



OPINION ARTICLE

REVISED On the development of B-Raf inhibitors acting through innovative mechanisms [version 2; peer review: 2 approved]

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Abstract

B-Raf is a protein kinase participating to the regulation of many biological processes in cells. Several studies have demonstrated that this protein is frequently upregulated in human cancers, especially when it bears activating mutations. In the last years, few ATP-competitive inhibitors of B-Raf have been marketed for the treatment of melanoma and are currently under clinical evaluation on a variety of other types of cancer. Although the introduction of drugs targeting B-Raf has provided significant advances in cancer treatment, responses to ATP-competitive inhibitors remain limited, mainly due to selectivity issues, side effects, narrow therapeutic windows, and the insurgence of drug resistance.

Impressive research efforts have been made so far towards the identification of novel ATP-competitive modulators with improved efficacy against cancers driven by mutant Raf monomers and dimers, some of them showing good promises. However, several limitations could still be envisioned for these compounds, according to literature data. Besides, increased attentions have arisen around approaches based on the design of allosteric modulators, polypharmacology, proteolysis targeting chimeras (PROTACs) and drug repurposing for the targeting of B-Raf proteins. The design of compounds acting through such innovative mechanisms is rather challenging. However, valuable therapeutic opportunities can be envisioned on these drugs, as they act through innovative mechanisms in which limitations typically observed for approved ATP-competitive B-Raf inhibitors are less prone to emerge. In this article, current approaches adopted for the design of non-ATP competitive inhibitors targeting B-Raf are described, discussing also on the possibilities, ligands acting through such innovative mechanisms could provide for the obtainment of more effective therapies.

Keywords

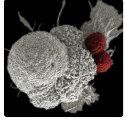
B-Raf, allosteric inhibitors, polypharmacology, drug repurposing, PROTACs, drug discovery and development, small molecule inhibitors.

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Any reports and responses or comments on the article can be found at the end of the article.



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REVISED Amendments from Version 1

In response to the Reviewers' very helpful suggestions, I made the following amendments to the manuscript. The manuscript was revised to better contextualize the development of innovative B-Raf targeting approaches, with respect to ATP-competitive inhibitors. Moreover, a description on the main elements characterizing the ATP-binding site of the kinases was also reported. This allowed also to better: (i) highlight how Ponatinib and PHI1 can be classified according to their experimentally derived binding mode, and; (ii) contextualize their example with respect to the type III B-Raf allosteric pocket mentioned within the manuscript. Moreover, the text was also revised to include additional insights on some of the experimental and *in silico* approaches that are expected to be of help for kinase allosteric drug design. A discussion on the main challenges that might be faced when rationally designing multi-kinase inhibitors was provided. Moreover, the main E3 ligases exploited so far for the design of PROTACs are also now listed, along with their full name. The text was carefully revised to remove typos and to improve its readability, while additional references to relevant literature data were also added in support of the revisions. The full name of the reported abbreviations was added where needed, in agreement to the Reviewers' comments. Finally, Figure 1 was revised to remove typos and to improve the image contents, as suggested.

Any further responses from the reviewers can be found at the end of the article

The Serine/Threonine protein kinase B-Raf is one among the most widely studied targets for cancer treatment.^{1,2} Under physiological conditions, this protein participates as key player in the Ras-Raf-MEK-ERK signaling pathway to the regulation of a number of cellular processes.^{3,4} In cancer cells, B-Raf is often upregulated, especially when it bears activating mutations, thus promoting oncogenic cellular processes as uncontrolled proliferation, tumor growth and metastasis.^{5–9} Of note, several studies have reported that B-Raf is frequently mutated in human cancers,^{6,10,11} with more than forty oncogenic mutations currently being described for this kinase.¹² For these reasons, *wild type* (WT) and mutant B-Raf proteins have gained remarkable relevance for the development of anticancer drugs over the last decades. Several drugs selectively targeting B-Raf proteins have also been approved for the treatment of metastatic melanoma,^{13,14} providing remarkable advantages in therapeutic regimens, and are currently under evaluation against colorectal cancer.¹⁵ However, the therapeutic use of the majority of these drugs is still hampered by drug resistance and side effects issues, resulting in responses that often remain temporary and rarely complete, the median time progression, *e.g.*, for Vemurafenib against melanoma being six to seven months.¹⁶ In particular, several findings have demonstrated that drug resistance often characterizing approved B-Raf inhibitors is mainly driven by feedback deregulation and over-expression of several other kinases.^{17–20} Nevertheless, clinical evidences have demonstrated that the use of selected B-Raf inhibitors in therapeutic regimens can also result in Raf paradoxical activation, which promotes cellular hyperproliferation of certain secondary skin lesions.^{21–26} In particular, it has been reported that when a B-Raf monomer is bound to specific inhibitors (*e.g.*, Dabrafenib and Vemurafenib), it can dimerize promoting the aberrant activity of a second drug-free protomer, which cannot be targeted due to protein conformational rearrangements.²¹ This event in turn promotes abnormal proliferation in cells harboring other oncogenic mutations, through the activation of the MEK–ERK pathway.²⁷ In this context, major research efforts have been devoted so far on the development of novel ATP-competitive kinase inhibitors binding to different α C-helix conformations of B-Raf (*i.e.*, α C-OUT and α C-IN) (Figure 1).^{13,21,22,28,29} Indeed, the vast majority of the reported B-Raf inhibitors reported so far act through the competitive binding to the ATP binding site of this protein, as for other kinases.^{30,31} Four different regions can be identified within the ATP binding cleft, which is highly conserved among kinases, *i.e.*: (i) a short hinge area containing a *hot-spot* residue (a cysteine in B-Raf) to which the adenine portion of the substrate bind to; (ii) a hydrophobic pocket adjacent to the hinge region; (iii) a further hydrophobic type-II pocket, located between the and the Asp-Phe-Gly (DFG) motif and the regulatory α C-helix, and; (iv) an allosteric site placed in correspondence to the α C-helix. To date, some privileged chemical scaffolds, and structural details in correspondence to the type-II pocket of the kinases potentially conferring kinase selectivity to ligands have been discovered.³² However, the obtainment of clinically safe ATP-competitive inhibitors showing high selectivity towards selected kinases often remains elusive. With regards to B-Raf, low efficacy deriving by non-optimal therapeutic windows and the establishment of allosteric priming could be observed, for example, for α C-IN binders, albeit they can abrogate aberrant Raf dimerization-derived activities, through the inhibition of both the monomer and dimer of the kinase.^{13,33,34} Examples of reported compounds binding to this type of conformation of B-Raf are the *pan*-Raf inhibitors RAF-265, MLN-2480, TAK-632, LY3009120, CCT196969 and CCT241161,^{35–37} some of them having been evaluated in clinical trials (*e.g.*, [ClinicalTrials.gov](https://clinicaltrials.gov) identifiers: NCT02014116, NCT01425008 and NCT00304525), also in combination with MEK blockers (*e.g.*, [ClinicalTrials.gov](https://clinicaltrials.gov) identifier: NCT01352273). On the contrary, α C-OUT B-Raf inhibitors demonstrated to provide good selectivity profiles and wider therapeutic windows compared to α C-IN binders.^{13,28} Examples of such compounds are, among the others, Vemurafenib, Dabrafenib and Encorafenib, which potently inhibit mutant B-Raf^{V600E} monomers, but resulted to be ineffective on tumor cellular contexts driven by aberrant Raf dimerization.^{13,28} Moreover, compounds binding to the α C-OUT conformation of B-Raf have also been reported to promote paradoxical activation.^{13,38} Besides, other studies have been focused on the development of compounds as PLX7904 and PLX8394, able to escape paradoxical activation of B-Raf (*i.e.*, the so-called “paradox breakers”) and to overcome some of known resistance mechanisms associated to previously reported

Raf inhibitors.^{39,40} For example, PLX8394 is currently being evaluated in Phase I/IIa clinical trials ([ClinicalTrials.gov](https://ClinicalTrials.gov/record/NCT02428712) identifier: NCT02428712). The development of paradox breaker compounds is expected to provide significant impact to anticancer therapy, as they allow to efficiently modulate the activity of mutant B-Raf^{V600E}, while circumventing protein dimerization.⁴¹ However, such compounds might also result in limited efficacy towards cancers with mutated Ras, according to literature data.⁴¹ Despite the advantages that can be observed in latest-generation ATP-competitive inhibitors of this kinase, several evidences encouraged a number of research groups to develop B-Raf modulators acting through innovative mechanisms. These include, for example, the modulation of the kinase activity of B-Raf with type III and IV highly selective allosteric binders, as well as through multi-target approaches (*i.e.*, polypharmacology) (Figure 1). The design of allosteric kinase inhibitors of B-Raf is particularly challenging, for example, due to missing activity data or the lack of crystallographic structures suitable for the investigations, whose *activation loop*, *activation segment* and *α C-helix* are often not clearly solved. However, recent advancements in crystallographic and *in silico* techniques will certainly help to overcome several of the issues currently encountered in kinase allosteric ligand design.^{31,42} Among them, for example, cryo-electron microscopy alone or in combination with X-Ray crystallography is expected to provide novel fruitful insights on the conformational flexibility of the proteins, which might be of help to, *e.g.*: (i) better understand the events occurring during the kinase activation and the ligand binding, and; (ii) circumvent some of the experimental limitations that could emerge during the crystal formation.^{43,44} Besides, *in silico* techniques based on machine and deep learning algorithms as AlphaFold have been recently developed to assist the prediction and refinement of reliable three-dimensional models of the proteins.⁴⁵ Moreover, other *in silico* methods based on molecular dynamics and hot spot analysis are also available to help investigating the mechanism of activation of the kinases and to facilitate the identification of novel druggable binding sites, respectively.^{46,47} The possibilities offered by such techniques are expected not only to help elucidating novel structures, and to identify additional potentially druggable kinase binding pockets, but also better assisting, for example, the design of allosteric ligands. The identification of allosteric inhibitors of B-Raf holds great promises in cancer therapy. Indeed, such targeting approaches are expected to help identifying ligands with higher selectivity towards B-Raf with respect to classic ATP-competitive binders, and to help to overcome drug resistance, similarly to as postulated for other kinases.^{48–52} Unfortunately, neither type III, nor type IV small molecule, allosteric modulators of B-Raf have been reported so far. However, potent type III inhibitors binding to an allosteric pocket in proximity to the regulatory α C-helix have been reported for several other kinases, such as BCR-ABL, MEK, EGFR and CDK2,^{53–59} providing valuable structural clues for the design of innovative B-Raf modulators. These results have also been fueled by the recent crystallographic resolution of type III allosteric inhibitors of mutant EGFR and MEK (*e.g.*, see references 52,56,60). In this regard, our research group has reported the design of previously unseen

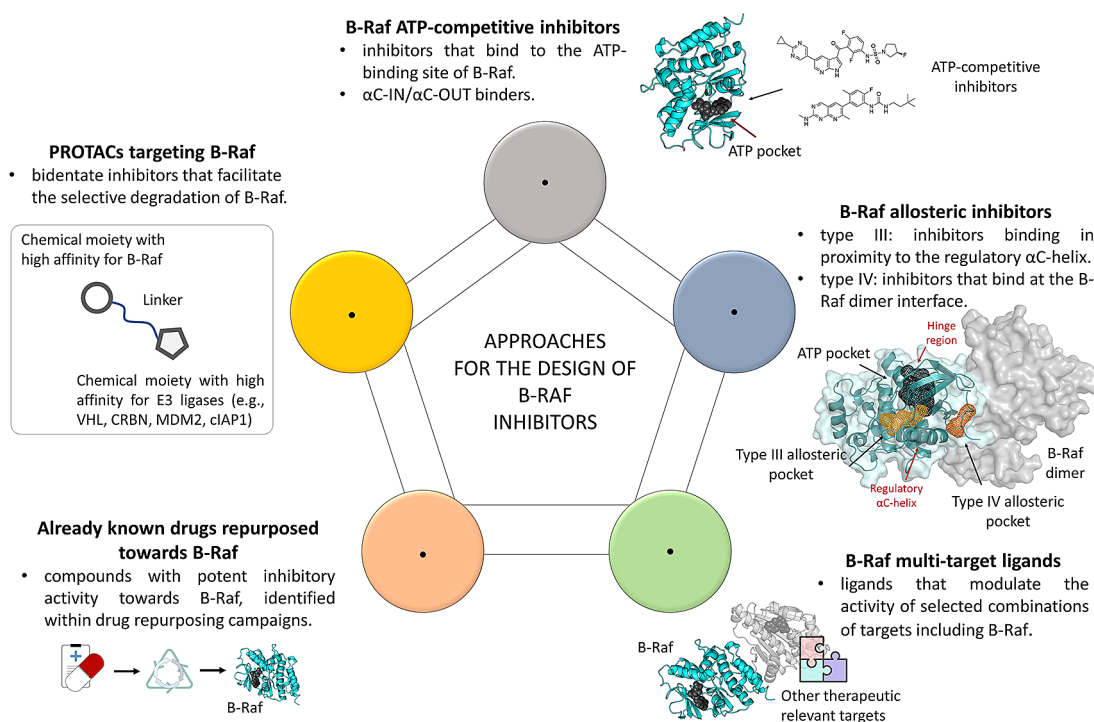


Figure 1. Schematic representation of the approaches currently pursued for the design of B-Raf ligands, discussed in the article.

type III allosteric inhibitors of the CDK2 kinase showing anticancer activity.^{53,61} Moreover, we have also reported the identification of structurally novel allosteric modulators of WT and double mutant EGFR, exhibiting inhibitory activity towards non-small cell lung cancer (NSCLC).⁵⁴ More recently, our research group has also demonstrated that B-Raf possesses an allosteric pocket similar to that of EGFR^{T790M}, adopting a DFG-IN/ α C-OUT conformation potentially druggable by type III modulators.⁶² Remarkably, the presence of such allosteric pocket in B-Raf has also been indirectly confirmed in a recent study by Cotto-Rios *et al.*⁶³ In particular, in their study the authors firstly identified Ponatinib as an inhibitor of B-Raf within a drug repositioning campaign. According to the experimentally derived binding mode, Ponatinib cannot be considered as an allosteric inhibitor of B-Raf. Indeed, it establishes interactions with the residues in the hinge region and the hydrophobic pocket outwards the DFG motif of the kinase. However, this compound presents a methyl-piperazine moiety that allocated into the allosteric site in proximity to the kinase regulatory α C-helix, thus indirectly confirming the presence of this region in B-Raf.^{62,63} The authors performed also medicinal chemistry optimizations on Ponatinib, identifying a compound (*i.e.*, PHI1) that showed similar binding mode, and selectivity towards B-Raf dimers in cancer cells.⁶³ Together with previous considerations and established literature data, the results of this study, to some extent, suggest that the design of allosteric inhibitors of B-Raf is feasible. Moreover, these results also suggest that the design of such allosteric ligands would also open to novel strategies enabling the full arrest of the B-Raf kinase activity, potentially either *via* single agents or combination therapies. Nevertheless, the results prospected in this study paved the way towards the identification of innovative B-Raf inhibitors among approved drugs (*i.e.*, drug repurposing) (Figure 1).^{63,64} Indeed, this approach has already been navigated also with natural products and clinically safe candidates, on different medicinal chemistry research areas.^{65–71} Similar considerations can also be argued for the design of allosteric ligands binding to the B-Raf dimer interface (*i.e.*, type IV). The interest on such type IV allosteric ligands for the targeting of both *wild type* and mutant B-Raf has steadily increased over the past few years, with a number of small polypeptides able to disrupt protein dimerization and transactivation being reported.^{50,51,72,73} In particular, Beneker *et al.*⁵⁰ were among the first to report the identification of a small set of polypeptides binding to the Raf dimerization interface. The results achieved in their study not only demonstrated that such a type of targeting is a feasible endeavor on B-Raf, but also that type IV allosteric ligands could provide remarkable results when used in combination with known mutant-selective ATP-competitive inhibitors promoting paradoxical activation of the ERK signaling.⁵⁰ On the same line, Gunderwala *et al.*⁵¹ more recently reported the identification of Braftide, a small polypeptide designed through a computational strategy blocking Raf dimerization. Notably, Braftide demonstrated to efficiently inhibit Raf dimerization and to provide degradation of the MAPK complex. Moreover, Braftide has also proved to synergize with Vemurafenib and Dabrafenib,⁵¹ further supporting the potential application of type IV allosteric B-Raf inhibitors on therapeutic regimens with approved ATP-competitive drugs. The possibility to identify allosteric ligands of this kinase could also open to novel therapeutic approaches promoting simultaneous blockade of B-Raf at different sites, for example, if used in combination with approved ATP-competitive drugs. Indeed, such a complementary therapeutic approach is being under study against EGFR-mutant lung cancer,⁵⁹ and it is expected to provide also valuable opportunities for B-Raf targeting. Allosteric inhibitors acting at a site different to those of the type III and type IV ligands described above have already been investigated for other kinases, in some cases with promising results.⁷⁴ In the specific case of B-Raf, the identification of these types of inhibitors is still at a preliminary stage, albeit remarkable therapeutic opportunities arising on these grounds could be envisioned for the near future.

Novel valuable opportunities could also arise from the identification of compounds exhibiting activity on B-Raf, in selected combinations of targets. The therapeutic advantages deriving by the simultaneous modulation of multiple targets involved in the physiopathology of a disease, either by using a combination of drugs, or with ligands endowed with tailored polypharmacology properties, have already extensively discussed in literature.^{75–78} Indeed, the use of approved B-Raf inhibitors is now mainly framed in combined regimens including modulators of other therapeutic relevant targets. For example, the B-Raf^{V600E} inhibitor Dabrafenib is now mainly used for the treatment of patients with unresectable or metastatic melanoma in combination with Trametinib (a MEK ATP-noncompetitive modulator).^{79,80} Similarly, Encorafenib (a B-Raf^{V600E} inhibitor) is used in combination with Binimetinib for the treatment of the same diseases, since their approval in 2018.⁷⁹ The importance of B-Raf as a therapeutic relevant target in polytherapies is also testified by the number of combinations including Vemurafenib, Dabrafenib and Encorafenib, with the MEK inhibitors Trametinib and Binimetinib, and Cetuximab that are currently under clinical evaluation, for example, against colorectal cancer (*e.g.*, [ClinicalTrials.gov](https://clinicaltrials.gov) identifiers: NCT03727763, NCT03693170 and NCT04673955). Besides, several clinical studies have also been reported on the investigation of mutant selective B-Raf inhibitors, with modulators of non-kinase proteins, one among the most studied being Hsp90 (*e.g.*, [ClinicalTrials.gov](https://clinicaltrials.gov) identifiers: NCT01657591 and NCT02721459).^{75–77} The selection of the most suitable targets for combination therapy approaches is generally driven by their involvement in relevant oncogenic processes. For example, several mechanisms by which tumor cells can exert drug resistance to B-Raf inhibitors derive by deregulation or overexpression of other oncoproteins,^{18–20,81,82} many of them being “*clients*” of Hsp90.^{83,84} Consequently, the simultaneous targeting of Hsp90 and B-Raf have represented an attractive strategy to overcome drug resistance to B-Raf inhibitors so far. Indeed, several biological studies and clinical evidence demonstrated that the inhibition of Hsp90 helps to overcome resistance to known blockers of B-Raf, and that their combined inhibition

provides synergistic effects in different cancer-related contexts.^{85–88} In line with the polypharmacology concept, further advantages can also be envisioned for cancer treatment on the design of ligands endowed with multi-target activity (Figure 1).

In the case of B-Raf, several studies describing the design of multi-target ligands have been reported so far.^{88–93} In particular, Anighoro *et al.*⁸⁸ reported in 2017 the identification of the first two compounds endowed with activity towards B-Raf and Hsp90, demonstrating that these targets share overlapping chemical spaces. The compounds reported in this study were identified by means of an integrated *in silico* strategy and represent valuable starting points for the development of innovative B-Raf/Hsp90 dual inhibitors, especially considering that they showed balanced multi-target activity and low molecular weight. Of note, Hsp90 and B-Raf belong to different protein families and present distinct binding site architectures, which makes the design of dual ligands of these proteins a difficult task. The interest around Hsp90 and B-Raf as partners in polypharmacology strategies has also been further explored more recently within an effort to identify ligands endowed with Hsp90/PDHK1/B-Raf multi-target activity.⁸⁹ The identification of compounds with such a tailored polypharmacology profile would enable the modulation of multiple pathways important to survival and proliferation of tumor cells, thus resulting in more effective anticancer therapies. However, the obtainment of Hsp90/PDHK1/B-Raf multi-target inhibitors is very challenging, as several, often conflicting, structural requirements should be taken into account in the ligand design process.

Besides, B-Raf has also been framed into multi-target ligand design projects including other kinases, such as VEGFR-2, p38 α and EGFR.^{90–93} The design of ligands modulating the activity of selected combination of kinases is particularly challenging. This mainly derives by the fact that the ATP-binding site of kinases is highly conserved in terms of shape and amino acids composition.³¹ However, structural peculiarities, and common anchoring points might also be identified and exploited for multi-kinase inhibitor design. In these cases, the design of ligands with a balanced multi-kinase activity profile is of primary importance to achieve good efficacy against, e.g., complex diseases, while retaining satisfactory safety profiles.⁹⁴ Moreover, the selection of the kinases to be considered for the multi-target ligand design should be guided by their therapeutic relevance and mutual involvement in the disease of interest.⁷⁵ In this regard, several studies have reported the identification of dual inhibitors of the B-Raf and VEGFR-2 kinases.^{92,95,96} The rationale behind the selection of B-Raf and VEGFR-2 for the development of multi-target inhibitors stands on the fact that these proteins fulfill complementary leading roles on processes related to cancer development and progression.^{97,98} For similar reasons, research efforts have also been performed for the design of B-Raf/p38 α dual inhibitors.^{93,99} Of note, continuous research has also been done for the targeting B-Raf and EGFR, either *via* combination of selective kinase inhibitors, or with polypharmacology ligands. For example, the inhibition of these targets has already been explored on colorectal cancer by means of combination of drugs, as drug resistance that derives by overexpression and activation of EGFR could be overcome through the blockage of B-Raf.^{20,100} The design of B-Raf/EGFR dual inhibitors has also been probed as a strategy to overcome drug resistance observed on melanoma and colorectal cancers to approved B-Raf^{V600E} drugs, providing promising results. Whereas reservations have been very recently raised on the dual inhibition of these targets as a therapeutic opportunity for patients affected by non-small cell lung cancer (NSCLC).¹⁰¹ Although particularly challenging, some possibilities on the identification of innovative B-Raf/EGFR dual inhibitors could also be envisioned on type III allosteric contexts. Indeed, B-Raf and EGFR present structurally similar type III allosteric pockets,⁶² which make them ideal candidates for the design of multi-target ligands, for example, by means of computational structure-based approaches as docking.^{102,103}

In recent years, increased research interests have also arisen around proteolysis targeting chimeras (*i.e.*, PROTACs) for targeting several therapeutic targets (Figure 1).^{14,104,105} Such an approach generally employs bidentate molecules bearing to two covalently bounded chemical moieties, one with high affinity towards the target of interest and the other recruiting specific components of the proteasomal degradation system (e.g., the E3 ligases Von Hippel-Lindau (VHL), cereblon (CRBN), murine double minute 2 (MDM2) and cellular inhibitor of apoptosis protein 1 (cIAP1)), to promote selective intracellular proteolysis.^{106,107} At present, PROTACs with high substrate specificity have been reported for targeting different protein kinases,¹⁴ including also mutant B-Raf.^{14,108,109} One example of such B-Raf mutant-selective PROTACs comes from a recent study by Alabi *et al.*,¹¹⁰ in which the authors designed a compound (*e.g.*, SJF-0628) showing high selectivity towards degradation of B-Raf^{V600E}. Although being still at their infancy, approaches based on the targeting of B-Raf with PROTACs technology are expected to provide novel valuable opportunities for cancer treatment and to help overcome drug resistances very often observed in marketed drugs, as recently discussed, for example, by He *et al.*¹¹¹ Indeed, such approaches allow to also promote complete removal of the protein scaffold other than blocking its catalytic functions, which might represent a valuable advantage over already reported ATP-competitive inhibitors.

Targeting protein kinases, such as B-Raf, has provided several therapeutic advantages in cancer treatment so far, as also testified by the number of approved drugs and clinical candidates modulating the activity of these proteins that are

currently under investigation.¹⁴ B-Raf has acted as a major player in this context, with some of its ATP-competitive inhibitors (*e.g.*, Sorafenib, Vemurafenib, Dabrafenib and Encorafenib) being approved for the treatment of patients with unresectable or metastatic melanoma in the last two decades.¹⁴ Although B-Raf^{V600E} inhibitors provided remarkable advantages in anticancer therapeutic regimens, several limitations could still be envisioned for these compounds, the most relevant being the establishment of drug resistance, paradoxical activation mediated by Raf dimerization and transactivation, and low efficacy towards Ras mutated cancers.⁴¹ Different strategies based on classic ATP-competitive single-target approaches are still being under study to overcome such limitations, some of them showing good promises.¹³ However, novel, and perhaps more valuable, opportunities can be envisioned on approaches targeting the allosteric sites of B-Raf proteins. Indeed, such approaches have already demonstrated to provide remarkable opportunities on other therapeutic-relevant kinases exhibiting high structural similarity with B-Raf.⁵⁹ Moreover, the activity of allosteric kinase inhibitors is less prone to be affected by insurgence of drug resistance deriving by site point mutations with respect to ATP-competitive binders,⁴⁸ this being a significant advantage in anticancer therapy. Importantly, the modulation of B-Raf by means of allosteric ligands would open to complementary approaches including also already reported ATP-competitive inhibitors to promote more efficient arrest the kinase activity, which is expected to result in improved therapeutic outcomes.^{50,51} In the near future, increasing research efforts will also be addressed towards the identification of multi-target inhibitors modulating B-Raf activity. Indeed, the importance of polypharmacology approaches for kinase targeting is well established,¹¹² as also testified by the increasing number of dual inhibitors targeting B-Raf reported in the literature over the last years.^{88–93} The selection of suitable combinations of targets for the rational design of B-Raf polypharmacology ligands is of primary importance in this context, the identification of those providing the highest therapeutic effectiveness being very often difficult. However, innovations on computational approaches are expected to aid on their identification.¹¹³ Similar considerations can also be drawn for the identification of inhibitors targeting B-Raf through innovative mechanisms among already approved drugs, as recently observed for Ponatinib.⁶³ Moreover, approaches based on PROTACs technology are also expected to bring significant chemical novelty on future B-Raf inhibitors design, as such compounds exert their activity through molecular mechanisms that are completely different with respect to those of approved drugs and compounds under investigation. The design of either allosteric, polypharmacology or PROTAC ligands targeting B-Raf proteins is challenging, especially with respect of classic kinase ATP-competitive binders. However, the recent advancements on understanding cancer cells biology and the improvements on experimental techniques and *in silico* approaches available for the analysis of information reported in public databases, will certainly facilitate the identification of novel B-Raf inhibitors acting through such innovative mechanisms.

Data availability

No data are associated with this article.

Author contributions

L.P.: Conceptualization, Writing – Original Draft Preparation, Writing – Review & Editing.

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Andrew Anighoro 

Relation Therapeutics LTD, London, UK

The author has appropriately and extensively addressed my previous review. I have no further comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Drug discovery, computational chemistry.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 15 March 2022

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Andrew Anighoro 

Relation Therapeutics LTD, London, UK

The author gives an overview of B-Raf as a known target for cancer related diseases. Then, provides an account of design strategies that may overcome some limitations of typical ATP competitive drugs.

- The author mostly refers to B-Raf as a target validated by "recent" evidence/studies, while it has been described in the literature as an oncogene at least from the 90s and Vemurafenib began clinical testing in 2008 to be approved in 2011 by FDA followed shortly by other drugs. I don't think this makes the article less interesting, but I feel that B-Raf should be referred sometimes in the text with adjectives such as "well established" rather than "recent".
- Would it be possible to give the reader an idea of how the α C-helix is structurally related to the ATP binding site? Maybe through a sentence in the text and/or marking it in Figure 1?
- When writing "*activation loop*", "*activation segment*" and " *α C-helix*" are italics and quotes necessary? α C-helix has already been used several times in the text up until then without quotes or italics.
- I think that the sentence "*recent advancements in crystallographic and in silico techniques will certainly help to overcome several of the issues currently encountered in kinase allosteric ligand design*" should be followed by at least mentioning some examples of techniques that the author is certain are going to help.
- Can it be explained more explicitly why Ponatinib/PHI1 do not qualify as allosteric inhibitors?
- Would it be possible to include a comment on the challenge of rationally designing kinase multitarget inhibitors given the selectivity issues mentioned by the author?
- The first time that the term PROTAC is mentioned, it should be by the full name followed by the abbreviation, proteolysis targeting chimera (PROTAC).
- "(e.g., an E3 ligase as VHL)" Probably, CRBN should be mentioned as well. Both should be mentioned by their full name first.
- Probably, "e.g." in Figure 1 when addressing E3 ligases can be removed as it makes it look like E3 ligases can be replaced by something else in the context of PROTACs. I am not aware if that's actually possible, but if that's the case, it should be mentioned in the text.
- In the abstract and in the main text the word premises is used a few times instead of promise, I believe.

Is the topic of the opinion article discussed accurately in the context of the current literature?

Yes

Are all factual statements correct and adequately supported by citations?

Yes

Are arguments sufficiently supported by evidence from the published literature?

Yes

Are the conclusions drawn balanced and justified on the basis of the presented arguments?

Partly

Competing Interests: No competing interests were disclosed.**Reviewer Expertise:** Drug discovery, computational chemistry.**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 16 Apr 2022

Luca Pinzi, University of Modena and Reggio Emilia, Modena, Italy

I would like to thank the Reviewer for the comments and the precious suggestions. The manuscript was revised to better contextualize the development of innovative B-Raf targeting approaches, with respect to established efforts (e.g., ATP-competitive inhibitors development). Accordingly, the descriptions referring to literature data were also revised under a more contemporary perspective.

A brief description of the main elements characterizing the ATP-binding site of the kinases was reported in the revised version of the manuscript. This helped to delineate how the regulatory α C-helix is related to the other structural regions in the kinases ATP binding site. Besides, Figure 1 was also revised to better highlight the position of the α C-helix, with respect to the hinge region in the B-Raf active site to which ATP-competitive inhibitors bind to, and to improve the image contents.

The text was also revised to provide further insights on some of the experimental techniques and *in silico* approaches that are (and are also expected to be even more in the future) of help for kinase allosteric drug design efforts. Moreover, additional references to relevant literature data were included in the revised version of the manuscript to better contextualize the application of some of these techniques, on both B-Raf related research and a more general perspective.

An explanation of how Ponatinib and PHI1 can be classified according to their experimentally derived binding mode is now provided in the text, as suggested by the Reviewer. As revised, the text is expected to help better contextualize the example reported on Ponatinib/PHI1, with respect to the type III B-Raf allosteric pocket mentioned within this article.

The main challenges that might be faced when rationally designing multi-kinase inhibitors are now discussed in the text. Moreover, the main E3 ligases that have been exploited so far for the design of PROTACs are now reported within the text.

The manuscript was carefully revised, to remove typos and to make it clearer to readers. Moreover, the full names of the reported abbreviations were added within the text where needed.

Competing Interests: No competing interests were disclosed.

Reviewer Report 04 March 2022

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Federico Falchi 

Laboratory of Computational Medicinal Chemistry, Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy

In this nice perspective, Pinzi summarizes the current state-of-the-art in the development of B-Raf inhibitors acting through innovative mechanisms. After a brief introduction about the traditional inhibitors (Atp-competitive) with their big limitations, Pinzi describes the most challenging approaches such as the design of allosteric modulators, the polypharmacology, and the PROTACs design with an eye to the computational strategies.

The manuscript is well understandable, offering both a biological and medicinal chemistry point of view. The references are adequate.

I do believe that work can be accepted certainly for indexing.

I have only a very few minor remarks:

1. The sentence below is too long:

Besides, other studies have been focused on the development of compounds as PLX7904 and PLX8394, able to escape paradoxical activation of B-Raf (i.e., the so-called “paradox breakers”) and to overcome some of known resistance mechanisms associated to previously reported Raf inhibitors,^{36,37} the latter ligand currently being evaluated in Phase I/IIa clinical trials (ClinicalTrials.gov identifier: NCT02428712).

Please change for example as:

Besides, other studies have been focused on the development of compounds such as PLX7904 and PLX8394, able to escape paradoxical activation of B-Raf (i.e., the so-called “paradox breakers”) and to overcome some of the known resistance mechanisms associated with previously reported Raf inhibitors.

PLX8394 is currently being evaluated in Phase I/IIa clinical trials (ClinicalTrials.gov identifier: NCT02428712).

2. The sentence below is too long:

Nevertheless, the results prospected in this study paved the way towards the identification of innovative B-Raf inhibitors among approved drugs (i.e., drug repurposing) (Figure 1), this approach being already navigated also with natural products and clinically safe candidates, on different medicinal chemistry research areas

Please change for example as:

Nevertheless, the results prospected in this study paved the way towards the identification of innovative B-Raf inhibitors among approved drugs (i.e., drug repurposing) (Figure 1). This approach is already been conducted also with natural products and clinically safe candidates in different medicinal chemistry research areas.

3. The abbreviation NSCLC is not straightforward, the first time an abbreviation is used it should be explained.
4. In the image:
 - a B in B-RAF is missing
 - the bullets points are not all the same
 - sometimes a period is used at the end of the sentence, other times not

Is the topic of the opinion article discussed accurately in the context of the current literature?

Yes

Are all factual statements correct and adequately supported by citations?

Yes

Are arguments sufficiently supported by evidence from the published literature?

Yes

Are the conclusions drawn balanced and justified on the basis of the presented arguments?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Drug design and drug development by means of computational techniques.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 16 Apr 2022

Luca Pinzi, University of Modena and Reggio Emilia, Modena, Italy

I would like to thank the Reviewer for their precious comments. The manuscript was carefully revised, and long sentences split to make the text clearer to readers, in agreement

to the Reviewer suggestions. Moreover, the full name of the reported abbreviations was added in the text. Figure 1 was revised to remove typos as suggested and to improve the image contents.

Competing Interests: No competing interests were disclosed.

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