not been assimilated into this report.
Overall, the weak analytical approach and the significant limitations, only some of which were mentioned by the authors, do not support the authors' conclusions, which could easily be misleading to the scientific community interested in the important topic of the pharmacological induction of hypothermia.

REVIEWER 2

Induction of Anapyrexia by Pharmacological Agents recaps the content of eight reviews of 18,808 reports describing drug-induced changes in thermoregulation by WG Clark published between 1979 to 1986. An innovative graphic illustrates the effects of specific drugs, grouped by classification, on the magnitude and direction of drug-induced changes in Tb. The author(s) define criteria for excluding thousands of reports and acknowledge several limitations of the method used to identify promising anyapyrexic agents through this review process. Figures 1 and 2 are highly effective graphics. Overall the review is a valuable resource to find information on the thermoregulatory consequence of a variety of agents.
A weakness of the manuscript is that it promises to identify the most promising agents in terms of anapyrexic potential based on the magnitude of drug-induced change in Tb, but in the end rules out all 8 compounds as viable candidates.
Major areas for improvements
Refocus the manuscript as a historical review of Clark's work or expand the review to include recent developments of drugs that target specific aspects of thermoregulation.
Be more specific about thermoneutral zone in terms of the neuroanatomic network that contributes to thermoregulation.
Include and defend criteria for characterizing a compound as affecting the thermoneutral zone.
Defend the assumption that the most "viable" candidate can be identified by magnitude of Tb change
Minor points for consideration
Include rationale for referring to hypoxia-induced anapyrexia as "regulated".

Authors' rebuttal:

Dr. Matteo Cerri Editor, Journal of Clinical and Translational Research
Re: Resubmission revised manuscript

Amsterdam, 7 July 2015

Dear Dr. Cerri,
Please find enclosed the resubmission of our manuscript entitled "Survey and critical appraisal of pharmacological agents with potential thermo-modulatory properties in the context of artificially

induced hypometabolism." We would like to thank the reviewers for critically reading the manuscript; they have raised several excellent issues. We have addressed the reviewers' comments point-by-point in red italics below and used the track changes function in the text to indicate the modifications.
Thank you for the opportunity to resubmit the manuscript.

Kindest regards,
Michal.

Complete rebuttal letter see pages at the bottom of this PDF.

2nd editorial decision:

Date: 10-Jul-2015

Date: 10-Jul-2015
Ref.: Ms. JCTR MC-220420015-1
Survey and critical appraisal of pharmacological agents with potential thermo-modulatory properties in the context of artificially induced hypometabolism
Journal of Clinical and Translational Research

Dear Author,

I'm pleased to inform you that your ms is now suitable to be published in JCTR.
The extend of your revision improved the overall quality of the ms, and the issues risen by the reviewers have been satisfactorily addressed. I will therefore not send the ms back to reviewers to be re-evaluated.

Minor observations:

in fig.3 legend "US government literature on laboratory animals.", please provide a reference (or delete the sentence).

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Dr. Matteo Cerri
Editor, *Journal of Clinical and Translational Research*

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Thank you for the opportunity to resubmit the manuscript.

Kindest regards,

Michal.

EDITOR

Dear Author,

after careful consideration, your ms can be eligible for publication in JCTR with major revision.
In particular, both reviewers have expressed some concern on the choice to use only the degree of Tb cooling, without normalization for body mass or Ta. This issue needs to be addressed, either justifying the choice or producing at least one kind of normalization (body mass for instance).

Dear editor,

We have addressed the reviewers' comments to the fullest possible extent and implemented changes in the manuscript accordingly.

Moreover, we have made several key modifications to the manuscript to improve the content in addition to the reviewers' comments. These include:

1) Numerous key references have been added to strengthen the premises of our arguments.
2) Mention of oher approaches to pharmacological hypothermia induction have been addressed, including the cutaneous cold receptor work by the Romanovsky Fever Lab.
3) An additional figure has been inserted containing the ΔT_b / body mass data, as requested by you and Reviewer 1.

REVIEWER 1

This manuscript aims to identify a group of drugs with a high potential for induction of anapyrexia (downward shift of the thermoregulatory set point or thermoneutral zone, as stated by the authors). The authors collected their information from a series of reviews (Clark, W.G., Neurosci Biobehav Rev) to identify drugs that produce anapyrexia. To identify such substances, the authors tabulated the temperature body changes, compounds and species tested. This type of data organization allowed them to identify eight agents with a strong potential to lead to a large Tbody decrease.
Even if the manuscript were well written, and the idea for such a report was of interest, I have major concerns about the approach used for the identification of these agents. Moreover, the approach used by the authors does not give any information about the ability of the selected drugs to have an anapyretic effect (downward shift of the thermoregulatory set point), but instead, it simply gives a different graphical representation of the data from Clark, W.G. concerning the pharmacologically-evoked decreases in Tbody.

Dear reviewer,

Many thanks for your useful comments, which we have attempted to implement in the paper to the fullest extent. We believe that your statements, and particularly your advice on normalization of ΔTb to body mass, have resulted in a considerably improved manuscript.

Indeed, one of the main aims of this review was to make the epic work of Dr. Clark accessible and comprehensible in a single view (hence the infographics) and to derive functional information from the compiled dataset. This was rather difficult with the information dispersed over 8 papers (which is why it took us a long time to analyze all the data).

Furthermore, by analyzing his data set, we could derive candidate anapyrexic agents that were subsequently appraised for their pharmacodynamic properties in the context of artificial hypothermia induction, thereby identifying their pharmacological/toxicological bottlenecks and knowledge gaps in the

framework of anapyrexic signaling (which has now been addressed in the main text in the newly added section 2).

Our approach constitutes the first step in a sequence of strategic papers as outlined in the response to point 2 of Reviewer 2.

Above all, these two main elements clearly distinguish our work from that of Dr. Clark's and add direction to future research with the candidate compounds.

Furthermore, the approach used by the authors is limited, and even less analytic than the compilation from Clark, W.G.

The approach used by us served the purpose of identifying potential anapyrexic agents from an ocean of tabulated data published in an era where the process of anapyrexia was not well-established. We therefore reorganized the data in terms of anapyrexia so as to identify candidate compounds, which is something Dr. Clarke did not do. In fact, and with all due respect for his tremendous work, no real analysis was performed on any functional parameters (graphically summarizing the number of citations and listing numerous parameters does not constitute 'analysis').

Major
As also mentioned by the authors, their analytical approach as strong limitations:
The number of animals in each report was not taken into account.
Tb was not corrected for the Ta
Differences in the dosage and administration route were not accounted for.
The manner in which experiments were performed was discounted.

Indeed, these limitations were specifically addressed so that readers may place our data in perspective. Nevertheless, the ensuing critical appraisal of the candidate compounds identified by our somewhat restricted yet otherwise valid analysis compensates for these analytical shortcomings. That is why some of the compounds were ultimately ruled out. In the end, several compounds were identified as potential anapyrexic agents following the critical appraisal, on the basis of which future directions could be addressed. The primary and secondary aims of the study were therefore achieved with full transparency and honest valorization of the analytical model.

On top of this, the authors didn't consider that the mass of the animal also contributes strongly to the thermal dispersion and the ability to lower the Tbody. In this regard, it is curious to notice that the group of drugs selected is, in fact, causing large Tbody changes in mice, but not in bigger species. Surely, if the authors normalize the changes in Tbody to the animal's mass, they will find a completely different result.

Excellent point. The data were reanalyzed and the following modifications were implemented in the text (section 5):

Nevertheless, a good illustration can be provided on the basis of mice (N = 62), rats (N = 96), and rabbits (N = 39) alone, as shown in Figure 3B-D, where the change in T_b was organized from greatest to lowest and plotted per compound following normalization to body weight (in kg). The upper limit and lower limit weights of these laboratory animals were used to demarcate the maximum and minimum boundaries of the T_b change per unit weight. This was done to semi-standardize the data because a considerable fraction of the articles from which the data were derived did not report the mean body weight or weight range of the animals used in the experiments. When normalized to body weight, the heat loss per kg body weight is most sizeable in mice and smallest in rabbits, confirming the observations in Figure 2 and clearly illustrating both principles described above. A complete list with the categorized variables is provided in Table S3.

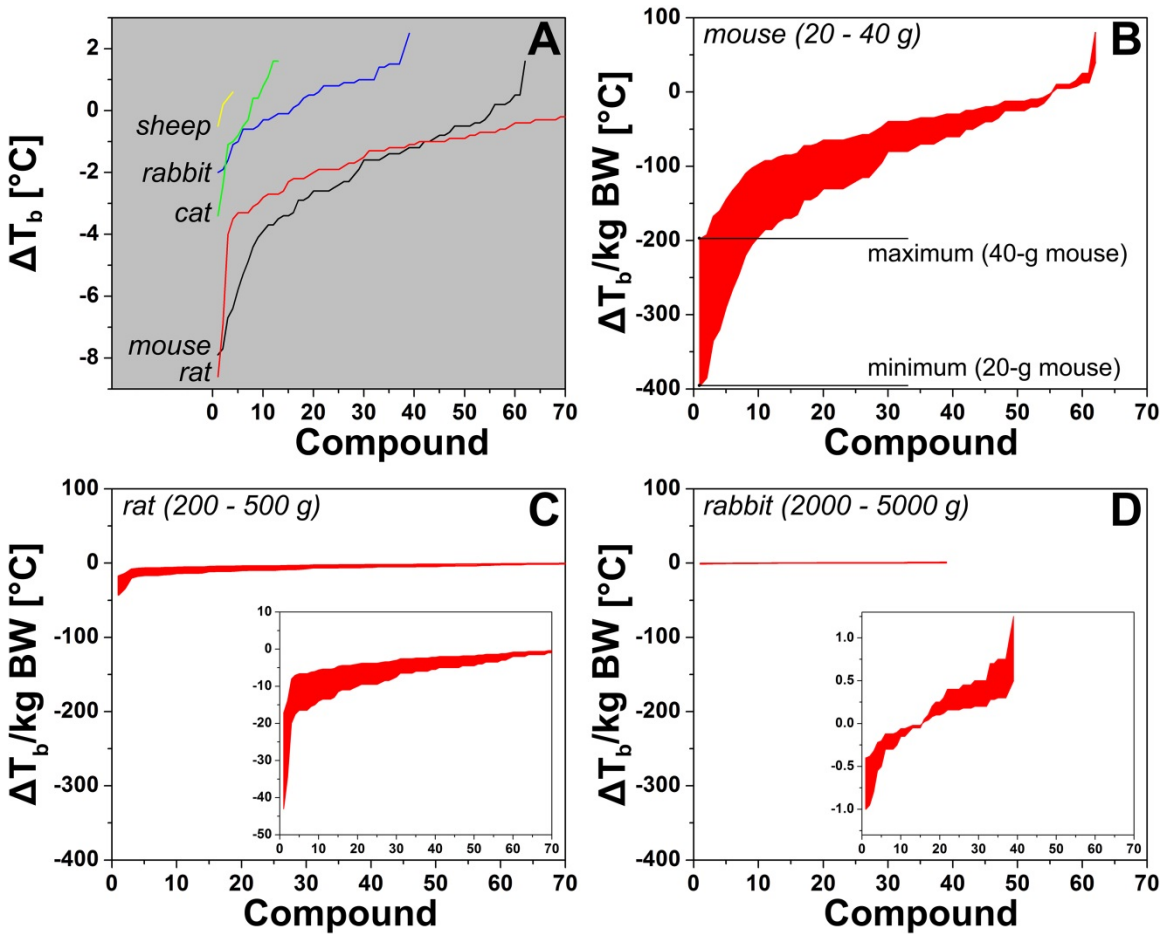

***Figure 3**. Change in core body temperature (ΔT_b) per species plotted as a function of compound with the most profound effect on T_b (1, x-axis) to the compound with the least effect on T_b (up to 70, x-axis). The ΔT_b represents the mean of all ΔT_bs reported for the respective compound in the respective species that were included in the analysis. Research data were included on the basis of the criteria described in section 3 and the legend of Figure 2. The complete data set containing all the species is provided in Table S3. The data were normalized to the maximum and minimum common body weights (BW) of laboratory mice (B), rats (C), and rabbits (D) and should be read vertically per compound, whereby the upper limit is the minimum ΔT_b for the heaviest animals. The actual recorded values fall between the upper and lower bounds per compound. Note the different y-axis scaling of the inset plots. Common body weights were obtained from the internet (e.g., laboratory animal providers such as Harlan and Charles River) and US government literature on laboratory animals.*

One of the drugs that the authors selected based on their calculation is acetaminophen. This is a COX1-2 inhibitor commonly used as an antipyretic. Even if cases of mild hypothermia have been reported, these occur only in patients in whom the thermoregulatory system is already activated, or functionally compromised, by bacterial and viral infections, and by clinical trauma, such as cancer or stroke [Samir S. A. et al., PNAS 2004]. Moreover this hypothermic effect has only been demonstrated in mice. Thus, it is unclear that this class of drugs would induce hypothermia in a normal subject. This is just one example of how important it is to consider the experimental basis used to obtain the data whose effects are then extrapolated in this review.

This is also a very useful point that has been addressed as follows (section 6.8):

"Moreover, the T_b-downmodulatory properties of acetaminophen may have been falsely ascribed in instances where the thermoregulatory system was already activated, or functionally compromised. These

instances include clinical trauma (such as cancer or stroke, such as cited in the previous paragraph [86]) and bacterial and viral infections [92]. Finally, the hypothermic effect of acetaminophen has only been demonstrated in mice. Rats exposed to increasing acetaminophen dosages did not exhibit heat-avoiding behavior, indicating that no pharmacological modulation of the Z_{tn} had occurred [93]. Accordingly, the effect of acetaminophen on behavioral thermoregulation pleads against its classification as an anapyrexic agent, and it is unclear whether this class of drugs would induce hypothermia in a normal subject."

Accordingly, ancillary factors that may affect acetaminophen-mediated hypothermia were included and the compound was dismissed as an anypyrexic agent.

In the "limitation" section, the author reports that experiments with helium and DMSO were performed at an average Ta of 10.0 ± 13.3 °C and 12.5 ± 10.3 °C. These large standard errors for Ta emphasize the large Ta range to which different mice were exposed in these experiments. It is not surprising that DMSO caused such a big change in Tbody in a small species like a mouse or rat when exposed to such a heavy cold thermal load.

The temperatures reported for all but one included mouse experiments with DMSO were very close to room temperature. For the rat the temperatures were indeed variable (derived from Clark WG, Clark YL: Changes in body temperature after administration of antipyretics, LSD, delta 9-THC, CNS depressants and stimulants, hormones, inorganic ions, gases, 2,4-DNP and miscellaneous agents. Neurosci Biobehav Rev 1981;5:1-136):

Mouse			**Rat**		
Ta	**ΔTb down**	**ΔTb up**	**Ta**	**ΔTb down**	**ΔTb up**
20-22	0.9		23	0.4-0.7	
31		1.2	23	1	
22.3	0.4-0.6		1.7	1.8	
4	2.1		23	2.1	
22-24	1.4		17	13.2	
22-24	2.1		24-26	3.6	
22-24		0.5	14-16	17	
			0-2	27	
			0-2	27	
			0-2	27	
			1-3	5.2	
			21-25	0	
			21-25	2.4-2.9	
			2-6	0	
			2-6	2.2-6.4	
			2-6	21.4	
			20-24	0	

Accordingly, the following text was added to section 6.2:

"In addition, the experiments performed in rats were performed in a temperature range of 0-26 °C [28]. The observation that DMSO caused such a large change in T_b in a small species such as a rat (Figure 3A, C) may in part be explained by the heavy cold thermal load."

None of the data reported provides any insight into potential changes in the thermoregulatory set point, indeed, if it was altered at all. The reported changes in Tbody could simply reflect a fall in Tbody that was due to inhibition of neurons in thermoregulatory centers in the brain or to effects on thermoeffector function. Indeed, many recent discoveries in the field of thermoregulation have not been assimilated into this report.

We kindly ask the reviewer to note that the reported data mainly served as a means to identify compounds with potential anapyrexic potential, which was subsequently explored in the critical appraisal section (section 6) to corroborate or refute the potential anapyrexic properties of candidate compounds. The critical appraisal was conducted in light of both thermos-effector functions and effects on the thermoregulatory center in the brain, albeit the latter is still difficult to determine directly and mainly gauged by the former. In the end, 3 compounds were identified as possibly true anapyrexic agents.

In our subsequent report (please see level 2 and 3 projects in the reply to point 2 of Reviewer 2) the most recent trends/discoveries in the physiological, biochemical, and neurological regulation of T_b will be elaborated. However, addressing these 'old' compounds in the 'new' context will be difficult due to the disconnect between the researched pharmacodynamic parameters and the more recently established parameters related to the thermoregulatory center in the brain.

Overall, the weak analytical approach and the significant limitations, only some of which were mentioned by the authors, do not support the authors' conclusions, which could easily be misleading to the scientific community interested in the important topic of the pharmacological induction of hypothermia.

We hope that the manuscript has reached an acceptable quality with the help of your comments and suggestions.

REVIEWER 2

Induction of Anapyrexia by Pharmacological Agents recaps the content of eight reviews of 18,808 reports describing drug-induced changes in thermoregulation by WG Clark published between 1979 to 1986. An innovative graphic illustrates the effects of specific drugs, grouped by classification, on the magnitude and direction of drug-induced changes in Tb. The author(s) define criteria for excluding thousands of reports and acknowledge several limitations of the method used to identify promising anyapyrexic agents through this review process. Figures 1 and 2 are highly effective graphics. Overall the review is a valuable resource to find information on the thermoregulatory consequence of a variety of agents.

Dear reviewer,

We are very grateful for your keen insights and valuable suggestions to improve the manuscript.

1) A weakness of the manuscript is that it promises to identify the most promising agents in terms of anapyrexic potential based on the magnitude of drug-induced change in Tb, but in the end rules out all 8 compounds as viable candidates.

You are correct in pointing out that the review ends rather anticlimactically. Accordingly, we have changed the title to "Survey and critical appraisal of pharmacological agents with potential thermo-modulatory properties in the context of artificially induced hypometabolism." By reformatting the framework in which the review has been written (i.e., a 'critical appraisal'), we now account for the possibility that some of the addressed compounds do not work as anapyrexic agents on the basis of the critical appraisal.

Moreover, please note that not all compounds were dismissed (e.g., see the abstract: "Of the agents that were discussed, reserpine, (oxo)tremorine, and (chlor)promazine may possess true anapyrexic properties based on their ability to either affect the thermoneutral zone or its effectors and facilitate hypothermic

signaling"). We merely cautioned readers that the anapyrexia-inducing potential of the studied compounds has not yet been unequivocally demonstrated, which was the main aim of the study (see reply to your point 2 and the newly added section 2 in the manuscript). Accordingly, we added the following text to the abstract to underscore the cautionary note:

"However, these properties are currently not unequivocal and warrant further examination in the context of artificially-induced hypometabolism."

That way the central message in the abstract is introduced early in the paper and reiterated in the second paragraph of the Concluding remarks ("As indicated in the sections on the pharmacological agents above, there are various agents such as reserpine, (oxo)tremorine, and (chlor)promazine that exhibit specific aspects suggestive of an anapyrexic potential. However, due to the primary research focus on aspects other than anapyrexia, the anapyrexic potential of these agents requires further examination").

Major areas for improvements

2) Refocus the manuscript as a historical review of Clark's work or expand the review to include recent developments of drugs that target specific aspects of thermoregulation.

This is an excellent proposal and in agreement with our longer-term research approach. Please know that we are employing a comprehensive, systematically layered strategy in this field of research that is organized as follows:

Layer 1: provide a critical appraisal of the most studied compounds with anapyrexic potential to characterize these agents against a backdrop of empirical evidence and to open avenues for new research in the context of artificial induction of anapyrexia. (THE CURRENT PAPER)

Layer 2: lay down a conceptual framework regarding the mechanisms of anapyrexic signaling in terms of hypometabolism-inducing pharmacological agents. This paper ("The physiology of artificial hibernation"), which has taken us over 3 years to compose, is currently under review at the Journal of Clinical and Translational Research and addresses mainly the physiological but also the biochemical and neurological basis for artificial hypometabolism induction by compounds with HIT-like properties (so-called hibernation induction triggers). A figure is included for illustrative purposes.

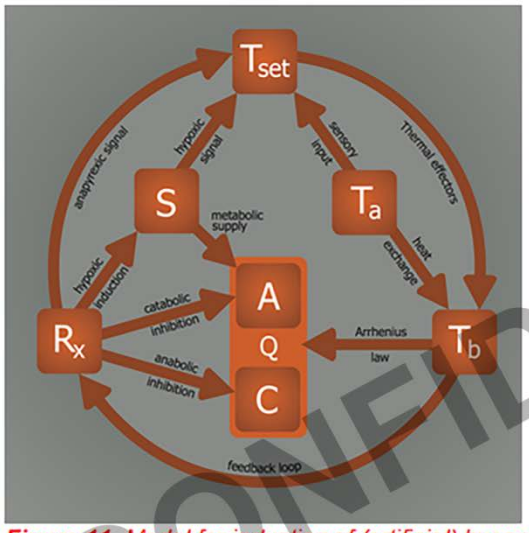

Figure 11. *Model for induction of (artificial) hypometabolism. Depicted parameters: Q, overall metabolism defined as chemical reaction speed (i.e., similar to k in Equation 1); C, catabolism; A, anabolism; T_b, core body temperature; T_a, ambient temperature; Z_{tn}, thermoneutral zone; S, substrate; R_x, (bio)chemical agent able to induce hypometabolism. The relationships: T_b—Q, Arrhenius law; T_a—T_b, heat exchange; Z_{tn}—T_b, thermogenesis and heat loss mechanisms; T_a—Z_{tn}, sensory input; S—Z_{tn}, hypoxic link; R_x—S, hypoxia/hypoglycemia induction; R_x—C, catabolic modulation; R_x—A, anabolic modulation; R_x—Z_{tn}, anapyrexic signal; S—A, metabolic substrate supply; T_b—R_x, positive/negative feedback loop.*

Layer 3: summarize the most recent developments and concepts in the pharmacological induction of hypothermia and hypometabolism through anapyrexic signaling. This paper, which we are currently finalizing, provides novel insights into, amongst other, hypoxic sensing via e.g., carotid and aortic bodies with respect to POAH-mediated thermoregulation, a mechanistic underpinning of these signaling axes, as well as an elaboration of compounds that play in on these mechanisms, which are different from the ones addressed in the current paper. A figure is included for illustrative purposes. PLEASE NOTE THAT THIS PAPER SPECIFICALLY ADDRESSES YOUR COMMENT. WE WOULD LIKE TO PUBLISH THIS SEPARATELY.

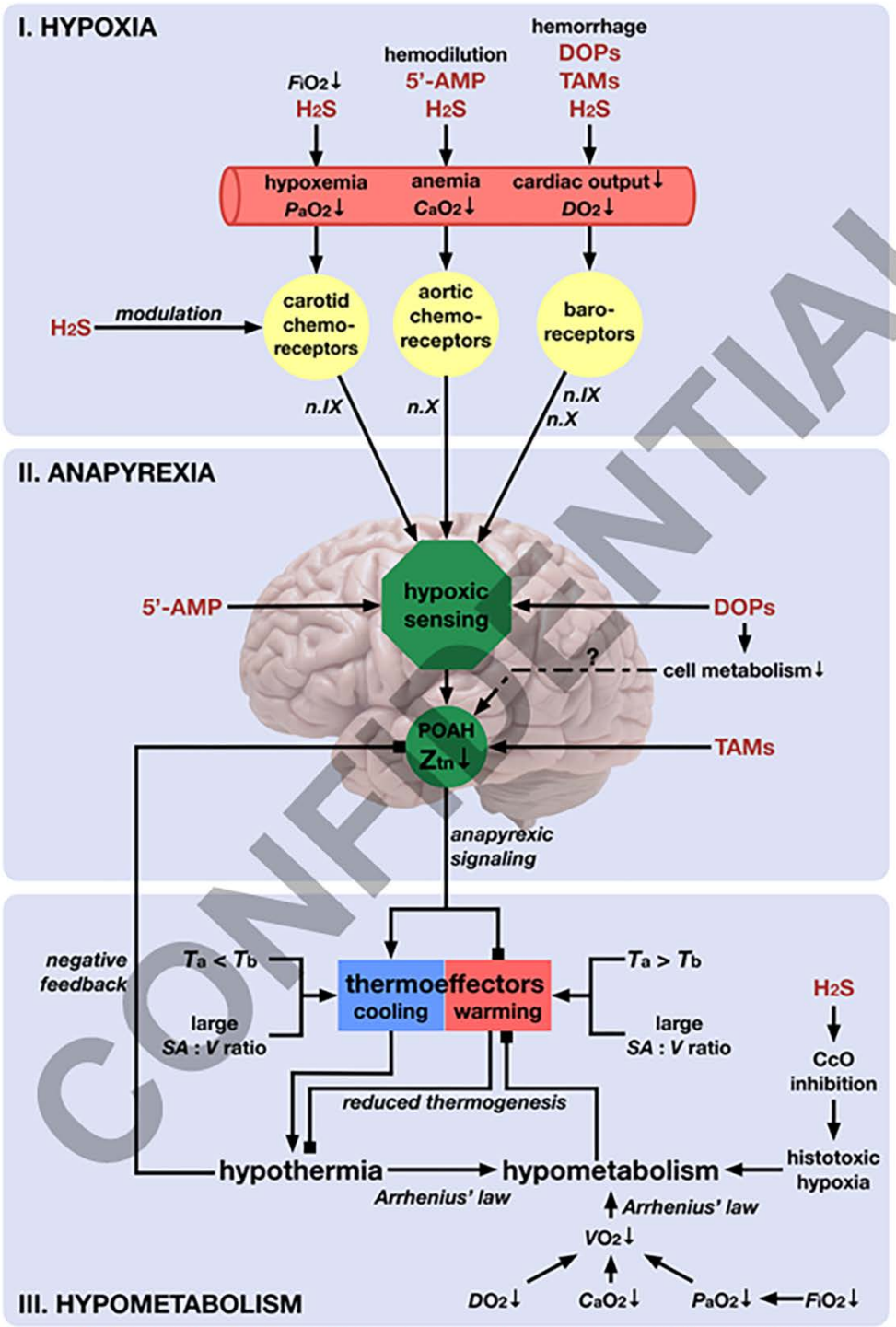

Figure 3. Schematic overview of hypoxia-induced anapyrexia and the possible targets of the HIAs discussed in this review (i.e., H_2S, 5'-AMP, TAMs and DOPs).

In order to be forthcoming and to inform the readers of the outlined strategy, we added an explanatory section (now section 2) in the manuscript that explicitly addresses the aims of the study:

"In order to provide an accessible summary of potentially useful pharmacological agents for the induction of anapyrexic signaling, we performed a review of literature and analyzed over a thousand

pharmacologically active compounds for their ability to induce anapyrexia in animals. The most viable candidates were identified on the basis of the magnitude of the reported heat loss and critically appraised in the context of the Z_{tn}-mediated heat loss mechanisms (Figure 1). In this study we focused specifically on the most studied compounds that potentially harness anapyrexic properties and addressed the candidate drugs against a backdrop of empirical evidence related to mainly pharmacodynamics and toxicology. The secondary purpose of this review was to guide novel research with 'old compounds' in the context of anapyrexic signaling by elaborating on the discrepancies in reported data and knowledge gaps. Subsequent reviews will focus on the physiological, biochemical, and neurological mechanisms of anapyrexic signaling in terms of hypometabolism-inducing pharmacological agents (manuscript submitted) and the role of hypoxic sensing via e.g., carotid and aortic bodies with respect to POAH-mediated thermoregulation (manuscript is preparation)."

Moreover, we addressed the existence of alternative strategies encompassing the artificial induction of anapyrexia in section 1:

"One interesting and potentially viable approach is pharmacological modulation of thermoregulatory cold receptors in the skin [23;24]. Studies published by Andrej Romanovsky's Fever Lab have demonstrated that selective inhibition of the transient receptor potential melastatin-8 (TRPM8) channel (cutaneous cold receptor) with M8-B effectively decreases the T_b in mice and rats via several thermoeffectors (thermopreferendum, tail-skin vasoconstriction, and brown adipose tissue) [23]."

23 Almeida MC, Hew-Butler T, Soriano RN, Rao S, Wang W, Wang J, Tamayo N, Oliveira DL, Nucci TB, Aryal P, Garami A, Bautista D, Gavva NR, Romanovsky AA: Pharmacological blockade of the cold receptor TRPM8 attenuates autonomic and behavioral cold defenses and decreases deep body temperature. J Neurosci 2012;32:2086-2099.
24 de OC, Garami A, Lehto SG, Pakai E, Tekus V, Pohoczky K, Youngblood BD, Wang W, Kort ME, Kym PR, Pinter E, Gavva NR, Romanovsky AA: Transient receptor potential channel ankyrin-1 is not a cold sensor for autonomic thermoregulation in rodents. J Neurosci 2014;34:4445-4452.

3) Be more specific about thermoneutral zone in terms of the neuroanatomic network that contributes to thermoregulation.

To address this point, a reference was made to a series of papers specifically dealing with this topic. We chose to redirect interested readers to these papers so not to distract the reader from the central message by drowning him/her in a pool of specialized and complex neuroscience and neurological material.

Accordingly, the following text was added (section 1):

"Readers interested in the neuroanatomical networks that govern mammalian thermoregulation via the POAH are referred to a panel of excellent papers by Morrison and Nakamura on this subject [10-13]."

10 Morrison SF, Nakamura K, Madden CJ: Central control of thermogenesis in mammals. Exp Physiol 2008;93:773-797.
11 Nakamura K: Central circuitries for body temperature regulation and fever. Am J Physiol Regul Integr Comp Physiol 2011;301:R1207-R1228.
12 Morrison SF, Nakamura K: Central neural pathways for thermoregulation. Front Biosci (Landmark Ed) 2011;16:74-104.
13 Nakamura K, Morrison SF: Central efferent pathways for cold-defensive and febrile shivering. J Physiol 2011;589:3641-3658.

4) Include and defend criteria for characterizing a compound as affecting the thermoneutral zone.

Perhaps this part was missed by the reviewer, but we feel that this has been adequately addressed in section 6, which now reads:

"A direct measurement of the Z_{tn} following administration of an agent would constitute the ultimate method to determine anapyrexic potential. However, due to the fact that we currently neither fully understand the body's temperature integration system nor have the means to monitor it, direct measurement of the boundaries that make up the Z_{tn} is impossible. Consequently, the gold standard in determination whether an organism is within the Z_{tn} boundaries is based the activity of thermal effectors (Figure 1).

Therefore, in the next sections the eight most promising agents are addressed in the context of their effect on thermogenic and heat loss effectors such as shivering, BAT activity, sweating, vasoconstriction/vasodilation, and behavioral accommodation (Figure 1)."

The list of thermal effectors is provided in the second paragraph of the Introduction (section 1).

Accordingly, no changes were implemented.

5) Defend the assumption that the most "viable" candidate can be identified by magnitude of Tb change

The following text was inserted at the beginning of section 6:

"The magnitude of T_b decrease was used as the standard parameter to gauge anapyrexic signaling potential insofar as a downward modulation of T_b is the most important hallmark of anapyrexic signaling and does not occur in hibernators and non-hibernators in the absence of a Z_{tn} adjustment under non-stimulatory circumstances (e.g., under conditions of normoxia, abundant food supply, $T_a \approx T_b$, etc.) [33;38]."

Minor points for consideration

6) Include rationale for referring to hypoxia-induced anapyrexia as "regulated".

The following section has been included in the introduction to justify this reference:

"A method to induce anapyrexia in small animals is by subjecting the animals to hypoxia, which triggers regulated hypothermia and corollary hypometabolism in some species as a countermeasure against the hypoxic, and thus potentially lethal, conditions [7-10]. One of the putative regulatory mechanisms is centered on carotid body sensing [11;12]. Carotid bodies are clusters of tissue composed of chemoreceptors and cells near the bifurcation of the carotid artery that detect changes in oxygenation-related parameters, including partial pressure of oxygen and carbon dioxide as well as pH and temperature [13;14]. When hypoxia is sensed, anapyrexia is induced through the inhibition of thermogenic effectors and activation of cooling effectors [1;9;15-17], which are under control of the POAH [5;6;18;19]."

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