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Invited Review

Biological targets and therapeutic strategies in the treatment of non-functioning pituitary tumors: state-of-the-art

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Abstract

Non-functioning pituitary neuroendocrine tumors (NF-PitNETs) represent one of the most common and heterogenous sellar pathology, and are nowadays still orphan of an effective pharmacological therapy that could substitute surgery or support in case of persistency/recurrence. In fact, despite NF-PitNETs are generally benign tumors, they can be characterized by supra- or extra-sellar growth with invasion of the cavernous sinus and/or involvement of the optical chiasm. For these reasons, lots of studies tried to unveil the molecular mechanisms underlying resistance and tumoral cells growth, so as to identify new possible therapeutic targets and novel drugs that could improve NF-PitNETs treatment.

This review aims to summarize the new biological targets and therapeutic strategies that have been proposed so far, trying to give rise to novel approaches and paving the way for personalized strategies for patients harboring NF-PitNETs.

In addition, further studies related to the complex mechanisms involved in NF-PitNETs resistance to the available therapies could provide the basis for the development of both prognostic biomarkers useful for patients' management and novel targets for the pharmacological treatment of these tumors.

Keywords non-functioning pituitary tumors (NF-PitNETs), pharmacological resistance, biomarkers, G-protein coupled receptors (GPCRs)

Non-functioning pituitary tumors

Pituitary neuroendocrine tumors (PitNETs) represent the most common pathology of the anterior pituitary, and the 10%-25% of all intracranial tumors, affecting up to the 5% of the general population.[1] Different subtypes are distinguished on the base of hypersecreted hormones, even if the 33% of PitNETs are classified as clinically non-functioning PitNETs (NF-PitNETs), so including all not hormonally active tumors. NF-PitNETs represent the most frequent subtype of PitNET after prolactin (PRL)-secreting ones that account for around 40%. PitNETs classification criteria lineage-restricted is based on pituitary transcription factors, as stated by the 5th WHO classification.[2] With respect to NF-PitNETs subtype, a preponderance of gonadotropic lineage, stated by steroidogenic factor 1 (SF1) positivity expression, was observed.[3] In fact, approximately 80%-90% of NF-PitNETs develop from gonadotrophic lineage, which account for 40%-50% of all pituitary macroadenomas.[4] However, while for secreting PitNETs the treatment with somatostatin receptor ligands (SRLs), dopamine agonists (DAs) or other drugs (eg, pegvisomant) represent a chance as additive pharmacological therapy, NF-PitNETs are still lacking of an effective pharmacological approach, and transsphenoidal surgery represents the only curative option to date. Moreover, excess mortality was observed in patients with NF-PitNETs, regardless of pituitary function, especially in women and in patients with a younger age at diagnosis[5] and since recurrence and relapse are very frequent events, the finding of novel therapeutic options is still needed together with a deeper understanding of molecular mechanisms underlying drug resistance.

Dopamine receptor type 2 and somatostatin receptors

Biological functions

The principal targets for PitNETs' pharmacological therapy are dopamine receptor type 2 (DRD2) and somatostatin receptors (SSTs). These are G protein-coupled receptors (GPCR) that lead to adenylyl cyclase inhibition with consequent decrease of cyclic AMP (cAMP) resulting in hormone secretion and cell proliferation decrease. Among the 5 dopamine receptor subtypes (DRD1–5),

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DRD2 is predominantly expressed in PitNETs. Two DRD2 isoforms generated by alternative splicing have been identified: D2S (short) and D2L (long)[6] with different physiological functions.[7-9] A role of DRD2 in mediating an inhibitory effect on NF-PitNET cells proliferation,[10,11] migration and invasion[12] was demonstrated. Specifically, DRD2-mediated antimitotic effects are exerted by both an increase in extracellular signal-regulated kinase (ERK)[13] and decrease of protein kinase-B (AKT) activity.[11]

Similarly, also SSTs family comprises 5 receptor subtypes (SST1-5), classified on structural properties and pharmacological profiles, including different affinity to SRLs. All SSTs mediate inhibitory actions on cell proliferation, secretion, and migration by association with inhibitory G proteins.[14-18] Among them, SST2 and SST5 represent primary targets for functioning PitNETs' treatment, since after ligand binding, both trigger several intracellular pathways.[19] Although all SSTs are detected in NF-PitNETs, the most abundantly expressed subtype is SST3.[20]

DRD2 and SSTs as pharmacological targets for NF-PitNETs therapy

DRD2 represents the main target of pharmacological therapy with DAs in PRL-PitNETs resulting effective in both normalizing PRL levels and obtaining tumor shrinkage, constituting the main first-line treatment, with only a 10% of patients resistant to DAs.[21]

The attempt to test the treatment with DAs in other DRD2 expressing-PitNETs subtypes has been carried out through several years, with lots of works present in literature especially reporting data for combined therapies with other drugs. An efficient combined treatment of the DRD2 agonist cabergoline and low-dose pegvisomant[22] or high-dose octreotide[23] in acromegaly patients has been unveiled. Moreover, a role of DAs in controlling remission in patients with mild Cushing disease was shown.[24,25]

Several attempts have also been made in NF-PitNETs, showing a potential broader application of DAs. Numerous studies, both in vitro[11,26,27] and in vivo,[28-30] have been published, as well summarized in a review by Cooper and co-authors.[31] Interesting data emerged from a 2-year randomized clinical trial involving 144 patients that demonstrated cabergoline treatment's effectiveness in reducing NF-PitNETs residual after surgery, with a high rate of tumor shrinkage, revealing potential usefulness in decreasing relapsing rate.[32] An interesting meta-analysis including 5 studies (187 patients) showed that cabergoline treatment determined NF-PitNETs tumor shrinkage only in 19% (95% CI 8%-38%, 4 studies, 108 patients), while prevention of tumor progression after surgery was seen in half of the cases (95% CI 35%-64%, 5 studies, 187 patients).[33]

Nevertheless, although some clinical benefits have been shown, a strong support for the use of DAs in NF-PitNETs is lacking. In fact, in striking contrast with the excellent therapeutic response in PRL-PitNETs and despite the majority of NF-PitNETs express functional DRD2,[20] published data are controversial in demonstrating DAs efficacy on tumor growth control,[22,31,34,35] facing poor feedback in clinical applications. Moreover, no correlation between DAs clinical responsiveness and DRD2 (or its isoforms D2L/D2S) expression was found. At this regard, it's important to state that the success of the therapy requires the proper signal transduction machinery functioning, as well as a correct DRD2 intracellular trafficking, suggesting the presence of post-receptor alterations underlying resistance.

A study conducted in a cohort of 113 NF-PitNET tissues revealed that the SST3 is the most abundantly SSTs expressed, even if SST2 and SST5 displayed appreciable expression levels, opening a window to combined therapies targeting different receptors subtypes.[20] SRLs treatment was tested in NF-PitNETs both *in vitro* and *in vivo*.[36] Indeed, there are data showing the SRL analog pasireotide (with high SST5 affinity) ability in reducing *in vitro* NF-PitNET cells viability and VEGF secretion.[37,38] These results were strengthened by the phase 2 trial PASSION-I, a single-arm study, that confirmed pasireotide ability in determining tumor mass shrinkage in 16.7% of patients [trial: NCT01283542].[39] Additionally, a double-blind clinical trial, named GALANT, in which patients with macro NF-PitNETs, either surgery-naive or with post-operative remnant ≥10 mm, have been randomized trough ^{68}Ga-DOTATATE PET/CT, showed that the SRL treatment was ineffective in reducing tumoral mass, in both surgery-naïve or operated patients.[40]

Likewise, few clinical trials have been conducted to evaluate octreotide potential effects in NF-PitNETs patients.[41-45] However, a significant reduction in tumor size was obtained in just 11%-13% of patients and a significant shrinkage (>30% of baseline size) was achieved in one study only,[44] with the majority having stable remnant tumors.[43] Another case-control study that needs to be mentioned is the one performed by Fusco and co-authors,[46] in which the effect of long-term octreotide treatment in 39 patients with NF-PitNETs was analyzed. The treatment with octreotide was associated with stabilization of postsurgical NF-PitNETs tumor residual in 81% of cases but with limited effect on tumor shrinkage.[46]

Nevertheless, no associations were found between SSTs and DRDs expression and relevant clinical or molecular features of NF-PitNETs.[20] Additionally, SRLs and DAs have been proposed to be considered as an adjunctive treatment to radiotherapy in aggressive gonadotropinomas in which surgery is not indicated nor curative.[47] The treatment of NF-PitNET primary cultures with the chimeric compound BIM-23A760, which binds with high affinity SST2 > SST5 and DRD2 showed antiproliferative[13,48,49] and/or pro-apoptotic action, by activating ERK1/2 and p38 pathways and caspase-3.[13] Furthermore, a case report showed in 20% of NF-PitNET's tumoral mass reduction induced by octreotide and cabergoline co-treatment.[50] However, despite NF-PitNETs expressing variable receptor levels, no effective pharmacological approaches involving the use of SRLs or DAs have matched clinical efficacy to date.

Molecular players involved in biological behavior and pharmacological resistance

There are multiple mechanisms underlying NF-PitNETs resistance to conventional treatments, and many works aimed to find successful pharmacological approaches highlighted the possible players involved in this tangled maze. The role of these different players is summarized here below (Figure 1).

β-arrestins

β-arrestin 1 and 2 are scaffold proteins involved in GPCRs desensitization, internalization, and signal transduction,[51-54] that are

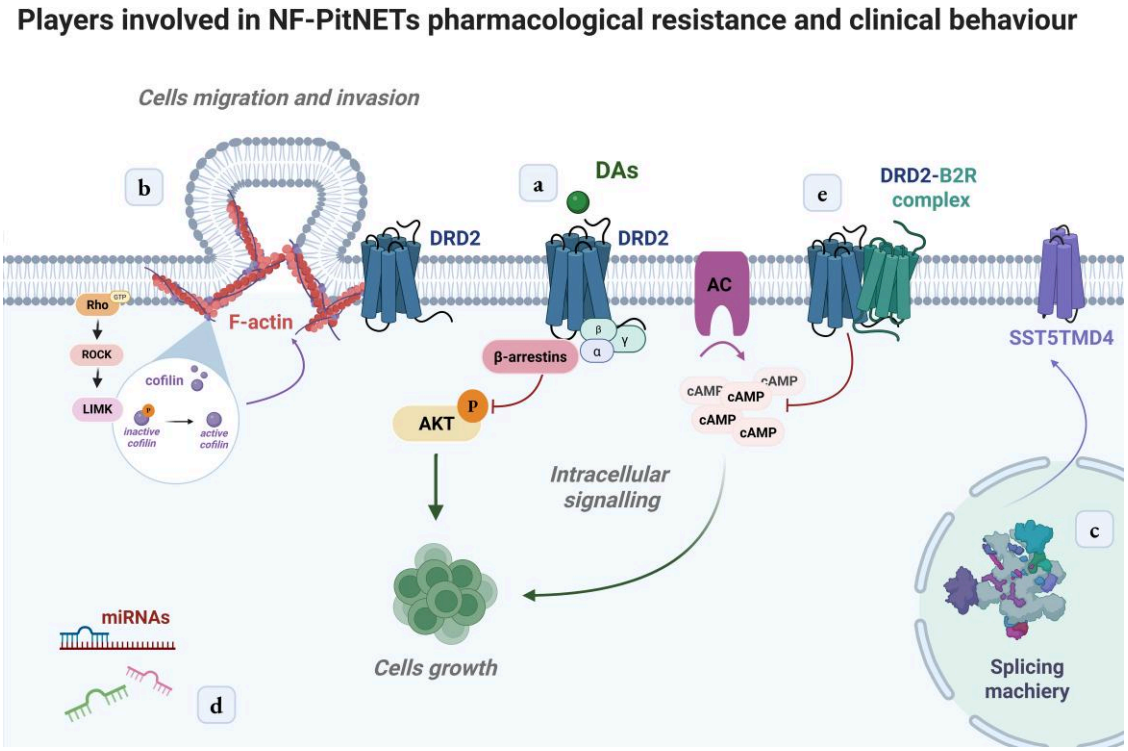

Figure 1 Different players involved in NF-PitNETs resistance and behavior. (A) NF-PitNETs cells proliferation inhibition after AKT inhibition through a β-arrestin 2-dependent mechanism after DRD2 activation. (B) Activated cofilin promoted NF-PitNETs cells migration. (C) splicing machinery involvement in the production of different SSTs variants. (D) miRNAs different expression levels related to different NF-PitNETs clinical features. (E) DRD2-B2R complex influence in NF-PitNETs intracellular signaling. Both the Figures have been made by the first author, that is the holder.

recruited to the plasma membrane by ligand-activated and phosphorylated receptors, leading to G protein mediated signal termination and receptor targeting for endocytosis.[51,55,56] Furthermore, β-arrestins are molecular mediators that lead to intracellular pathway activation, encouraging the study of correlations between their expression and treatment's responsiveness in NF-PitNETs.

At this regard, β-arrestin 2 has been proposed as molecular marker associated with *in vitro* NF-PitNETs responsiveness to DAs[11] (Figure 1A). It was identified as fundamental player leading to cabergoline antimitotic action, through DRD2 activation in NF-PitNETs primary cultured cells (Figure 2A). In fact, the lack of β-arrestin 2 prevented DRD2 inhibitory effect on AKT pathway with a consequent resistance to DAs antimitotic action. This effect was restored by β-arrestin 2 overexpression in tumors that were previously lacking. More specifically, NF-PitNETs have been divided in 2 different subgroups, based on AKT activity's inhibition after DAs treatment. DRD2 inhibited AKT phosphorylation in about 30% of "responsive" tumors, resulting ineffective in the "resistant" group. Western blot analysis showed that β-arrestin 2 was expressed by the 80% of "responsive" NF-PitNETs, whereas it was undetectable in the majority of "resistant" ones.[11] Furthermore, β-arrestin 1 protein expression was also analyzed, resulting expressed in all NF-PitNETs samples tested ($n = 41$), strengthening the specific involvement of β-arrestin 2 in DRD2-mediated AKT dephosphorylation.[11]

β-arrestins expression was also previously tested at the mRNA level in a smaller NF-PitNETs cohort and very high β-arrestin 2 mRNA expression, compared to β-arrestin 1, has been detected.[57]

Overall, these studies suggested that β-arrestin 2 could represent a novel biomarker predicting NF-PitNETs responsiveness to DAs. However, further analysis is needed to assess its expression by immunohistochemistry in a large cohort of patients in order to correlate these data with clinical responses and validate its usefulness.

Cofilin

Another player that has been demonstrated to be involved in PitNETs pharmacological resistance is the cytoskeleton and its associated proteins, such as the actin-binding protein cofilin.[19] Cofilin is a small protein that promotes new actin filaments polymerization leading to cells migration, by both increasing the number of free barbed ends and by providing actin monomers.[58] A possible correlation between cofilin expression and PitNETs invasive behavior has been investigated. At this regard, since cofilin activity is tightly and negatively regulated by phosphorylation on Ser3, expression levels of active/inactive cofilin were studied. It was shown that a low ratio of phosphorylated cofilin (p-cofilin) correlated with NF-PitNETs local invasiveness, leading to a reduction of surgery's success and increased tumor recurrences. In fact, immunohistochemistry analysis showed that p-cofilin levels were higher in non-invasive NF-PitNETs with respect to the invasive ones, in which very low or absent cofilin was detected.[12] Another work analyzed at immunohistochemistry level cofilin and p-cofilin expression in a large number of NF-PitNETs ($n = 55$). Considering the overall cohort, the increase of cofilin

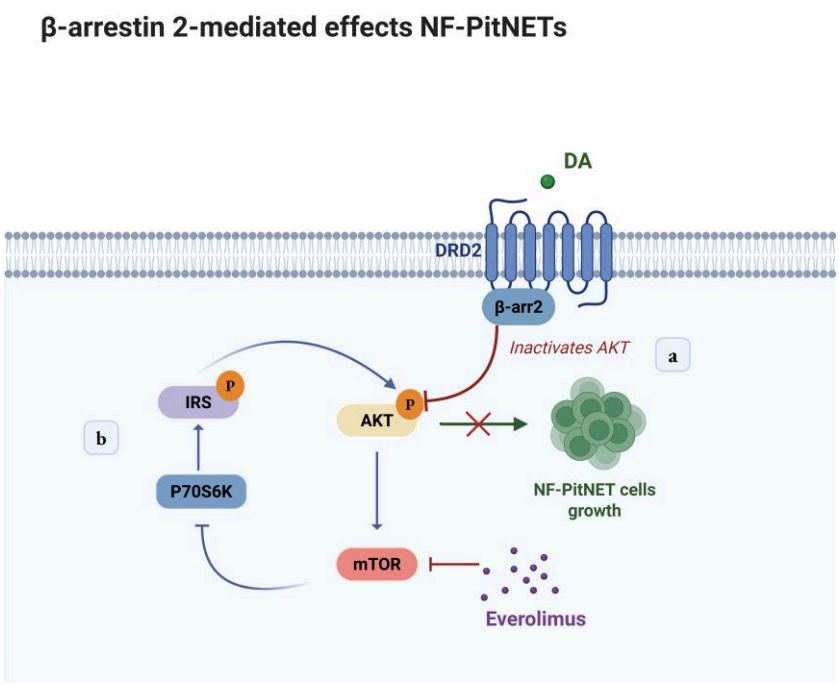

Figure 2 Role of β-arrestin 2 in mediating NF-PitNETs responsiveness to DAs. (A) β-arrestin 2 inhibitory effect on AKT pathway with a consequent NF-PitNET cells proliferation inhibition, after DRD2 activation. (B) β-arrestin 2-mediated AKT upstream inhibition to block the escape mechanism induced by everolimus treatment in NF-PitNET cells. Both the Figures have been made by the first author, that is the holder.

expression correlated with tumor size, but not with invasiveness. On the other hand, considering the subgroup of invasive tumors, an association with recurrence and increase of cofilin expression was found.[59] Therefore, p-cofilin was proposed as potential molecular marker associated with NF-PitNETs invasive behavior (Figure 1B).

Splicing machinery

Lately, an involvement of the splicing machinery and its associated factors in tumor formation and progression emerged. A severe dysregulation of splicing machinery components in all PitNETs subtypes compared to normal pituitary was observed and possible alterations that might underline pharmacological resistance or intracellular pathway alterations have been investigated in NF-PitNETs. A study by Vasquez-Borrego simultaneously analyzed 42 splicing machinery's components in a large NF-PitNETs cohort ($n = 88$) and a marked dysregulation of several splicing factors in tumoral samples with respect to normal pituitary was detected.[60] A significant downregulation of 4 major and 2 minors spliceosome components, other than 12 splicing factors (among which SRSF1), was found. Specifically, a direct correlation with SRSF1 mRNA levels and aberrant splicing variant of SST5 (SST5TMD4), generated by non-canonical SST5 splicing was unveiled.[60,61] It was shown that SST5TMD4 contributes to oncogenesis, determining an increase of malignant features and hinders response to SRLs treatment in gonadotrophs[62] (Figure 1C). Notably, the analysis of clinical features of the tested NF-PitNETs samples highlighted an association between the splicing factor SRRM4 and higher chiasmatic compression. The detection of these alterations opened a window on testing the effects of small molecules regulating splicing components.

Bradykinin type 2 and 5-hydroxytryptamine receptor 2B *receptors*

Another challenging contribution in understanding the mechanisms involved in NF-PitNETs resistance to standard therapies was given by a recent study on bradykinin type 2 receptor (B2R) found overexpressed in NF-PitNETs[63] and its broad involvement in pituitary tumorigenesis was suggested. B2R is a GPCR ubiquitously expressed in different cells of the nervous systems that leads to an increase in intracellular Ca^{2+} essential for the initiation of signal transduction.[64] Interestingly, B2R-DRD2 complex formation in human NF-PitNETs was demonstrated,[65] implying a B2R activation interference with DRD2 response to DAs in NF-PitNETs promoting pharmacological resistance. In B2R-DRD2 complexes, B2R stimulation profoundly alters DRD2 signaling, disrupting DRD2-mediated inhibition of cAMP accumulation. In this perspective, the authors suggested that DRD2 signaling could be restored by blocking B2R activation, contributing to fill a possible lack of knowledge in DA-resistance in NF-PitNET, thus opening perspectives of potential new therapies in these tumors[65] (Figure 1E).

One more interesting study unveiled the role of 5-hydroxytryptamine receptor 2B receptor (HTR2B), a GPCR found upregulated in SF1 positive- NF-PitNETs.[66] Particularly, HTR2B was associated with NF-PitNETs growth other than an enhancement of cabergoline's inhibitory effects on PitNET tumoral cells viability. In this direction, Lin and coauthors proposed HTR2B as a novel therapeutic target for NF-PitNETs, particularly elucidating that its inhibition might improve cabergoline's efficacy in NF-PitNETs.[66]

miRNAs

Another chapter on which a light was shed is represented by micro-ribonucleic acids (miRNAs), whose role and involvement in therapeutic outcome has been largely studied in different types of tumors, among which PitNETs. miRNAs are small non-coding RNAs that regulate post-transcriptional protein-coding genes expression by targeting mRNA. A narrative review greatly summed up the 106 miRNAs found dysregulated in NF-PitNETs and linked them back to target genes involved in both cell cycle regulation or signaling pathways.[67] Specifically, a study identified the involvement of differentially expressed miRNAs in NF-PitNETs pathogenesis, showing their implication in pathways related to tumoral cells apoptosis and proliferation.[68] Since miRNAs expression downstream effects were unveiled,[69] deeper studies on their association with clinical features could open novel perspectives for patients with NF-PitNETs. A miRNA microarray analysis identified different methylome profiles between invasive and non-invasive tumors, highlighting hsa-miR-191-3p upregulated expression in invasive tumors.[70] A deregulation in miR-210-3p, miR-149-3p, and miR-29b-3p in invasive compared to non-invasive NF-PitNETs was detected.[71] On the contrary, miR-145-5p overexpression negatively correlating with NF-PitNETs invasiveness.[72] Of note, the same authors showed how CircOMA1 promotes NF-PitNETs progression by acting as the sponge of tumor suppressor miR-145-5p to regulate the tumor protein translationally controlled 1 (TPT1) signaling pathway, revealing it as a therapeutic target in preventing tumorigenesis. Another study found a significative correlation between miR-508-5p deregulation and aggressive clinical behavior[68] and miRNA-17, miR-19, miR-106A, and miR-20 have been correlated with NF-PitNETs recurrence.[71] Specific miRNA alterations were detected between GH-, ACTH-, PRL-secreting PitNETs, and NF-PitNETs in which miR-149-3p, miR-130a-3p, and miR-370-3p were significantly under expressed as compared to functioning tumors.[68] Furthermore, some miRNAs expression was demonstrated to be helpful in monitoring tumor regrowth and recurrence.[73] In this context, their use as biomarkers through liquid biopsy was proposed to detect early stage NF-PitNETs[67] or the use of miRNA sponges or competing endogenous RNA, thereby blocking their regulatory effect.[74]

To resume, the differentially expressed miRNAs may provide a new insight as biomarkers for NF-PitNETs (Figure 1D), uncovering the potential of their expression to tumorigenesis, recurrence and treatment. Applicative ways to regulate miRNAs expression have been summarized in a review article,[75] in which all the methods available to date have been clearly listed helping to develop novel applicative pharmacological strategies. Undoubtedly, this represents a challenging alternative starting point for deeper studies aimed at pharmacologically targeting or inhibiting miRNA expression.

Tumoral microenvironment

Another fundamental player that was unveiled to be involved in regulating responsiveness to pharmacological treatment is tumoral microenvironment. More specifically, several tumoral microenvironment's components, such as tumor-associated macrophages (TAM) and fibroblasts, tumor-infiltrating lymphocytes, folliculostellate cells, angiogenesis, as well as the extracellular matrix (ECM) and its remodeling, have been demonstrated to have an intricate role in PitNETs. Of interest, they have been linked to PitNETs hormone secretion, dimension, invasion, proliferation, progression, recurrence, other than therapeutical response. A detailed review by Ilie and co-authors carefully described main tumoral microenvironment's components in PitNETs.[76] Interestingly, the same group deeply study the role of folliculostellate cells in gonadotroph tumors, elucidating the importance of SB100-expressing cells, associating them with lower proliferative rate and positively correlated with estrogen receptor alpha (ERα) expression.[77] Moreover, their spatial associations suggest that S100B + folliculostellate cells could play a role in gonadotroph tumorigenesis, giving a contribute to the maintenance of tumoral cells in a low proliferating, FSH+/ERα + differentiated state.[77] In this connection, other evidences outlined that differences in gonadotroph tumors, mainly related to FSH-LH positivity, may be implicated in resistance to treatments.[78] Specifically, SST2, SST5, and ERα differential expression in gonadotroph subgroups was analyzed, opening the door to consider whether the histological subtype may be a predictor of response to SSTs ligands treatment. Furthermore, ERα moderately correlated with SST2 expression and with FSH % in all tumor analyzed.[78]

Additionally, the cytokine network and the immune cell infiltrates within PitNETs tumoral microenvironment have been characterized. Specifically, it was outlined that NF-PitNETs secreted more cytokines and had 9 times more neutrophils than somatotropinomas; moreover, NF-PitNETs did not show increased CD3+, CD8+, CD4+, and FoxP3+ infiltrates compared to secreting-PitNETs.[79]

In addition, some specific associations related to NF-PitNETs invasive behavior have been highlighted: NF-PitNETs cavernous sinus invasion was associated with higher number of TAM, specifically those CD163+, higher FoxP3/CD8 ratio, and increase of programmed death-ligand 1 (PD-L1) expression. Another characteristic of invasive NF-PitNETs that has been observed was high expression of VEGF-A, VEGFR1, and associated immunosuppressive microenvironment, suggesting that novel targeted therapies could be applied.[80]

TAMs, that polarize to M1 and M2 subtypes exerting antitumoral and pro-tumoral effects, respectively, have also been analyzed in NF-PitNET: M2-like/M1-like TAMs ratio >1 was identified, suggesting that their recruitment and polarization into the pro-tumoral M2 subtype drives NF-PitNET proliferation and invasion.[81] Accordingly, cultured M2 macrophages promoted greater invasion and proliferation of primary NF-PitNET cultured cells.[81]

Accordingly, a challenging association that need to be mentioned is the one outlined by Principe and co-authors: they unveil a link between gonadotroph tumors invasiveness and macrophages.[82] Specifically, they demonstrated that gonadotroph PitNETs drive the recruitment of macrophages and their subsequent polarization to an M2-like phenotype and present an increased CD68+ macrophage signature with respect to secreting-PitNETs, unveiling specific infiltrating M2 macrophages-associated markers (CD163, MRC-1, ARG1, and CSF1R M2). Moreover, the association between infiltrating CD68+/CD163+ macrophages and the invasiveness of gonadotroph tumors points to macrophage-targeted immunotherapies being a potent strategy to limit the progression of gonadotroph PitNETs.[82] The percentage of CD68+ TAMs was also positively correlated with NF-PitNETs size.[83]

Additionally, invasiveness of hormone-negative PitNETs was associated with high expression of the glycoprotein endothelial cell-specific molecule-1 (ESM-1).[84]

Another interesting chapter that faced interest in studying NF-PitNETs' invasiveness and responsiveness to medical treatment are markers associated with epithelial-mesenchymal transition (EMT). EMT is a phenomenon that was showed to be more frequent in NF-PitNETs compared to somatotropinomas and has been linked to the clinical course of these tumors.[79,85] Furthermore, it has already been described that EMT genes correlate with invasive PitNETs as well as with angiogenesis and extracellular matrix (ECM) degrading genes.[86] Furthermore, EMT has been described as a cause of resistance to medical treatment in PitNETs, in particular to SRLs,[87] thus, the evaluation of biomarkers associated to EMT in PitNETs, such as SNAIL1, PITX2 E-cadherin, or vimentin, would seem to be recommendable in case of considering novel challenging medical treatments.[85,88,89]

With respect to ECM remodeling, numerous studies have looked at matrix metalloproteinases, enzymes that degrade and remodel the ECM, specifically MM-P9 expression that has been associate with angiogenesis and progression, was associated with NF-PitNETs recurrence.[90]

Novel approaches for NF-PitNETs treatment

DRD2-biased agonists

An additional alternative approach to overcome NF-PitNETs pharmacological resistance could be the use of DRD2-biased agonists, able to selectively activate β-arrestin or G-proteins pathway.

Since β-arrestin 2 role in exerting antimitotic action through DRD2 activation was demonstrated,[11] a recent work explored the possible use of specific DRD2-agonists, UNC9994 and MLS1547, able to preferentially activate β-arrestins and G-proteins pathway, respectively.[27]

In tumoral lactotrophs, UNC9994 was more effective in reducing cell proliferation and PRL secretion compared to cabergoline, which activates both β-arrestin and G-proteins pathway, hypothesizing that its effectiveness is due to the activation of the β-arrestin pathway, with consequent reduction of p-AKT. In PRL-PitNETs, cAMP exerts an inhibitory role on cell proliferation,[91] and the inhibitory G protein pathway activated by cabergoline might counteract the antiproliferative effect induced by β-arrestin 2.

On the other hand, in NF-PitNETs, DRD2-biased agonists did not exert a more efficient antimitotic action with respect to cabergoline. Indeed, it was demonstrated that both unbiased (cabergoline) and biased DRD2 ligands reduced cell proliferation to similar extents in 4/23 NF-PitNETs, whereas 13 tumors were resistant to all treatments. In the remaining tumors, a heterogeneity of responsiveness was observed.[27] The best result was obtained with cabergoline, which induced an inhibition of cells proliferation in about 43% of tumors, in contrast with the 26% of tumors responsive to UNC9994. No correlations have been found between ligand efficacy and lineage derivation, nor DRD2 and β-arrestin 2 transcript expression.[27] Nevertheless, this study suggests that the identification of novel biomarkers predictive of responsiveness to biased DRD2 agonists might be useful to develop more personalized therapies. In particular, agents such as UNC9994 or other highly specific biased DRD2 agonists may offer therapeutic benefits for a subset of patients with NF-PitNETs who are likely to respond to their effects.

Drugs targeting somatostatin receptors

Since previous analysis showed SST3 predominant expression in NF-PitNETs, this receptor was also investigated as pharmacological target for NF-PitNETs. A SST3 peptide agonist (BIM-355) inhibited NF-PitNET cells viability and growth, although it resulted effective just in tumors characterized by high SST3 expression.[60] Furthermore, a pancyclic hexapeptide SRL with affinity to all SSTs subtypes, ITF2984, has been identified and showed a 10-fold improved affinity for SST3 compared to octreotide or pasireotide.[92] Two different clinical studies in healthy patients showed that ITF2984 was safe and well tolerated. In addition, the drug was also tested phase II clinical trial in acromegalic patients.[92,93]

Moreover, it was showed that ITF2984 displays antitumor activity in MENX (homozygous mutant) NF-PitNETs rat model,[92] and a sex-related difference between SST3 expression and ITF2984 responsiveness was highlighted.[92] In fact, authors stated that ITF2984 showed a significative tumor response and a compensatory upregulation of SSTR3 mRNA only in female rats, while no antitumoral effect has been observed in male rats with lower SSTR3 levels.

In addition, a recent study showed its antiproliferative and proapoptotic *in vitro* effects in all primary cultured NF-PitNET tested.[94] All samples expressed SST3 and median SST3 mRNA expression level was comparable to that of SST2 and SST5.[94] Nevertheless, with respect to the gender-related differences highlighted above, while one study showed a non-significant trend for higher SST3 expression in NF-PitNETs removed from female patients,[95] no gender-related difference was found in receptor expression or in ITF2984 responsiveness in the study by Di Muro,[94] accordingly with the observation that gender was not a predictor of SRLs treatment response.[96]

Another chapter that needs to be mentioned is nuclear medicine, specifically peptide receptor radionuclide therapy (PRRT) directed to high SSTs-expressing tumors faced application in aggressive or metastatic NF-PitNETs. PRRT efficacy have been mainly related to reach a stable disease in patients with NF-PitNETs and has been extensively reviewed.[97]

Combined therapies with mTOR inhibitors and DAs or SRLs

Mammalian target of rapamycin mTOR inhibitors, like everolimus or rapamycin, have been proposed as therapeutic strategy for tumors resistant to conventional drugs, displaying successful results in a wide spectrum of NETs. PI3K-AKT-mTOR pathway is known to have a fundamental role in regulating cell proliferation and survival and was found overactivated in PitNETs as compared with normal pituitary.[98-101] *In vitro* studies demonstrated that mTOR inhibitors reduced cultured NF-PitNET cells growth and viability.[38,102-106] Nevertheless, the use of everolimus in clinic is limited by the development of resistance, that was reported both *in vivo* and *in vitro*. Sensitivity to everolimus is actually reduced by an escape mechanism that induces an upstream increase of p-AKT, leading to pro-survival pathway activation.[107] Even if the mechanisms at the bases of resistance are incompletely understood, combined therapies to block mTOR function and prevent AKT activation may improve antitumoral activity. A

promising strategy has been proposed by combining everolimus with cabergoline, that resulted effective in reducing cell proliferation in primary cultured NF-PitNET cells through AKT activity reduction, as discussed in paragraph 1.1[11,106] (Figure 2B). Everolimus unresponsive NF-PitNETs developed a substantial enhancement of p-AKT/total-AKT ratio when treated with everolimus alone, in agreement with the escape mechanism mentioned. It was showed that the co-treatment with cabergoline strongly reduced the increase of p-AKT correlating with its antimitotic effect. Therefore, we might assume that resistance to everolimus might be caused by the loss of the negative upstream feedback on AKT.[107,108] A recent work from our group highlighted the involvement of β-arrestin 2 in mediating cabergoline inhibition of p-AKT activity and cells proliferation after everolimus co-treatment, through genetic silencing experiments in tumoral lactotrophs, in which the lack of β-arrestin 2 reverted the antimitotic effect induced by the combined treatment.[106] This study showed that cabergoline co-administration might improve mTOR inhibitors antitumoral activity, paving the way for a potential combined therapy in β-arrestin 2 expressing NF-PitNETs.[106]

Another strategy might be represented by the co-administration of mTOR inhibitors and SRLs. A study supported the additive effect exerted by the mTOR inhibitor RAD001 and the SRL analog, pasireotide, in NF-PitNETs showing a significant reduction of NF-PitNETs cell viability and a decrease of VEGF secretion.[38]

This alternative approach in NF-PitNETs was strengthened by another work in which octreotide conferred sensitivity to rapamycin treatment in 70% of primary cultured cells tested.[104]

Targeting the splicing machinery

Since evidences on splicing machinery alterations emerged,[60] the choice to target specific splicing factors with selective drugs might be a challenging alternative approach for NF-PitNETs treatment. Accordingly, based on previous bioinformatic analyses results and since several studies reported its efficacy in exerting antitumoral activity in different subtypes of tumors, Vazquez Borrego and co-authors tested the effects of Pladienolide-B in NF-PitNETs. Pladienolide-B is a compound able to directly target SF3B1, a key component for spliceosome assembly, leading to its inhibition.[109] Pladienolide-B showed a significative antimitotic action in NF-PitNETs cultures. However, its inhibitory effect was not maintained on chromogranin-A secretion.[60] Based on these evidences, further studies on splicing components and possible correlations with NF-PitNETs pharmacological resistance are needed, together with the development of selective drugs targeting specific splicing factors, since evidences recently emerged in GH-PitNETs.[110]

Tumor microenvironment-targeted therapies options

Since relevant microenvironment's role emerged in last years, some targeted therapies have been proposed also as innovative pharmacological chance for NF-PitNETs. From a therapeutic perspective, immune-checkpoint inhibitors, anti-PDL1 and anti-VEGF drugs have been tested in aggressive or metastatic PitNETs, and the use of other tumor microenvironment-targeting drugs can be evaluated.[76]

For instance, the monoclonal antibody that blocks VEGF, bevacizumab, has already shown efficacy in aggressive PitNET and pituitary carcinomas,[78] as well as immune checkpoint inhibitors, which effect was extensively reviewed by Ilie and co-authors.[111] Moreover, anti PDL-1 drugs need to be mentioned as innovative approach. Their effect was studied in a small cohort of 4 aggressive/metastatic NF-PitNETs: 2 patients showed partial radiological response to treatment while 2 stable disease.[112]

Conclusions

To conclude, this review summarizes the potential pharmacological targets for the treatment of NF-PitNETs, paving the way for combined therapeutic strategies that simultaneously target DRD2 and SST3, both of which are highly expressed in these tumors, ultimately supporting a more personalized approach to patient care. Moreover, we emphasize the possibility that small molecules may serve as biomarkers of NF-PitNETs responsiveness to different therapeutic modalities and may correlate with specific clinical behaviors. Certainly, studies employing *in vivo* NF-PitNETs models, together with the development of novel compounds, will enhance our understanding of the mechanisms underlying resistance to current pharmacological approaches and help broaden the range of effective therapeutic options for this heterogeneous group of tumors.

Authors' contributions

Federica Mangili (Conceptualization [lead], Data curation [lead], Formal analysis [lead], Investigation [lead], Project administration [lead], Supervision [lead], Writing—original draft [lead], Writing—review & editing [lead]), Donatella Treppiedi (Conceptualization [equal], Data curation [equal], Investigation [equal], Methodology [equal], Writing—original draft [equal]), Genesio Di Muro (Writing—review & editing [equal]), Nicole Panseri (Data curation [equal], Writing—original draft [equal], Writing—review & editing [equal]), Luca Bosoni (Investigation [equal], Writing—original draft [equal], Writing—review & editing [equal]), Erika Peverelli (Conceptualization [lead], Resources [lead], Supervision [lead], Writing—original draft [lead], Writing—review & editing [lead]), and Giovanna Mantovani (Funding acquisition [lead], Project administration [lead], Supervision [lead], Validation [lead], Writing—original draft [lead], Writing—review & editing [lead])

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