Biological and transcriptional effects of RTKs inhibition; EGFR, FGFR and IGFR as new therapeutic targets for MCC

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Abstract

Merkel Cell Carcinoma is a rare neuroendocrine tumor of the skin with an increasing incidence. It is a very aggressive type of cancer with a high mortality rate (30% of its patients). There are two main etiologies, MCPyV + tumors, characterized by the presence of the integrated Merkel Polyoma virus (MCPyV) and low mutational index, and MCPyV- tumors displaying high mutational burdens with a typical UV mutational signature, caused by exposure to UV radiation. Today, treatments for MCC are based on surgical excision along with adjuvant radiation therapy or chemotherapy, which shopw only limited effects. There is a current lack of knowledge about the driver mechanisms of MCC carcinogenesis, which is reflected in the lack of biomarkers for diagnosis and targeted therapy. Some recent studies have focused on characterizing MCC tumors to discover the mechanisms that drive this disease. As part of these initiatives, activated CREB has been shown as an independent factor of mortality caused by MCC. Continuing with the mechanistic characterization of MCC tumors, numerous mutations in receptors with tyrosine kinase activity (RTK) have been detected in MCC tumors. Therefore, to study the role of RTKs-dependent signaling in a CREB context, we decided to carry out a study on the different biochemical (CREB activation) and biological effects (proliferation and apoptosis) caused by different RTK inhibitors in a collection of MCC cell lines (MCPyV- and MCPyV +). In this regard, we have taken advantage of the compounds M666-15 (a selective CREB inhibitor, CREBi), dacomitinib (anEGFRi), BGJ 398 (FGFRi), and BMS-754807 (which inhibits IGF-1R/InsR and TrkA/B). These were highly effective at inhibiting MCC cell proliferation. Moreover, dacomitinib, BGJ-398, and BMS-754807 induced apoptosis in MCC cell lines, as well as reducing binding of CREB and P-CREB to specific DNA elements. Therefore, our study shows that deregulated RTK activities towards CREB activation can be crucial in promoting MCC carcinogenesis and therefore constitute and important mechanisms of MCC tumorugenesis that we can target wich specific inhibitors.

1. Introduction:

1.1. Clinical and mechanistic insights Merkel cell carcinoma

Merkel Cell Carcinoma (MCC hereon) is a rare neuroendocrine malignant cutaneous tumor. Tumor cells resemble Merkel cells in the basal layer of the epidermis (Pulitzer 2017). Merkel cells are important in tactile stimulation and also have neuroendocrine functions, being able to express cytokeratin 20 and synaptophysin (Jürgen C. Becker et al., 2017).

MCCs are very aggressive with high mortality rates, specially those with advanced age, immunosuppressed or suffering from other skin tumors (Schandendorfl et al., 2017).

There are two main mechanisms that can explain MCC tumorugenesys. One is by clonal integration of tha Merkel cell polyoma virus (MCPyV) in cells, and the other by chronic exposure to ultraviolet light and acquisition of C>T or CC>TT mutations in the cellular genome.

1.1.1. Epidemiology of Merkel cell carcinoma:

The mean age at diagnosis is 75-80 years, and survival depends on the stage at diagnosis and its location. Among the risk factors to highlight, the main one is chronic exposure to UV, due to the number of cases of MCC with advanced age, with chronic exposure to the sun and with a history of other cancers associated with exposure to the sun. In addition, injuries tend to appear on the most exposed parts of the body, such as the face or neck. Another risk factor is immunosuppression, which favors the development of MCC. This process is favoured by mutations caused by chronic UV exposure, and also by the integration of the virus, MCPyV (virus of the polyomaviridae family). In immunosuppressed patients the disease develops and progresses faster, so it can be seen that the efficiency of the immune system is crucial to control the disease.

As mentioned above, there are two main causes of MCC. Carcinogenesis can start from clonal integration of the MCPyV virus or from DNA damage due to chronic UV exposure. Although exposure to the sun, it can promote virus integration due to local immunosuppression through inflammatory mechanisms. About 50-80% of MCC cases are positive for the presence of the virus, it depends on the cohort of patients studied. The incidence in the presence of the virus changes from one country to another, in Asia the 80% of cases are positive for MCPyV, while in Austalia it is 60%. In addition, in Australia they also have a higher incidence of MCC MCPyV- than for example in the United Kingdom (Nirenberg et al., 2019). Also, MCPyV is usually integrated into the host genome, although they normally remain asymptomatic (Becker et al., 2019).

MCPyV is a virus of the polyomaviridae family and its primary infection does not cause symptoms. It is normally acquired in childhood and is present in most healthy people. MCPyV expresses early transformation genes, the large (LT) and small (ST) T antigens, both help the integration of the viral genome into the host genome. Also MCPyV, express ST49, which has an important oncogenic activity, being able to promote gene expression changes such as aerobic glycolysis of fibroblasts, important for malignant tumor cells that has glycolytic rates 200 times higher than normal tissues (Becker et al., 2019).

MCPyV-positive MCC tumor cells do not usually have many mutations or copy number differences, but MCPyV-negative do, due to UV exposure. Mutations generally present in MCPyV- MCC generally affect RB1 (Jürgen C. Becker et al., 2017), an important for regulating the cell cycle, while MCPyV + has intact RB1. The functions of RB1 are stopping the cell cycle and repressing the E2F factors that replicate DNA and start the S phase, therefore the inactivation of RB1 is important for carcinogenesis, which can be achieved through mutations, or by binding from LT. MCPyV- also shows mutations in TP53 and although MCPyV + does not show mutations, its activity is reduced, because large LT and small LT antigens inhibit both TP53 and RB. Other mutations in MCC MCPyV- are found in signalling pathway genes such as NOTCH, which is important in the repair of DNA damage and the modification of chromatin. Loss-of-function mutations in NOTCH1 and NOTCH2 have been reported in MCPyV− MCCs (Harms et al., 2015). Although the same mutations do not exist in MCPyV +, LT and ST are likely to

disturb different pathways, making the appearance of mutations unnecessary for carcinogenesis (Goh G et al., 2016).

Both etiologies present the deregulated Pi3K / AKT / mTOR pathway, produced through mutations or other mechanisms (Jürgen C. Becker et al., 2017).

Furthermore, MCC is capable of inhibiting the immune response, causing T cells to progressively lose their function, or inhibiting cellular responses from PD1 or ligand 1 PD1 signalling, or HLA defects (Jürgen C. Becker et al., 2017).

1.2. Diagnosis and prevention:

MCC is a fast growing tumor and the most characteristic lesions are red or violet nodules, or even the appearance of ulcers, although they are less frequent. It is composed of small, monomorphic, round and oval cells, with a vesicular nucleus and little cytoplasm. Neoplastic cells are large and pleomorphic with multiple nucleolus, and there is usually a lot of necrosis (Jürgen C. Becker et al., 2017). The diagnosis of MCC is usually difficult and confused with other diseases, so it is necessary to make a histopathological analysis, through a biopsy in the lymph nodes since it quickly spreads through them and later to the brain, lungs, bones etc. Through this sample, immunological markers are analysed for a better diagnosis.

There are four stages of MCC: stage 0 (*in situ*), stage I (localized disease, primary lesion ≤2 cm), stage II (localized disease, primary lesion> 2 cm), stage III (lymph node spread), and stage IV (disease metastatic beyond local nodes). Survival depends on when the diagnosis is made, at 5 years it is 62.8% of patients in stage I, between 34 and 54% in stage II, 26-40% in stage III and 13.5% in stage IV. They are normally diagnosed in stage I or II, although a large tumour thickness, high mitotic rate and an infiltrative growth pattern is associated with an increased risk of metastasis and an unfavourable prognosis (Jürgen C. Becker et al., 2017).

As for the immunohistochemical markers used for diagnosis, they are type I and II cytoskeletal keratins, especially CK20 (Rastrelli et al., 2018) and also CK8, CK18 and CK19, while neoplastic cells express synaptophysin. One third of MCC stained positively for p63 related to a worse prognosis. They are also usually negative for CK7 and TTF1.

Once the disease is overcome, it is necessary to carry out a surveillance of the MCC patients, at the most frequent beginning and if there is no recurrence of the disease, the reviews are extended over time. A physical examination, imaging, and analysis of blood biomarkers are usually performed to check the patient's condition and whether or not there is a recurrence.

To carry out the diagnosis, a biopsy is necessary, if it is positive for MCC, it is necessary to examine the lymph nodes to know if they are positive or negative, and to know which treatments are best for the patient.

1.3. Different strategies for therapy:

Once the diagnosis has been made and the primary tumour has been located, the first treatment to take into account is its excision. It is usually performed up to 1 or 2cm from the muscular fascia, and the margins are analysed to know that it has been completely removed. It is usually supplemented with adjuvant wide-field radiation therapy, in the same way as if there has been metastasis to the lymph nodes, as well as a complete lymph node dissection (Villani et al., 2019).
Another treatment used is chemotherapy, for metastatic MCCs that cannot be operated, although it is not usually very effective and there is a great variability of response between patients (20-61% response rate). It is important to take into account the adverse effects that the treatments can produce, since normally the patients tend to be elderly people with other pathologies, and less tolerant to multimodal treatments. Therefore, it is important to take into account the quality of life of the patient.

An effective treatment is immunotherapy, specifically the PD1-PDL1 immune checkpoint to reactivate the immune response. Clinical trials with antibodies against PD1 or PDL1 have had long and high response rates, greater than for chemotherapy. They also had a higher progression-free survival rate at 6 months, accounting for 67% versus 23% of chemotherapy. Thus, immunotherapy can benefit advanced patients and is better than other current treatments (Nghiem et al., 2016).

As we can see, nowadays there is no really effective therapy for MCC patients. The only treatments to consider are excision, which cannot be carried out in all cases as it depends on the location and the staging of the tumor. Other treatments are radiotherapy and chemotherapy, which have also not shown great effectiveness and have numerous side effects. There is no targeted therapy for patients with MCC, so it is important to study this disease to know how it works and to locate new therapeutic targets that can be used to develop targeted therapy, which together with immunotherapy achieves real effectiveness for MCC patients and manages to increase the survival percentage. Therefore, some studies have been devoted to performing parallel massive sequences (MPS) and the entire exome sequence (Gonzalez-Vela et al., 2017) to search for genetic mutations and alterations in somatic copy number in tumors from MCC patients with the aim of better characterizing the disease and the carcinogenic mechanisms of MCC, in order to develop effective targeted therapy in the future.

1.4. Relevant signalling pathways in Merkel cell carcinoma

In previous studies of our group, we studied the complete exome of a cohort of patients (Gonzalez-Vela et al., 2017),, as well as biomarkers, to detect a series of signalling mechanisms with the potential to participate in the development of MCC, and results showed that MCPyV + and MCPyV- tumours can share certain mechanisms of carcinogenesis such as NFAT, P-CREB and P-STAT. Although MCPyV- tumours are capable of exclusively developing other disease mechanisms such as C-MYC and LEF1, they are not frequently found in MCPyV positive tumours.

In this work, our group studied different genes and pathways to find out which were the similar or divergent mechanisms between both etiologies that could have relevance in the development of MCC,

and divided them into different sections: focal adhesion and extracellular matrix, metabolism, transcriptional regulation etc. We can see below the presence of the different mutations in the patient cohort used for the study in Figure 1.

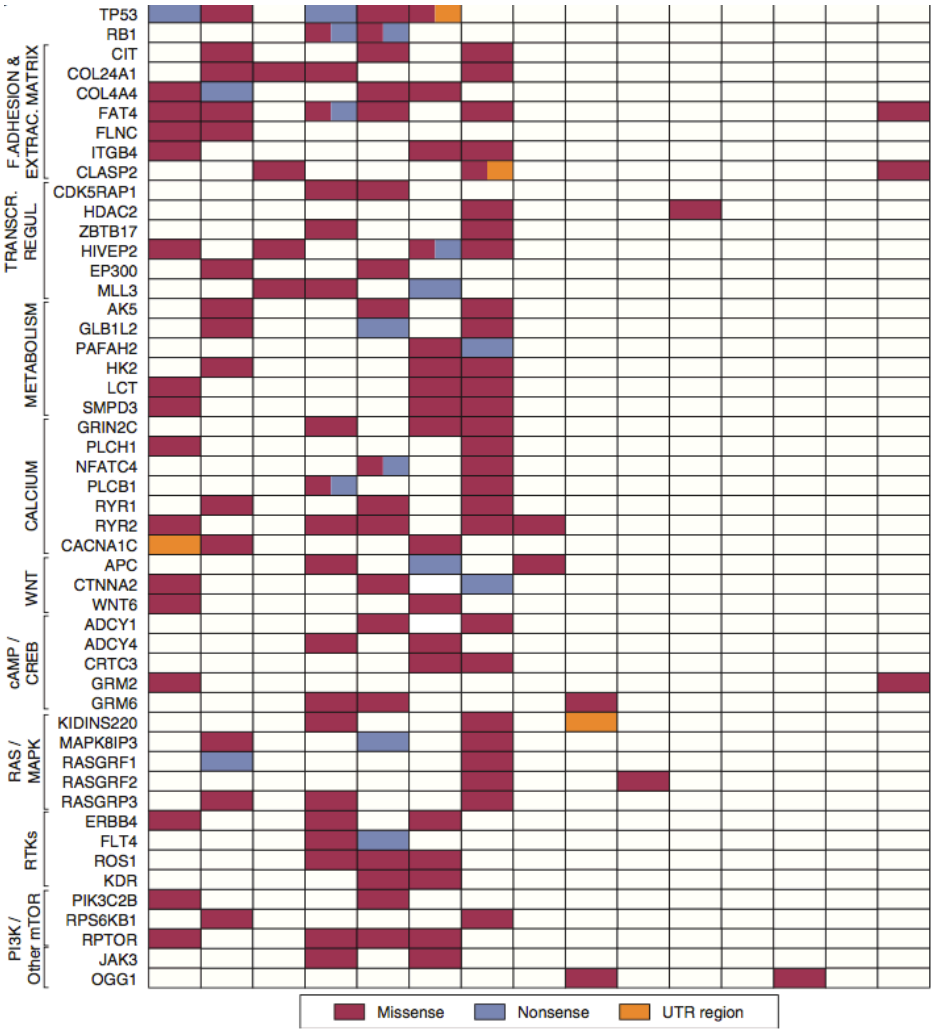


Figure 1. Mutations present in the patient cohort used. We can see the mutated genes on the left and each column corresponds to a patient. The color of the quadrant shows the type of mutation: red for missense mutations, purple for nonsense and orange for UTR regions. C, cytosine; CREB, CRE-binding protein; EXTRAC, extracellular; F, focal; MAPK, mitogen-activated protein kinase; MCPyV, Merkel cell polyomavirus; MI, mutational index; PI3K, phosphatidylinositol 3 kinase; REGUL, regulation; RTK, receptor with tyrosine kinase activity; SNV, single nucleotide variation; SSM, somatic single-base mutation; T, thymine; TRANSCR., Transcription; UTR, untranslated region (Gonzalez-Vela et al., 2017).

The expression of biological markers was studied such as MCPyV, p63, RB, and TP53, as well as transcriptional factors such as b-catenin, LEF-1, P-CREB, NFAT, C-MYC, and P-STAT. They found that for MCPyV + they had a homogeneous expression of all except for P-STAT, which was found in a low number of patients, and were negative for LEF-1 and C-MYC. For the MCPyV- samples they were more heterogeneous, RB was lost in 50% and C-MYC and LEF-1 were expressed in half of them. Almost all MCPyV- tumors have mutations in RB1 and TP53, while in MCPyV + it remains intact although the

activity of TP53 is reduced (Wong et al., 2015). As explained previously, MCPyV- tumors have high mutation rates, and various signaling pathways can be found such as NOTCH1, FAT1, PI3K (PIK3CA, AKT1, PIK3CG) and MAPK (HRAS, NF1) (Harms et al., 2015). Thus, it is possible that both etiologies share disease mechanisms, although mutations in MCPyV- cause it to develop other additional molecular characteristics.

The presence of mutations or amplified genes of receptors with tyrosine kinase (RTK) activity has been observed, which affects the proper functioning of their signalling (figure 2), so the RTK pathway may be relevant in MCC carcinogenesis.

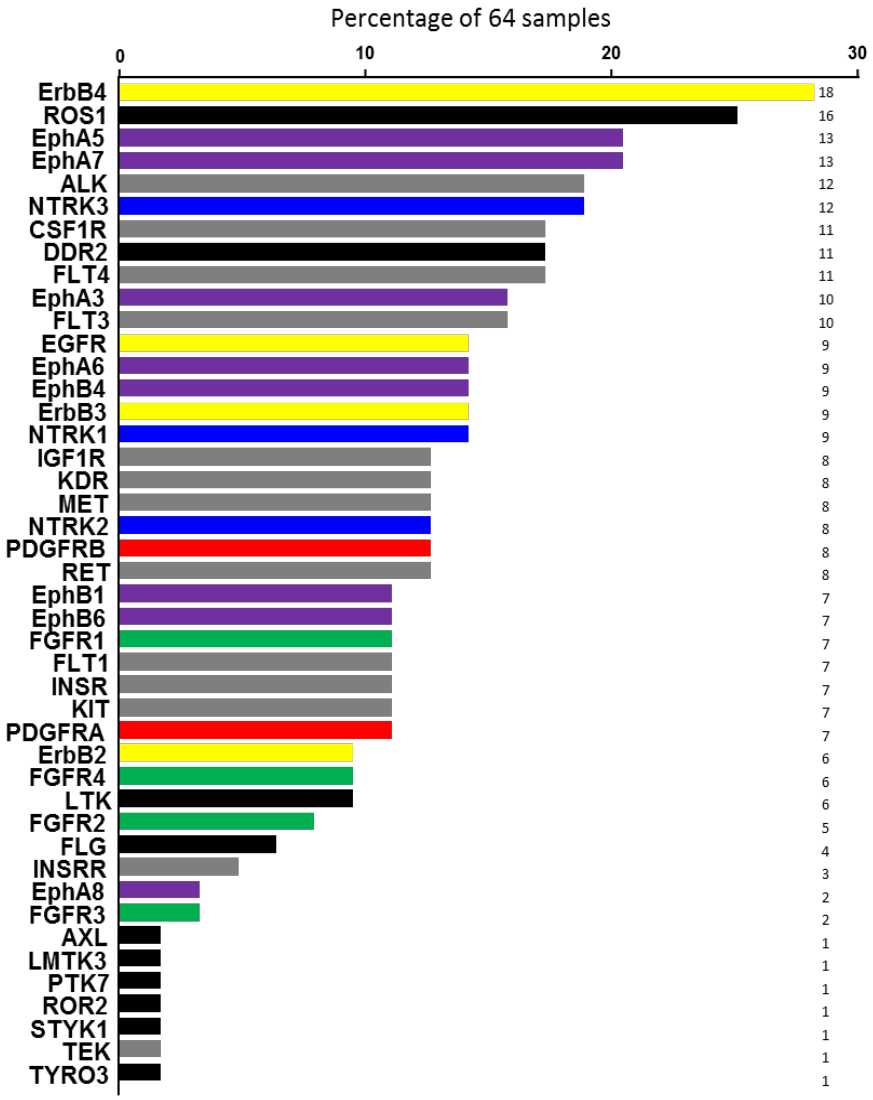
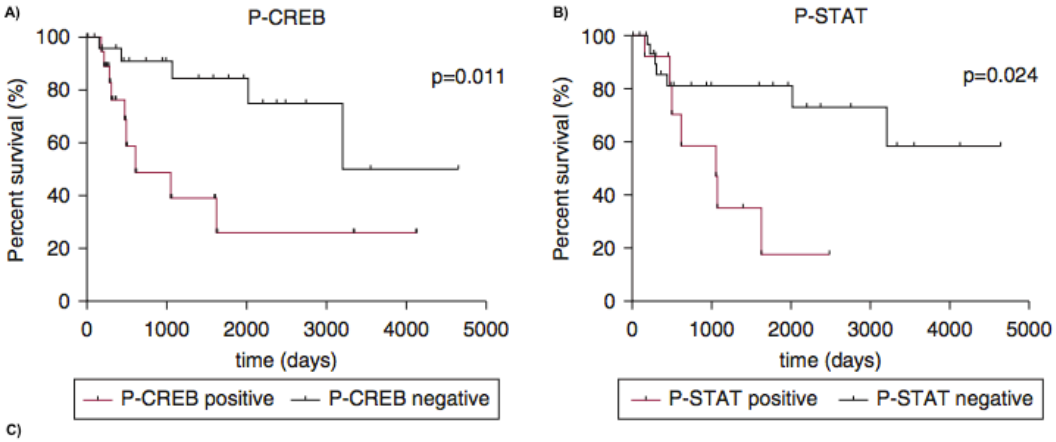


Figure 2. Genetic and signalling mechanisms alterations in MCC. Graph shows mutations from whole exome sequencing data of 64 MCC patients obtained from publications (meta-analysis); each colour represents a different family of receptors: platelet-derived growth factor receptors (PDGFRs in red) epidermal growth factor receptors (EGFRs or ErbBs in yellow), the insulin growth factor receptor (IGFR) and the fibroblast growth factor receptor (FGF 1/2/3/4, in blue). (Ruso-Julve et al., data non-publish). The data has been collected through different literature: (Harms et al., 2015) (González-Vela et al., 2017) (Wong et al., 2015).

Our group also analysed the death events caused by MCC in different patients and found that the cases with the expression of P-CREB or P-STAT showed a shorter survival (figure 3). Finally a multivariate study that took into account age, virus, and tumour stage helped identify P-CREB as an independent predictor of mortality (González-Vela et al., 2017) with a HR of 5.56 (Figure 3).



C)

Hazard Ratios for P-CREB and STAT markers, in relation to mortality

	Vital status Death N=15	Survival N=33	HRc[a]	(95%	CI)	HRa[b]	(95%	CI)
P-CREB								
Negative	5	20	1	--		1	--	
Positive	9	12	3.89	1.28	11.87	5.56	1.22	25.33
P-STAT								
Negative	7	24	1	--		1	--	
Positive	7	8	3.37	1.1	10.3	1.65	0.22	12.35

a HRc = Crude Hazard Ratio.
b HRa=Hazard Ratio adjusted for P-CREB, P-STAT, MCPyV status, sex, age and stage.

Figure 3. Relationship between survival of MCC patients with specific characterization. Kaplan-Meier curves show the percentage of survival of MCC patients, positive in red and negative in black. A) P-CREB (P 1⁄4 0.011) and (b) P-STAT (P 1⁄4 0.024). C) Crude hazard ratio and hazard ratio adjusted for P-STAT3, P-CREB, MCPyV status, sex, age, and stage, with 95% confidence intervals. CI, confidence interval; HRa, adjusted hazard ratio; HRc, crude hazard ratio; MCC, Merkel cell carcinoma; P-CREB, phosphorylated CRE-binding protein; P-STAT, phosphorylated signal transducer and activator of transcription (González-Vela et al., 2017).

Thus a number of signalling pathways have been identified in MCPyV- MCC, such as LEF1, C-MYC and RB. The most common mutations for C-MYC were nonsense mutations and truncation. Furthermore, C-MYC mutations were normally accompanied by mutations in adenylate cyclase and G protein-coupled receptors, which are involved in CREB activation. Mutations for RB were normally truncations, which impede the expression of its protein, assuming a worse prognosis. These results suggest that the high mutational load present in MCC MCPyV- tumours causes them to acquire specific biological properties

and activate other oncogenic pathways such as C-MYC or LEF1 that are not as present in MCC MCPyV + cases (Figure 4).

Interestingly and alongside these, convergent pathways between the two etiologies were identified like for example inactivated TP53 and certain deregulated signalling mechanisms, as indicated by the detection of biomarkers such as P-STAT, P-CREB, and NFAT. Another marker shared by both etiologies is p63, whose expression also supposes a worse prognosis (Figure 4).

From what we can see how MCPyV- and MCPyV + can develop similar disease mechanisms with clinical implications for the survival of patients, as well as the potential to develop a therapy. Furthermore, thanks to the discovery that P-CREB can be useful for predicting survival factor, we can make a better diagnosis and predict poorer results and explore approaches for other more specific therapies.

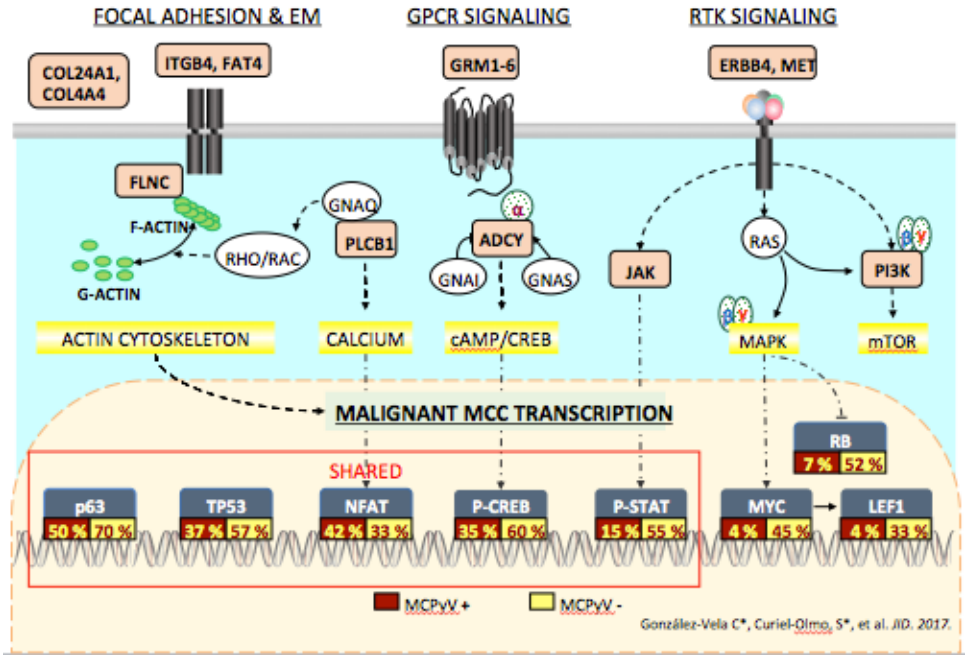


Figure 4. Convergent and divergent mechanisms between both etiologies of MCC. In the scheme we can see the different signalling pathways altered in MCC. We can see the expression percentage of each biomarker, in red for MCPyV + and in yellow for MCPyV-. GPCR, G protein-coupled receptor; MCPyV, Merkel cell polyomavirus; NFAT, nuclear factor of activated T cells; P-CREB, phosphorylated CRE-binding protein; MAPK, mitogen-activated protein kinase; P-STAT, phosphorylated signal transducer and activator transcription; PI3K, phosphatidylinositol 3 kinase; RTK, receptor with tyrosine kinase activity (Gonzalez-Vela et al., 2017).

1.4.1. Tyrosine kinase receptors (RTKs)

Tyrosine kinase receptors (RTKs) are receptors located on the cell surface that bind to different ligands, such as growth factors, hormones and cytosines. They are important to carry out many cellular functions such as cell growth, cell division and apoptosis, but also in the development of many types of cancers,

since their mutations produce the activation of their signalling pathway, producing a series of changes in the expression of different genes, as well as the expression of proteins (Trenker R., et al., 2020). Therefore, they play a fundamental role in angiogenesis and tumour progression.

There are 58 members of the RTK family, many of them are represented in our analysis like for example the insulin receptor (INSR or IGFR), fibroblast growth factor receptor (FGFR), vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR), insulin growth factor receptor type 1, platelet growth factor receptor (PDGFR). Most RTKs are formed by single subunits, although there are some types that can form dimers. Each monomer is made up of a hydrophobic transmembrane element, made up of 25-40 amino acids, an extracellular N-terminus region, and an intracellular c-terminal region. The N-terminal end has ligand binding sites, which bind to extracellular ligands, such as hormones or growth factors, while the c-terminal end has more conserved ends, which are responsible for kinase activity. of receptors, catalyzing autophosphorylation of the receptor and activating its signalling cascade.

When the binding of a ligand with one of the receptors present in the membrane occurs, the rapid dimerization of several RTKs present in the membrane occurs and as a consequence the activation of its kinase domain causing its autophosphorylation. Certain proteins that affinity to these now phosphorylated sites will be able to bind to the receptor and in turn be phosphorylated, causing the start of the downstream signal transduction pathway.

Through this process, through different mutations in the RTK itself or in some of the proteins in its signalling pathway, this pathway can have a great implication in the development of different cancers since it produces changes in the expression of many genes to the that regulates. The use of receptor kinase inhibitors has been found to be an interesting treatment for various cancers, as has been found in gastrointestinal stromal tumours, ERBB2 breast cancers, etc. (Yamaoka T., et al., 2018).

1.4.1.1 Role of RTK signalling pathways in MCC

In the study that we have seen previously (González-Vela et al., 2016), they studied the different mutations present in a cohort of MCC patients, we can see it in figure 1, where the samples from 15 patients and the mutations found in them, such as mutations in the RTKs, in the calcium pathway, in genes related to metabolism, in MAPK, CREB, etc. We can verify that a large number of these mutations belong to the receptors with tyrosine kinase activity (figure 2), as well as in the elements that make up this pathway, therefore our team in the laboratory performed a meta-analysis of the published exomes of MCC samples, and found that many of them had mutations in the RTKs or in their signalling pathway (data not shown).

Furthermore, given the biological importance that CREB activity in MCCs, we estudied CREB phosphorylation in response to RTKs agonists (activating the most represented RTKs in our study) in MCC cells.

In tables 1 and 2, we can see the effects that specific RTK agonist exert over ERK1/2 (mitogen-activated protein kinases related to signal transduction and CREB phosphorylation (Steven et al., 2016)). Different

ligands capable of activating RTKs were used alongside Forskolin (used as control for AC activation) to study the degree of phosphorylation of both ERK and CREB in different MCC cell lines (MCPyV-: MCC13, MCC14 / 2, MCC26; MCPyV +: MKL1) and thus check whether the pathway of RTKs had a relationship to CREB activation. It can be seen how these ligands: forskolin, EGF, FGF, PDGF among others, are capable of increasing the degree of ERK phosphorylation, and therefore its activation, as well as causing it in CREB. So the activation of RTKs is directly related to CREB. Therefore, mutations in the RTK pathway can cause a malfunction in the cascade of signals that activate CREB, causing its overexpression and accelerating the process of angiogenesis and tumor progression.

Table 1. ERK 1/2 phosphorylated-dependent of RTKs activation. Relative phosphorylation levels of ERK 1/2 in MCC 13, MCC 14/2, MCC 26 and MKL-1 cells incubated for 15 min with the respective ligands: forskolin, EGF, FGF, PDGF, NGF, BDNF, NT-3 and IGF. All experiments were done independently in triplicate. FC means fold change from P-ERK/ERK ratios. Data are mean ± SEM; unpaired Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs control condition.

Ligands	MCC 13				MCC 14/2			
	FC mean	SEM	p-value	Signif.	FC mean	SEM	p-value	Signif.
Forskolin	1,862	0,090	< 0.0001	***	2,059	0,264	0,0015	**
EGF	1,724	0,316	0,0348	*	2,459	0,252	0,0001	***
FGF	8,734	2,290	0,0053	**	1,423	0,139	0,0077	**
PDGF	1,683	0,108	0,0006	***	1,446	0,130	0,0093	**
NGF	2,197	0,163	0,0003	***	1,092	0,168	0,5409	ns
BDNF	1,069	0,126	0,6144	ns	1,316	0,181	0,0537	ns
NT-3	1,041	0,073	0,6078	ns	1,415	0,174	0,0171	*
IGF	1,046	0,211	0,7906	ns	1,071	0,101	0,3721	ns

Ligands	MCC 26				MKL-1			
	FC mean	SEM	p-value	Signif.	FC mean	SEM	p-value	Signif.
Forskolin	1,055	0,029	0,0815	ns	2,583	0,998	0,1879	ns
EGF	1,580	0,174	0,0292	*	1,427	0,125	0,0035	**
FGF	1,586	0,106	0,0012	**	4,812	0,654	0,0002	***
PDGF	1,415	0,110	0,0062	**	2,945	0,812	0,0352	*
NGF	1,151	0,057	0,0124	*	3,076	0,780	0,0245	*
BDNF	1,220	0,058	0,0063	**	1,336	0,207	0,1108	ns
NT-3	1,303	0,087	0,0086	**	1,200	0,192	0,3365	ns
IGF	1,404	0,043	0,0011	**	0,970	0,070	0,6886	ns

Table 2. CREB phosphorylated-dependent of RTKs activation. Relative phosphorylation levels of CREB in MCC 13, MCC 14/2, MCC 26 and MKL-1 cells incubated for 15 min with the respective ligands: forskolin, EGF, FGF, PDGF, NGF, BDNF, NT-3 and IGF. All experiments were done independently in triplicate. FC means fold change from P-CREB/CREB ratios. Data are mean ± SEM; unpaired Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs control condition.

Ligand	MCC 13				MCC 14/2			
	FC mean	SEM	p-value	Signif.	FC mean	SEM	p-value	Signif.
Forskolin	8,895	1,891	0,0041	**	2,169	0,348	0,0283	*
EGF	1,411	0,133	0,0141	*	3,741	0,830	0,0299	*
FGF	1,881	0,138	0,0006	***	2,265	0,358	0,0242	*
PDGF	2,489	0,447	0,0106	*	0,962	0,057	0,5397	ns
NGF	2,493	0,528	0,0197	*	1,181	0,140	0,2674	ns
BDNF	1,038	0,231	0,8761	ns	1,152	0,048	0,0134	*
NT-3	1,083	0,133	0,5658	ns	1,310	0,104	0,0159	*
IGF	1,091	0,048	0,1325	ns	1,500	0,101	0,0078	**
Ligand	**MCC 26**				**MKL-1**			
	FC mean	**SEM**	**p-value**	**Signif.**	**FC mean**	**SEM**	**p-value**	**Signif.**
Forskolin	4,271	0,892	0,0072	**	2,678	0,592	0,0082	**
EGF	3,353	0,943	0,0307	*	2,663	0,581	0,0188	*
FGF	2,414	0,675	0,0267	*	1,726	0,232	0,0181	*
PDGF	2,461	0,692	0,0528	ns	1,607	0,134	0,0105	*
NGF	2,033	0,479	0,0244	*	1,482	0,141	0,0269	*
BDNF	0,959	0,164	0,7747	ns	1,845	0,140	0,0001	***
NT-3	1,047	0,319	0,8659	ns	1,253	0,023	< 0.0001	***
IGF	1,237	0,069	0,0049	**	0,955	0,059	0,3334	ns

As explained above, the RTK medium signaling pathways that are key to promoting tumorigenesis, such as proliferation, differentiation, angiogenesis, cell survival, migration, apoptosis, etc. Mutations that affect RTKs or their signaling pathway below cell transformation that we can see in different types of cancer such as EGFR mutations in breast cancer, or PDFG mutations in gastrointestinal stromal cancer, which reduce patient survival and suppose a worse prognosis, as well as an increase in angiogenesis and vasculogenesis (Tarik Regad 2015). But a number of RTK inhibitors and antibodies have been developed that are used as therapy and have been found to be efficient, such as monoclonal antibodies Cetuximab and Panitumumab that attack EGFR ligand binding in patients with metastatic colorectal cancer, or inhibitors. from RTKs such as Sorafenib or Sunitinib that inhibit a VEGFR that have improved the overall survival of patients with renal cell cancer and gastrointestinal stromal tumor (Tarik Regad 2015).

Therefore, the in-depth study of the progression pathways of the MCC, such as the sea through the RTKs, help to better understand the development of the disease, find a useful therapeutic objective for the development of targeted therapy

1.4.2. CREB role in Merkel cell carcinoma

1.4.2.1. Molecular structure of CREB

In human and mouse, the CREB (cAMP response element-binding protein) genes are located on chromosome 2q33.3 and consist of 11 exons and 3 isoforms (α , β and Δ) produced through alternative splicing with identical function (Ichiki 2006). The full-length sequence of CREB can be divided in four functional domains, in which kinase inducible domain (KID) necessary for the activation of CREB (Sun et al. 2016), containing Ser133 (target of multiple protein kinases), and Q2 domain for constitutive activation, responsible for binding with RNA polymerase II initiation complex through the recognition and binding to the canonical CRE (5'-TGACGTCA-3') (Altarejos and Montminy 2011). The upstream protein kinases activating CREB include protein kinase A (PKA), protein kinase B (PKB, also known as Akt), protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CaMKII), p90 ribosomal S6 kinase (p90RSK), casein kinase I and casein kinase II (Trinh et al. 2013). PKA phosphorylation does not alter the secondary structure of CREB, and therefore, has no effect on the binding of CREB to DNA (Richards et al. 1996).

CREB is found in the nucleus and is a transcription factor that binds to certain DNA sequences through which it can increase or decrease the transcription of the genes it regulates, such as c-Fos, BDNF and nerve growth factor. To activate CREB it is necessary for it to be phosphorylated, and for this it is necessary for some of its receptors to be activated by extracellular signals. CREB can be activated by three different pathways, from receptors bound to G proteins, tyrosine kinase receptors, or by calcium, and can be activated by their binding to neurotransmitters (which bind to receptors coupled to G proteins), calcium (which binds to ion channels) or growth factors (which bind to tyrosine kinase receptors). These once active pathways will activate different kinases such as protein kinase A (PKA), calmodulin-dependent protein kinase (CaMK), mitogen-activated protein kinases (MAPK), as well as other kinases, to carry out phosphorylation of CREB, the most common phosphorylation site being ser133. Once CREB is phosphorylated it can bind to DNA and the CBP protein (CREB binding protein) is recruited and thus P-CREB can carry out its function of activating the transcription of a certain gene (Johannessen M., et al., 2004) .

1.4.2.2. Biological functions of CREB in carcinogenesis.

The function of CREB is very important for the proper functioning of cells and the body, since it regulates critical processes such as glucose homeostasis, cell growth, memory, among many others. For this reason, its function as a proto-oncogene and its involvement in different types of cancers have begun to be studied, since it could be related to the signalling that initiates and produces the malignance transformation of cells and metastasis.

CREB overexpression and its role in carcinogenesis have been extensively studied for different cancers, like for example in acute myeloid leukemia (Deepa B. et al., 2005). High CREB levels are associated with an increased risk of relapse and decreased cancer-free survival. Furthermore, CREB promotes abnormal proliferation and survival of myeloid cells through the regulation of specific target genes, which is why we verified the relationship of CREB in the transformation of myeloid cells in acute myeloid leukaemia. It is just one example of the many other studies that correlate CREB overexpression with different cancers, such as prostate, breast, lung cancer, etc. In contrast, the down-regulation of CREB in different cancer cell lines produced an inhibition of proliferation and the induction of apoptosis. So CREB may be a target to consider for developing new cancer therapies, in fact, certain molecules have already been found that are capable of inhibiting CREB phosphorylation, or inhibiting CREB binding with CBP (Jiang M. et al., 2019).

As we have previously seen, CREB is one of the elements in common that the two etiologies of MCC possess, and it has also been verified that the presence of active P-CREB in samples from patients with MCC is a predictor of mortality and a worse prognosis. Furthermore, CREB overexpression may be related to the process of carcinogenesis, as we have seen that it occurs in other cancers. CREB can be activated or over-activated by three different pathways, but one of them is especially important in MCC cases, which is the RTK pathway, since many patient samples show a large number of mutations in this pathway. These mutations could contribute to CREB overexpression, and with it the onset of disease, metastasis etc.

In the present work, we evaluate the biological effects (proliferation and apoptosis) of a number of pharmacological drugs which are specific RTKs inhibitors in an *in vitro* context, using different MCC cell lines with all etiologies of the cancer. Thus, we evaluate the role of RTK and CREB in the tumour proliferation taking advantage of the pharmacological inhibitors available to target these pathways.

2. Hypothesis and objectives

2.1. Hypothesis

A RTKs/CREB signalling network can act as a malignant mechanism driving tumorogenesis and progression of MCPyV-positive and -negative MCCs. Upon detection and functional analysis, the key members of this network can serve as potential novel markers for diagnosis and targeted therapy.

2.1. Objectives

1) Analyse the biological role of CREB in MCC cells ex vivo and in vivo.
2) With special focus on EGFR and FGFR, study the activity of RTK inhibitors in MCC cells in a panel of MCC cell lines:

2.A) Over cell proliferation and apoptosis.

2.B) Over CREB phosphorylation and DNA-Bonding capability

3. Materials and methods

3.1. Maintenance of cell cultures

The cell types used in this study are MCC14 / 2, MCC13, MCC26 and MKL1, which were obtained through the company Sigma Aldrich (MO, USA). The different cell lines have been obtained through MCC tumours from human patients, in which the presence of the MCPyV virus in one of them, MKL1 has also been clonally integrated into its genome. Although all cell lines are from MCC, they have their differences. MKL1 is the only cell line positive for MCPyV and which grows in suspension, while the rest are adherent cells and MCPyV- (MCC14 / 2, MCC13, MCC26).

They also have differences in their morphology and in the expression of different genes. As we can see in table 3.

Regarding morphology, MKL1 cells are irregular, normally they form large floating cell aggregates, in which the cells that are further inside die due to lack of supply of nutrients. In contrast, MCC14 / 2, MCC13 and MCC26 are large low-contrast adherent cells.

Table 3. Immunohistochemical markers used in MCC cell lines. Cells lines MCPyV- (MCC13, MCC14/2 and MCC26) and MCPyV+ (MKL1) were cultured in growing conditions in presence of heat inactivated FBS. MCPyV: Merkel cell polyomavirus; (+) means positive and (-) means negative for the respective antibody.

Cell line	Markers											
	MCPyV	RB	TP53	P16	P-STAT3	P-STAT5	P-CREB	NFAT	C-MYC	YAP	PDL1	PD1
MKL-1	+	+	+	+	-	-	+	+	+	+	-	-
MCC13	-	+	+	+	-	-	+	-	-	+	+	-
MCC14/2	-	+	+	+	-	-	+	+	+	+	+	-
MCC26	-	+	-	+	+	-	+	-	-	+	+	-

When carrying out the maintenance of cell lines, it must be keep in mind that not all grow in the same way or in the same media. On the one hand, we have MKL1 cells growing in suspension in RPM1 medium with 10% heat-inactivated FBS, while the rest are adherent and use DMEM medium with 15% FBS IH. In both media we add the antibiotic penicillin-streptomycin at 5% final concentration. Suspended cells have lost the ability to bind to the cell matrix and grow more slowly, so they do not have to be passed as often. Instead, the adherent cells grow in monolayer and faster, so it is necessary to pass them every 2 or 3 days. All of them grow at 37ºC in a humidified atmosphere with 5% CO2.

To pass the adherent cells, we aspirate the medium and wash with a phosphate buffered saline (PBS). We remove the PBS and trypsinize with 1X trypsin (stock at 10X 5g / L). To raise the cells we allow to

incubate with trypsin for about 5 minutes in the incubator, then we pipet to separate the cells and carry out the necessary dilutions with the corresponding medium to re-seed.

The cells in suspension are treated differently, since they do not grow adhered to the plate but they do form large aggregates. It is necessary to separate the cells through intense pipetting and then centrifuge at 700g for 5 minutes. We remove the medium that is the supernatant and add trypsin to the pellet (where our cells are located), to get them separated and leave to incubate for 5 minutes. We pipet again. After this process we can add medium and make the corresponding dilutions to sow.

3.2. Drugs and pharmacological agents

In this study we use different drugs for different purposes. On the one hand, to calculate the IC50 of the different cell lines we used ponatinib (AP24534), H89 dihydrochloride, Selumetibib (AZD6244) and ruxolitinib, all of them are inhibitors, in table 4 we can see that they inhibit in more detail, and they were purchased at through Selleck Chemicals (Texas, USA). They were used in two stocks of 0.1, 1 and 10 mg / ml, to achieve ascending concentrations from 0 to 5000nM, 20000nM or 45000nM, depending on the case.

For cytometric experiments, the drugs okadaic acid and adriamycin were used as positive controls for apoptosis, obtained through Sigma Aldrich Company (MO, USA), and were used in a stock of 10mg / ml. The treatments used were BMS-754807, dacomitinib and BGJ 398 that were used in stocks 1 and 10 mg / ml, to achieve final concentrations of 1 and 10 μM. For the elisas the same drugs were used and they were obtained from Selleck Chemicals (Texas, USA).

All drugs were dissolved in DMSO (VWR, Germany) at concentrations of 0.1, 1, and 10 mg / ml, except adrimycin and okadaic acid, which were dissolved in water. To achieve the final concentrations for each experiment, DMEM or RPMI medium was used (depending on the cell line, for all the experiments with adherent cells we used DMEM, except for those in which we used the MKL1 cell line that we used RPMI) and DMSO vehicle so that all the samples had the same volume.

Table 4. Targets of RTK inhibitors. On the left we see the different inhibitors used, and on the right their corresponding targets.

INHIBITOR (μM)	TARGET
Selumetibib (AZD6244)	MEK1, MEK2, ERK1/2
H89 dihydrochloride	PKA, ROCKII, MSK-1, others
Dacomitinib	EGFR, ErbB2, ErbB4
BGJ 398	FGFR
BMS-754807	IGF-1R/InsR, Trk A/B, others
Ruxolitinib	JAK1/2
Ponatinib (AP24534)	PDGFRa, VEGFR2, FGFR1, ScR

3.3. Cell viability assays

The cells were seeded in 96-well plates, with a density of 1000 cells/well at different concentrations of inhibitors with DMSO (vehicle) and medium. As we know, IC50 is a quantitative measure that indicates the amount of drug or drug that we need to inhibit proliferation by 50%, therefore we need to use a series of increasing concentrations. The concentrations used were increasing concentrations from 0nm to 20,000nm for MCC13, MCC14 and MCC26 with the treatments for Selumetinib (AZD6244), H89 dihydrochloride, ruxolitinib and ponatinib (AP24534). Increasing concentrations from 0 to 45000nm and with H89 dihydrochloride, from 0 to 5000nm were used for MKL1 with ponatinib (AP24534), ruxolitinib and Selumetinib (AZD6244).
When we use a cell line, with a specific treatment, two 96-well plates are prepared, one will be measured at 0h as an untreated control, and the other at 48h as treated. Once the two 96-well plates are seeded, they are left to grow for 24 hours in a humidified atmosphere at 37º and 5% CO2. Subsequently, the plate corresponding to 0h is measured and the 48h plate is treated with the drug (triplicates of each concentration are performed) and the necessary concentrations, and we will leave them in the incubator for 48h.

Cell proliferation is mediated from a CellTiter-Glo Luminencent Cell Viability Assay kit provided by Promega (MA, USA). This is a luminescent assay that determines the number of viable cells based on the amount of ATP present as a metabolic indicator of living cells. The reagent is added directly to the wells (100µl) once the control has been allowed to incubate for 24 h without treatment, and the plate is treated after 48 h. Let it rest for 10 minutes. It prod uces cell lysis and ATP passes to these available in the medium, where it will bind to luciferin and throuh UltraGlo rluciferase enzyme a luminescent signal will be generated proportional to the amount of ATP pr esent in each well, which in turn is directly proportional to the number of cells in the present.
The luminescent signal is measured with the Spark multimode microplate reader (Tecan Trading AG, Switzerland). In this way, through the control plate that we measured at 0h, and with the treated plate that we measured at 48h, we can see the proliferation in the presence of the drug and compare the different concentrations, in order to calculate the IC50.

The maximum mean inhibitory concentration or IC50 was estimated in 48h of pharmacological treatment using the GraphPad Prism 6 software.

3.4. Apoptosis assays

We used the MCC14 / 2 and MKL1 cell lines with dacomitinib BMS-754807 and BGJ 398. And as positive apopotosis controls for all experiments, okadaic acid and adriamycin.
Adriamycin is an intercalant of DNA and prevents the nucleic acid replication process, as well as the transcription process. On the other hand, okadaic acid is a phosphatase inhibitor, specifically PP1 and PP2A proteins. These proteins are involved in the regulation of multiple cellular processes through the

modulation of the degree of phosphorylation or defophorilation of proteins. PP1 regulates processes of glycogen metabolism, cell division, and protein synthesis, while PP2 phosphatase controls glycolysis, lipid metabolism, cell growth and division, apoptosis, transcription, and protein synthesis. Both drugs produce cellular apoptosis in a short time of treatment and their effectiveness has been proven in different studies, which is why we use them as apoptosis controls in our trials (Dietrich et al., 2020 and Lee KS et al., 2020).

To measure apoptosis on the cytometer we used the Flowcellect annexin Red kit (EMD Millipore, MA, USA). Through which we have to carry out a series of stains and have three controls, the blank without any staining, positive control for annexin V, and positive control for 7AAD.

Annexin V is a calcium-dependent phospholipid binding protein with a high affinity for phosphatidylserine found on the cell plasma membrane, on the inside of the cell. Annexin V is conjugated to a CF647 red dye that is excitable at 670nm and can be detected with the cytometer. Cells with an early or late apoptosis in which the stability of the plasma membrane is lost, will have an increase in fluorescence for annexin with respect to the target, since annexin V will be able to bind to phosphatidylserine on the inside of the plasma membrane, because it will be compromised, as one of the physiological changes due to apoptosis that occurs more quickly.

7-aminoactinomycin (7-AAD) is a 488nm laser excitable membrane waterproofing DNA intercalator. So all living cells will be negative for 7-AAD, as well as cells in early apoptosis, since the nuclear membrane will not be compromised yet. Those cells that are in late apoptosis will be positive for 7-AAD and annexin V. Cells that are only positive for 7AAD, but not annexin V, would mean that they are dead cells without a membrane, but we cannot confirm that these cells come from the induction of apoptosis caused by drugs. With this technique we can differentiate both living cells and early and late apoptosis quickly and efficiently.

We plated the cells in 6-well plates at a cell density of 100,000 cells / well, except for MKL1, which planted around 1,000,000 cells / well since they are more sensitive and during the preparation of samples and stains we lost a large number of cells. The next day after sowing, the respective drugs were treated with stock concentrations of 1 and 10mg / ml, to obtain final concentrations of 1 and 10μM plated the cells in 6-well plates at a cell density of 100,000 cells / well, except for MKL1, which pl antokadaic acid and one for adriamycin) and three wells for the staining controls (one for the blank witho ut any staining, another only positive for Annexin V, and another only positive for 7AAD).

We let the inhibitors work 24h, and 2h before finishing, we treated the positive controls with adriamycin and okadaic acid at a stock concentration of 10mg / ml, to obtain a final concentration of 10 μgr/ml.

After 2h of the positive controls, we removed the medium and washed with 1ml of PBS, removed and added 1ml of trypsin, and left to work for a couple of minutes in the incubator and scraped to extract all the cells from the wells (alone with the adherent cell lines, that is, MCC14 / 2), we then centrifuged 5-10 minutes at 1000g, and discarded the supernatant.

The suspension cells, MKL1, are treated differently during the first steps, we transfer the corresponding cells from each well to cytometry tubes and centrifuge for 5 minutes at 1000g. We discard the supernatant

and add 1X trypsin to the pellet, and leave it to work for about five minutes in the incubator. We again centrifuge again to the same conditions as before and discard the supernatant. At this point we are in the same situation for all cell types, both adherent and suspended, so the continuation of the protocol will be the same for all cell types.

We resuspend the cells in 100 microliters of assay buffer (belonging to the kit) at 1X. In turn, we added 100 microliters of anexin red working solution to all samples, except for the blank and the 7AAD positive control. We leave it to work for 15 minutes in the incubator and we centrifuge again under the same conditions, again removing the supernatant. We wash with 200 microliters with the 1X assay buffer and centrifuge again, again removing the supernatant. We added 195 microliters of 1X Assay Buffer and 5microliters of 7AAD, except for the blank sample and the annexin V positive control.
Once the staining protocol is finished, we can already measure the samples on the flow cytometer, and we can differentiate 4 populations:

- Non-apoptotic cells: Annexin V (-) and 7-AAD (-)
- Early apoptotic cells: Annexin V (+) and 7-AAD (-)
- Late apoptotic cells: Annexin V (+) and 7-AAD (+)

3.5. CREB transcription factor ELISA-based binding assays

The Elisa was carried out to detect and quantify the binding capacity of the transcription factor CREB and phosphorylated CREB to the consensus binding sites of DNA, using a kit of TransAM CREB transcription factor assay (Active Motif, CA, USA), following manufacturer's instructions.

We used a Merkel carcinoma cell line, MCC14, and treated the cells with the RTK inhibitors dacomitinib, BGJ-398 and BMS-754807 at two final concentrations of 1 and 10 µM. We performed two elisas, one to see total CREB, and another to see P-CREB. First we seed the cells in p10 plates and let them grow for 24h. The next day we performed the treatments with the inhibitors, at two different concentrations for each inhibitor. We leave the treatment to work for 24 hours, always leaving a couple of controls untreated.
As we have mentioned, we perform two elisas, one to see total CREB, and another to see P-CREB. The process is the same, the only thing that changes are the antibodies used for one or the other. To see CREB, we will use a specific primary antibody for CREB and its corresponding secondary, and for P-CREB the same. The antibodies used are provided through the TransAM CREB transcription factor assay kit, as well as all the solutions and buffers used in the protocol.
We carried out the cell lysate through nuclear extract kit (Active motif), following the manufacturer's instructions. Protein concentration was determined using another kit, DC protein assay reagent kit (Bio-rad).
We added 30 microliters of complete binding buffer to each well, which will facilitate the binding of CREB and P-CREB present in the samples to their binding with the consensus sequences present in the wells. Subsequently, we added 5 μ g of sample (MCC14 treated with inhibitors) or positive control (from

the kit) to the wells, each sample was made in duplicate, diluted in 20 μl of complete lysis buffer and allowed to incubate for three hours at room temperature with mild agitation (rocking platform). For the blank, no sample was added, simply 20 µl of the complete lysis buffer in the well.

After incubation, each well was washed three times with 1X wash buffer, and the plate was emptied into the sink. The next step is to incubate with the corresponding primary antibody (CREB 82B514; reference 40961 Active motif, 1: 1000, for the Elisa in which we want to see CREB) (phosoho-CREB ser133; reference 43096, Active motif, 1: 500, for the Elisa in which we want to see P-CREB) diluted in 100 μl of 1X antibody binding buffer for one hour, at room temperature without stirring. Subsequently, the wells are washed three times with 1X wash buffer as above, and we incubate for one hour at room temperature with the corresponding secondary antibody (anti-mouse IgG for CREB and anti-rabbit IgG for P-CREB), diluted in 100 μl of antibody binding buffer. We wash the wells four times and add the developing solution, allow to incubate for 10 minutes in the dark so that the blue colour of each reaction occurs in each well. Then, we add the stop solution to each well, which in the presence of acid, the blue colour turns yellow, and we read the absorbance in Spark Multimode Microplate Reader (Tecan Trading AG) for 5 minutes at 450nm.

3.6. Statistical analyses

Unless otherwise specified, all experiments were done independently in triplicate and all numerical data were summarized as the mean ± SEM (standard error of the mean) using GraphPad Prism software. Each global mean was compared using unpaired Student's t-test (α = 0.05).

4. Results

4.1. Biological effects of CREB and RTKs inhibition in MCC proliferation and apoptosis.

As commented above, our group had previously reported a network of somatic mutations affecting genes with potential to affect receptor tyrosine kinase (RTKs) proteins and CREB in MCC patients, among others (González-Vela et al., 2017). Thus, the meta-analysis based on all the open whole exome sequencing data published (64 MCC patients) detected a number of recurrently mutated genes affecting EGF receptors, ephrin receptors, insulin-like growth factor receptors, neurotrophic tyrosine kinase receptor, Platelet-derived growth factor receptors and other receptors (see introduction).

Focusing on the potential role of the transcription factor CREB and the somatic mutations detected in related RTKs genes, we take advantage of the different specific inhibitors of the activity of these proteins in order to study their role in biological processes, such as proliferation and apoptosis in MCC cell lines.

4.1.1. Antiproliferative effects of RTKs and CREB pharmacological inhibition in MCC cells.

To explore this possibility in MCC using targeted inhibition of RTKs and CREB signalling, we decided study them in a panel of MCC cell lines. These cells were representative of the two main etiologies in

MCC: MCPyV- (MCC 13, MCC 14/2 and MCC26) and MCPyV+ (MKL-1); and showed positive expression for P-CREB (data not shown). Considering this, MCC cells were incubated with increasing doses of the indicated specific inhibitors which caused a dose dependent inhibition of cell proliferation that enable us to calculate the IC_{50} concentration in each case. These inhibitors were: selumetibib (AZD6244) (specific MEK inhibitor), H89 dihydrochloride (specific PKA inhibitor), M666-15 (specific CREB inhibitor), dacomitinib (EGFR, ErbB2, ErbB4 inhibitor), BMS-754807 (specific inhibitor of IGF-1R/InsR and Trk A/B), BGJ 398 (FGFR inhibitor), ruxolitinib (JAK1/2 inhibitor) and ponatinib (AP24534) (inhibitor of PDGFRa, VEGFR2, FGFR1) (see table 4 in materials and methods).

To verify the proliferation effects of the different RTK inhibitors, the different IC_{50}s were calculated. On the one hand we used the cell lines MCC13, MCC14 / 2, MCC26 and MKL1 with ruxolitinib and ponatinib (AP24534), and on the other hand, we used the lines MCC26 and MKL1 with selumetibib (AZD6244) and H89 dihydrochloride. As we have explained in the materials and methods section, increasing concentrations of each drug were used in order to calculate the respective IC_{50}, and see the effect that inhibitors have on cell proliferation. The table 5 shows the IC_{50} of each cell line for each treatment used and the specific target of each inhibitor.

Table 5. RTKs inhibitors IC50 values in MCCs cell lines.. IC50 (concentration of treatment necessary to inhibit 50% of cell growth) values in Merkel cell carcinoma cell lines used in this study (MCPyV-: MCC13, MCC14 / 2, MCC26 and MCPyV +: MKL1) incubated for 48 h, using control vehicle and increasing concentrations of M666-15, H89 dihydrochloride, selumetinib (AZD6244), dacomitinib, BGJ 398, BMS-754807 and ponatinib (AP24534), respectively (n=6). Novel data provided by this study is the IC50 marked by orange color, the rest of the data has been provided by other collaborators of the project. Data are IC50 dose (calculated for each condition, µM) ± SD.

INHIBITOR (µM)	**TARGET**	**IC50 (µM)**			
		MCC 13	**MCC 14/2**	**MCC 26**	**MKL-1**
Selumetibib (AZD6244)	MEK1, MEK2, ERK1/2	> 20.00	> 20.00	2.73	1.19
H89 dihydrochloride	PKA,	> 20.00	> 20.00	> 20.00	0,94
M666-15	CREB	0.28	0.25	0.33	0.02
Sorafenib	Raf-1, B-Raf, VEGFR-2FGFR1, others	4.98	7.32	3.56	7.52
Dacomitinib	EGFR, ErbB2, ErbB4	1.29	1.82	1.22	0.84
BGJ 398	FGFR	3.90	1.11	0.73	3.90
BMS-754807	IGF-1R/InsR, Trk A/B, others	1.52	0.62	0.17	3.82
Ruxolitinib	JAK1/2	4.69	7.87	7.04	17,09
Ponatinib (AP24534)	PDGFRa, VEGFR2, FGFR1, ScR	3.87	3.36	0.89	26,6

Results show that all the cell lines are very sensitive to treatment with M666-15, but especially MKL1s are 10 times more sensitive than the rest of the cell lines used. Dacomitinib inhibitor values show to be very effective, and its response in cell lines resembles M666-15, being, once again, more potent for MKL1. The targets of this inhibitor are certain receptors with tyrosine kinase activity present in the plasma membrane, such as EGFR (epidermal growth factor receptor). On the other hand, BGJ-398 and BMS-754807 show to be effective for all cell lines, although to a lesser extent for MKL1, in contrast to selumetibib and H89 dihydrochloride inhibitors, which show practically no effect for adherent cell lines , but MKL1 do. Finally, all cell lines show to be much less sensitive to treatment by sorafenib, ruxolitinib and ponatinib.

4.1.1.1. Ponatinib (AP24534)

MCC14, MCC26, MCC13 and MKL1 cell lines were incubated with increasing concentrations of ponatinib (Figure 5).

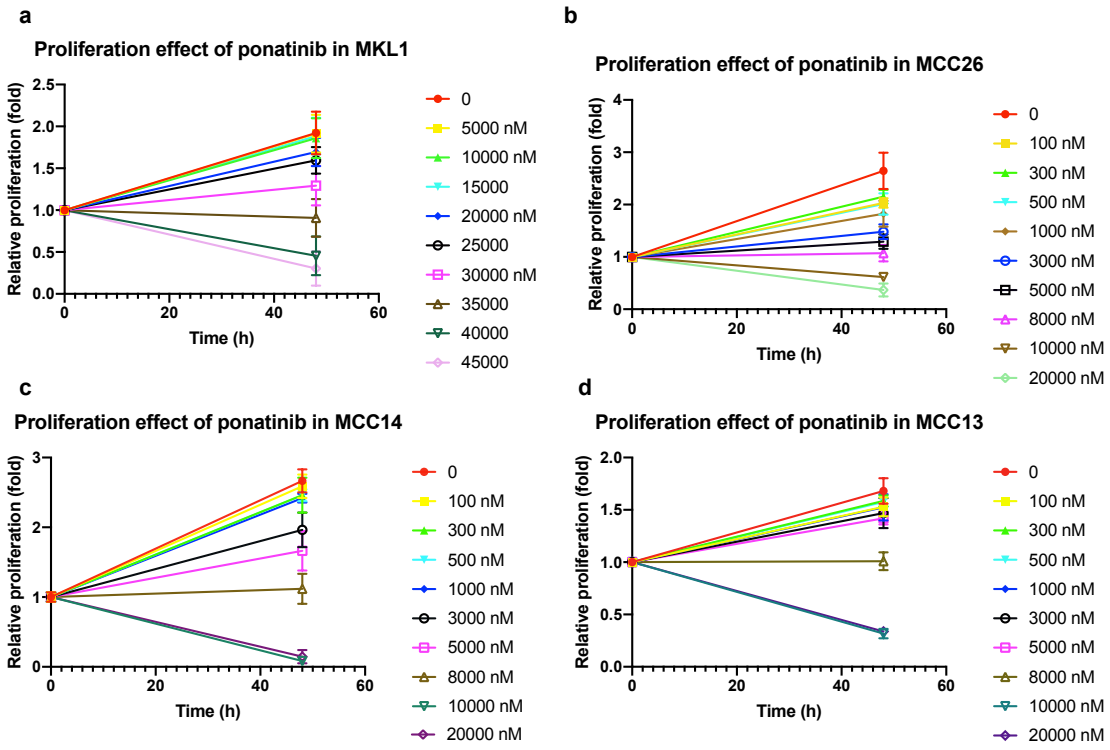


Figure 5. Proliferation of lines MCC14 / 2, MCC26, MKL1 and MCC13 treated with increasing concentrations of ponatinib. IC50 for each of them; MCC14 / 2 3.36 μ M; MCC13 3.87 µM; MCC26 0.89 µM; MKL1 26.6 μ M.

Ponatinib is an RTK inhibitor that targets certain RTK receptors such as vascular endothelial growth factor (VEGF) receptors, platelet-derived growth factor receptor A (PDGFRa), or fibroblast growth factor

receptor 1 (FGFR1). Results show that the negative cell lines for MCPyV are more sensitive than MKL1 for this drug.

4.1.1.2. Ruxolitinib

MCC14, MCC26, MCC13 and MKL1 cell lines were incubated with increasing concentrations of ruxolitinib (Figure 6).

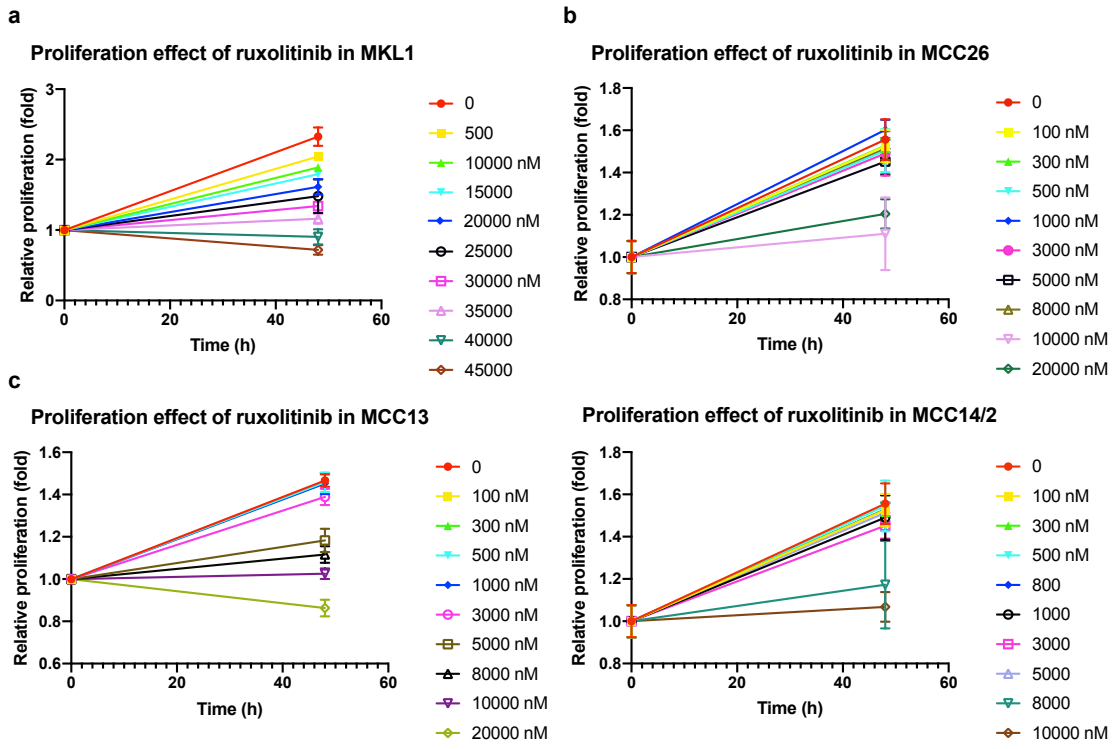


Figure 6. Proliferation of lines MCC14 / 2, MCC26, MKL1 and MCC13 treated with increasing concentrations of ruxolitinib. IC50 for each of them; MCC14 / 2 7.87 μ M; MCC13 4.69 μ M; MCC26 7.04 μ M; MKL1 17.09 μ M.

Ruxolitinib is an inhibitor of JAK1 / 2, kinases present in the RTK pathway to carry out signal transduction and thus be able to modify gene expression. As in the previous case, we can verify that MKL1 cells are less sensitive than the rest of the cells, with a IC50 of 17,09µM an is an inhibitor of JAK1 / 2, kinases present in the RTK pathway to carry out signal transducti on. Although in this case, this drug also has no special effect on the rest of the cell lines since their IC50s are 7,87, 7,04 and 4,69 μ M. So inhibiting proliferation by blocking JAK1 / 2 does not appear to be very efficient.

4.1.1.3. Selumetibib

MCC26 and MKL1 cell lines were incubated with increasing concentrations of selumetibib (Figure 7).

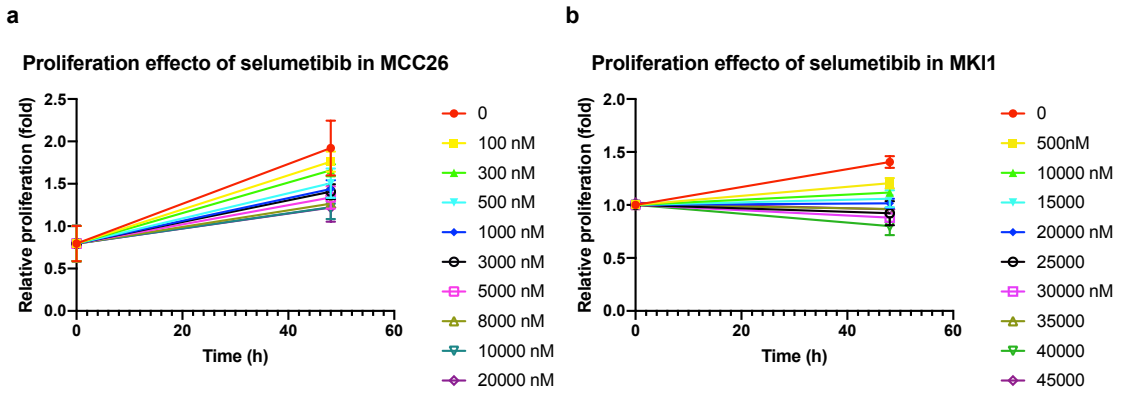


Figure 7. Proliferation of MCC26 and MKL1 lines treated with increasing concentrations of selumetibib. IC50 for each of them; MCC26 2.73 μ M; MKL1 1.19 μ M.

Selumetibib is an inhibitor of the RTK pathway through MEK1 and MEK2, kinases that are responsible for carrying out signal transduction. This treatment has effect in both MCC26 and MKL1 cells, although do not in the rest. In this case, the effectiveness of the treatment does not differ between positive and negative cells for the virus, as in the previous cases. For lines MCC14 / 2 and MCC13 it is absolutely not effective, since their IC50s are greater than 20 μ M, in order to reduce the proliferation of these lines, using this drug we would need to use very large concentrations. In contrast, for MCC26 and MKL1 its effectiveness is significant, since their IC50s are around 1 and 2 μ M.

4.1.1.4. H89 dihydrochloride

MCC26 and MKL1 cell lines were incubated with increasing concentrations of H89 dihydrochloride (Figure 8).

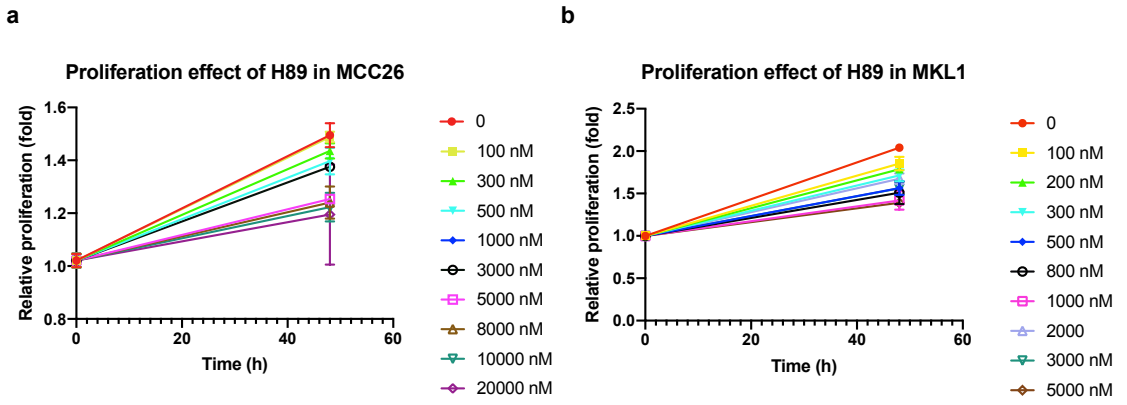


Figure 8. Proliferation of MCC26 and MKL1 lines treated with increasing concentrations of H89 dihydrochloride. IC50 for each of them; MCC26> 20 μ M; MKL1 0.94 μ M.

H89 dihydrochloride is a highly selective PKA pharmacological inhibitor. Results show that there are two groups within the effectiveness of this drug, on the one hand the MCPyV- cell lines show IC50 values

above 20 μ M. This shows a reduced efficacy of this drug over these cells.. On the other hand MKL1(MCPyV + cells) show a high anti-proliferative response to H89 that shows an IC50 of 0.94 μ M.
As we have been able to verify in all the experiments presented, the increasing concentration of the drug produces a reduction in the proliferation, which are the expected results. In general, many of the inhibitors are capable of greatly reducing cell proliferation, therefore RTK pathway is important in the proliferation of MCC cells. The most effective treatments are M666-15, dacomitinib, BGJ-398 and BMS-754807, but especially M666-15, the CREB inhibitor, since the IC50s of all cell lines are very low, and they need a very little amount of drug to drastically reduce cell proliferation.

4.1.2. Pro-apoptotic effects of RTKs pharmacological inhibition in MCC cells.

Having evaluated the effectiveness of RTK inhibitors in cell proliferation of MCC lines, we wanted to evaluate other biological process which RTK inhibitors can produce such as the induction of apoptosis. For this assay, we performed using flow cytometry technology, taking advantage of a commercial kit based on annexin V staining, which allows us to differentiate the different cell populations (living cells, and cells in early and late apoptosis) (see more information in Methods section).
For this, we used two cell lines which were representative of the two main etiologies in MCC: MCPyV- (MCC 14/2) and MCPyV+ (MKL-1), and three specific inhibitors: dacomitinib (EGFR, ErbB2, ErbB4 inhibitor), BMS-754807 (specific inhibitor of IGF-1R/InsR and Trk A/B) and BGJ 398 (FGFR inhibitor), which showed the most effectiveness inhibiting proliferation in these MCC cell lines. The cells were incubated with the respective inhibitors, at range concentrations that include IC50 values for each drug (1 and 10 μ M), for 24 hours. Each sample was carried out in triplicate. It is important to highlight that experimental controls were carried out: positive apoptosis controls(10 µM okadaic acid and 10 µM adramycin), stainingcontrols (blank, in which we will not add any staining, positive for annexin V, and finally another control for 7AAD).

4.1.2.1. MKL1 treated with dacomitinib

Dacomitinib is an inhibitor which target tyrosine kinase activity such as EGFR, and some tyrosine kinases like ErbB2 or ErbB4. It was shown to be highly effective in inhibiting cell proliferation in all cell lines, having an IC50 of around 1 μ M, although MKL1 were somewhat more sensitive, having an IC50 of 0.84 μ M. It is also effective in inducing apoptosis in MKL1 cells, as we can verify in the results shown in figure 9.
The differences between the cells without treatment, and the cells with treatment at 1 μ M are practically non-existent, but when we compare with the results with a treatment of 10 μ M, the result shows a significant increase of MKL-1 cells in late apoptosis (Figure 9). Furthermore, the number of cells in early apoptosis decreases, with respect to the treatment with 1 μ M, it may be due to the fact that with such a strong treatment they have advanced more rapidly in the apoptosis process, whereas with the 1 μ M treatment, there are more cells in early apoptosis than late, since it has not given them time to advance as much in the apoptosis process. It must be taken into account that the IC50 of the cell lines with this

treatment are around 1 μ M, so it is possible that this concentration is capable of stopping proliferation, but it does not produce a great effect on cell death, on the other hand with a higher concentration, it achieves that effect.

Annexin V negative and 7AAD positive cells are dead cells that lack a plasma membrane, but it does not know if they have died from treatment or other reason.

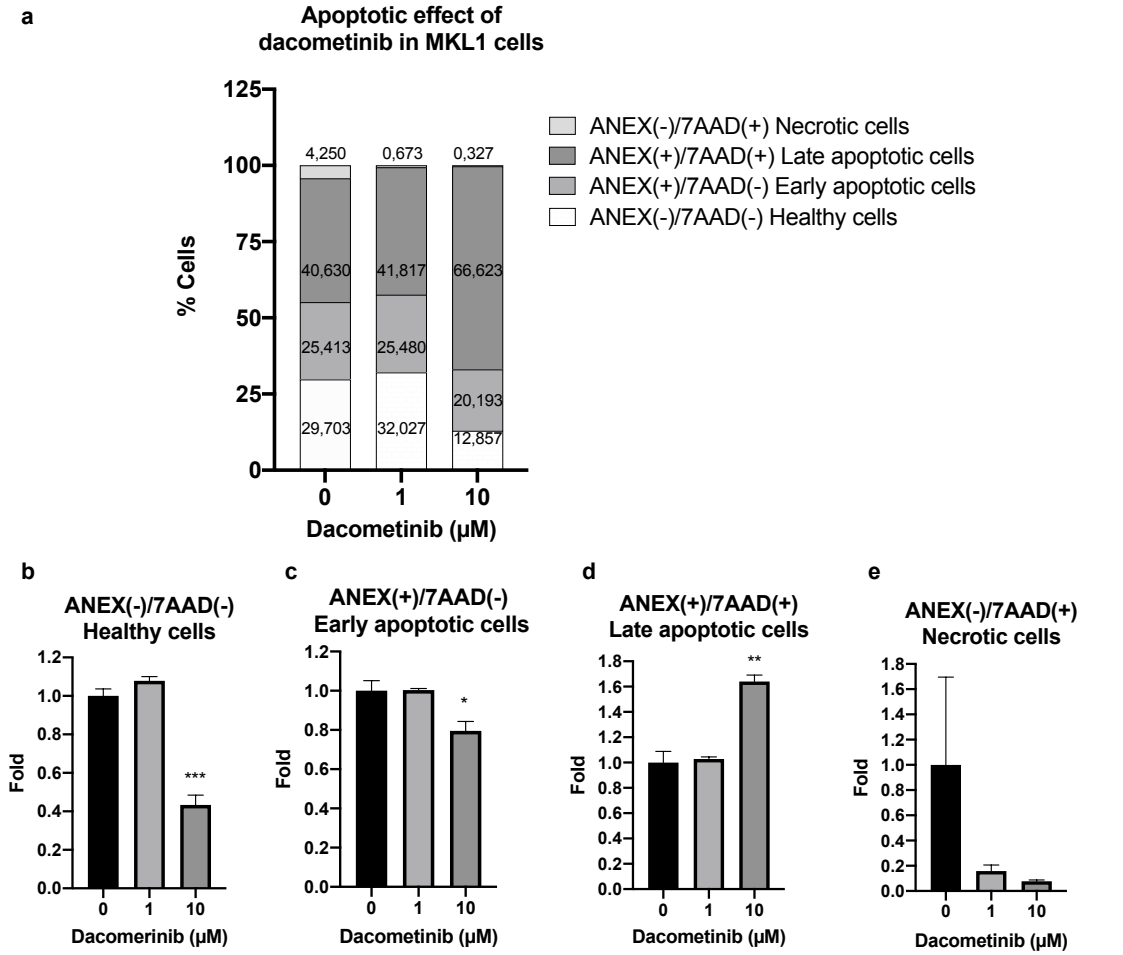


Figure 9. Pro-apoptotic effects of dacomitinib in MKL1 cells. MKL1 cells previously incubated for 24 h with dacomitinib 1 and 10 µM, or untreated cells (control). A) Percentages of cells grouped according to the sample (untreated, treated with 1µM or treated with 10µM). B) Healthy cells of each sample C) Cells in early apoptosis of each sample D) Cells in late apoptosis of each sample. Data are mean ± SEM; Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs control condition.

4.1.2.2. MCC14/2 incubated with dacomitinib

MCC14 / 2 cells treated with dacomitinib, show similar results than MKL1 cells. The differences between the cells without treatment, and the cells treated with 1 μ M are practically non-existent, and with a more powerful treatment, 10 μ M, healthy cells decrease and cells in late apoptosis increase. Results show that this treatment is effective in inducing apoptosis, in both cell lines MKL1 and MCC14 / 2 (Figure 10).

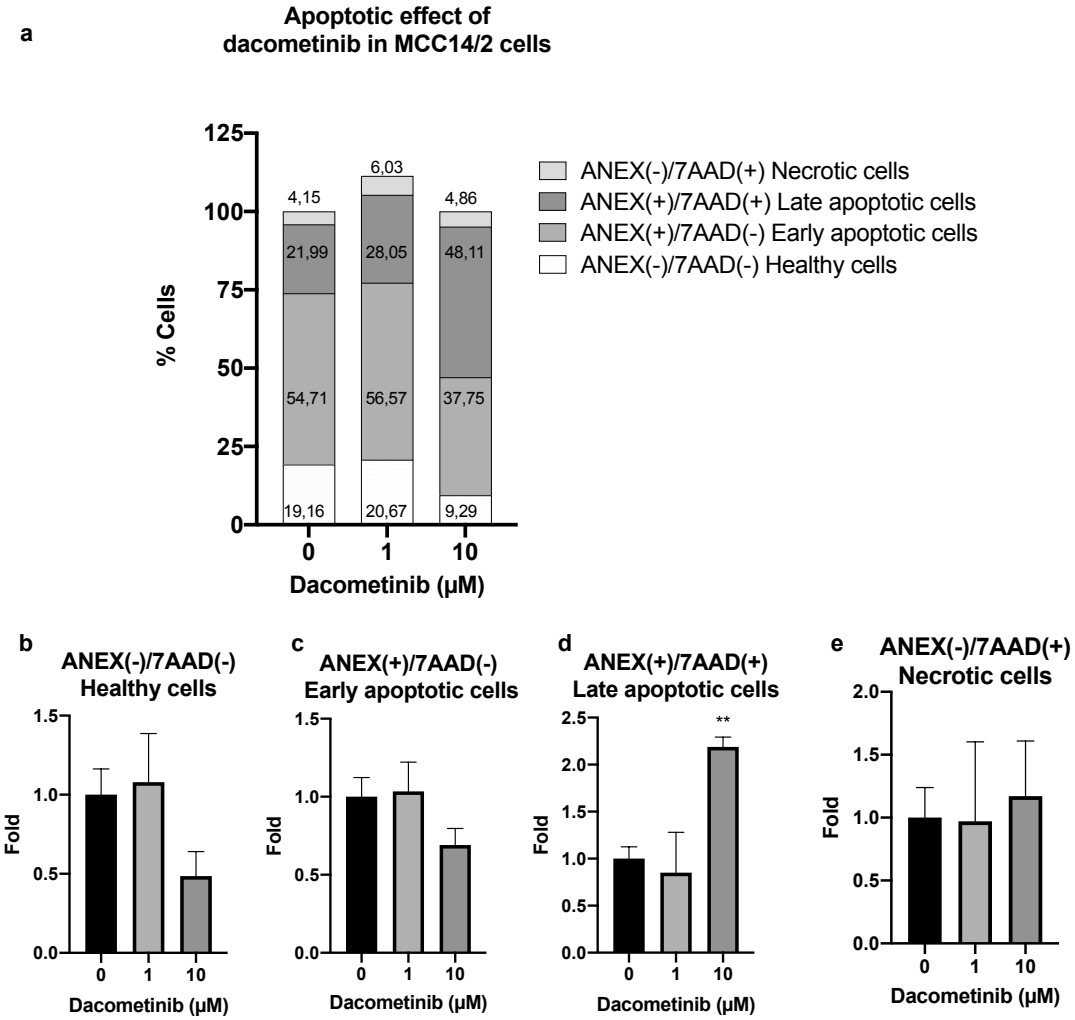


Figure 10. Pro-apoptotic effects of dacomitinib in MCC14/2 cells. MCC14/2 cells previously incubated for 24 h with dacomitinib 1 and 10 µM, or untreated cells (control). A) Percentages of cells grouped according to the sample (untreated, treated with 1µM or treated with 10µM). B) Healthy cells of each sample C) Cells in early apoptosis of each sample D) Cells in late apoptosis of each sample. Data are mean ± SEM; Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs control condition.

4.1.2.3. MCC14/2 treated with BMS-754807

BMS-754807 is an RTK inhibitor whose primary target is the insulin growth factor receptor (IGF-1R) as well as the insulin receptor (INSR). This treatment proved to be very effective in inhibiting the cell proliferation of all the lines used, although MKL1 was somewhat less sensitive. In these tests to measure the induction of apoptosis in MCC14 cells, it shows that it effectively produces cell death. Furthermore, in this case, it can observe the differences from the 1 μ M treatment with respect to the untreated sample (figure 11). The results show how the population of cells in late apoptosis increases significantly, as well as the population of healthy cells decreases. Cells in early apoptosis remain more or less constant. In contrast, with the 10 μ M treatment, the cells in early apoptosis increase, and the population remains in

late apoptosis and healthy cells with respect to the 1 μ M treatment. The IC50 of this treatment for this cell line was 0.64 μ M, so the effects in inducing cell death can be seen from the 1 μ M treatment, and it is not necessary to use as much concentration as in the previous cases to see the effects.

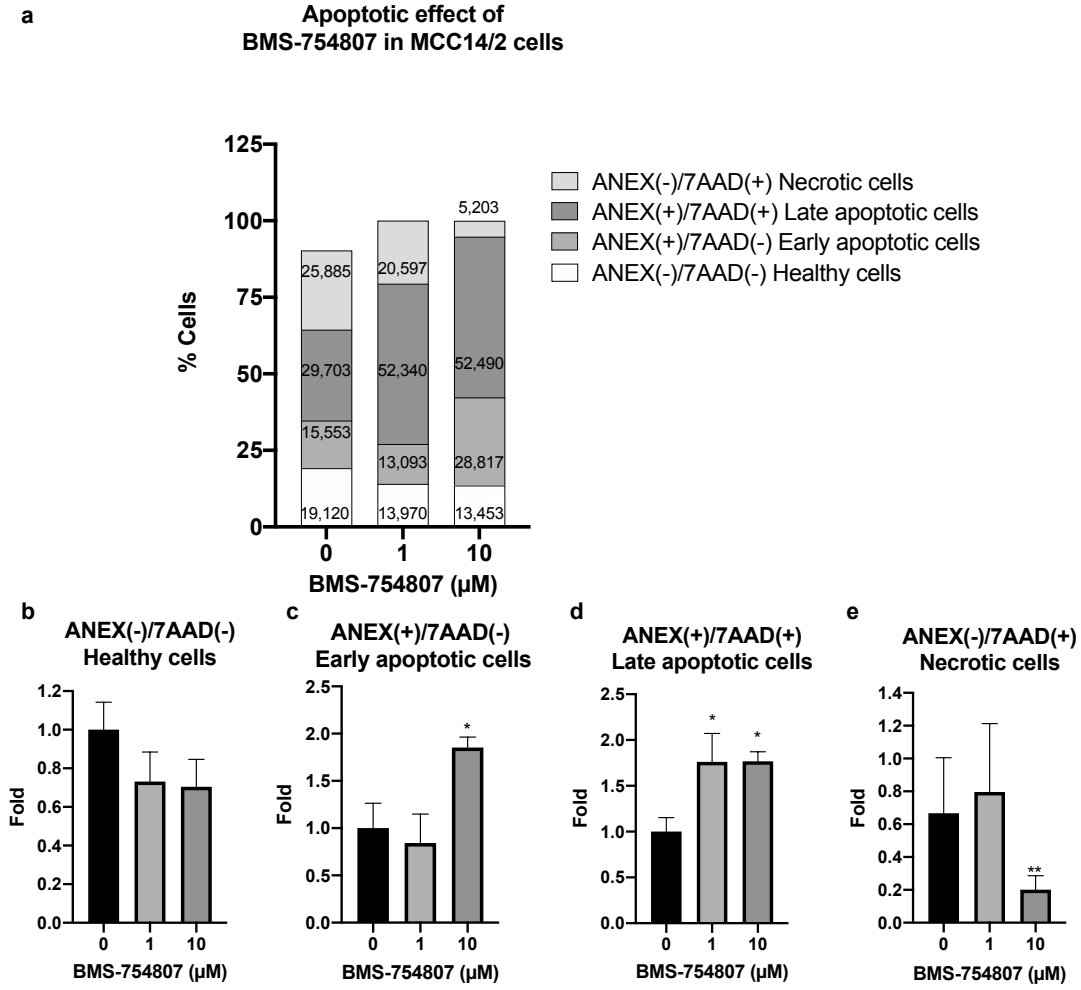


Figure 11. Pro-apoptotic effects of BMS-754897 in MCC14/2 cells. MCC14/2 cells previously incubated for 24 h with BMS-754807 1 and 10 µM, or untreated cells (control). A) Percentages of cells grouped according to the sample (untreated, treated with 1µM or treated with 10µM). B) Healthy cells of each sample C) Cells in early apoptosis of each sample D) Cells in late apoptosis of each sample. Data are mean ± SEM; Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs control condition.

4.1.2.4. MKL1 treated with BMS-754807

MKL1 cells were treated with BMS-754807 and results show a decrease in the population of living cells, also population of cells in apoptosis increases (early and late apoptosis) with the treatment (figure 12). But this approach failed to show large differences between the 1 and 10 μ M treatment. The IC50 of MKL1 with BMS-754807 is approximately 4 μ M, we expected not to see great effects in the treatment with 1 μ M, but we expected to see them increasing the concentration. It must also be taken into account that in all cases, in the untreated samples, the number of living cells is generally very small, because in

the processing and staining of the samples, we lose a lot of cells and many die. It is important to keep in mind that the concentrations that inhibit a biological effect, such as proliferation, do not have to be the same for another biological process, such as apoptosis.

But in general, our results show the reduction of the population of living cells, as well as the increase in apoptosis, in MKL1 cells.

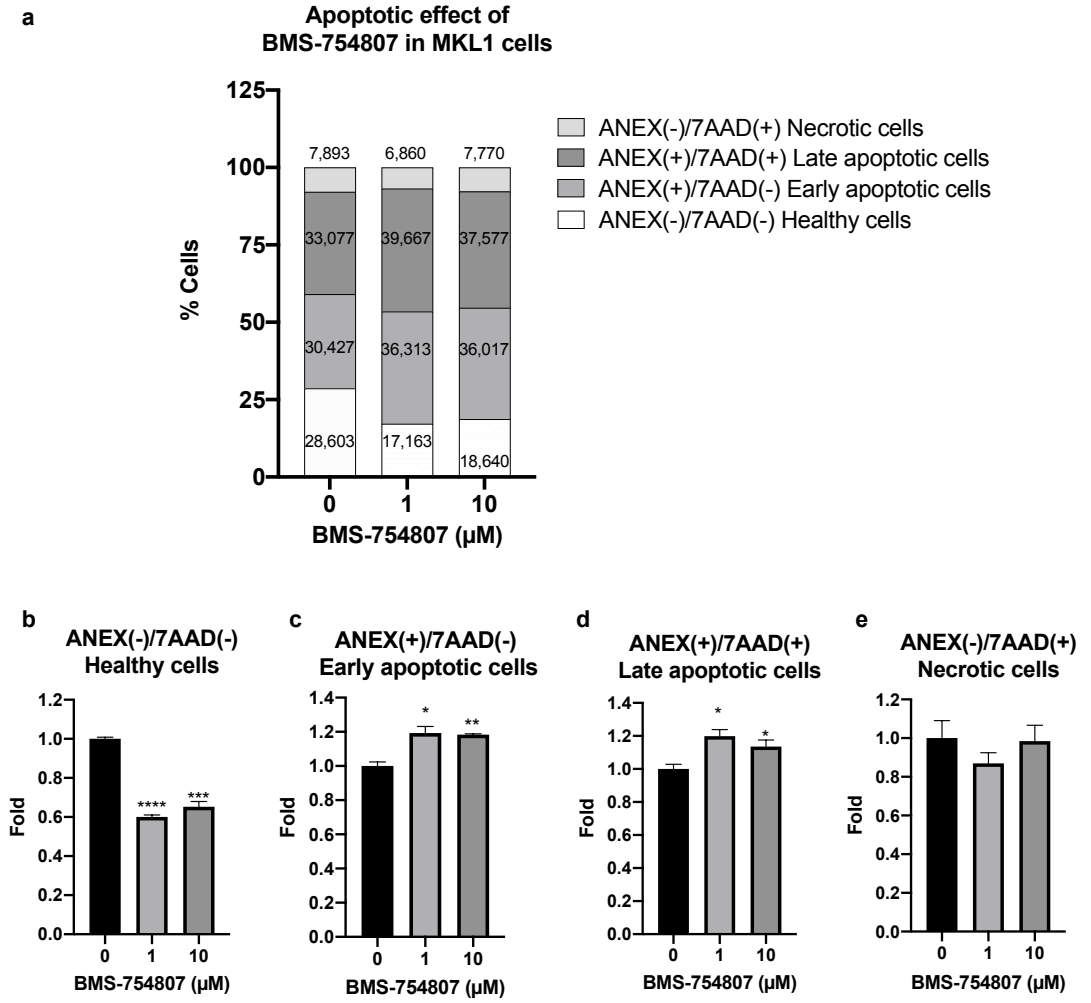


Figure 12. Pro-apoptotic effects of BMS-754897 in MKL1 cells. MKL1 cells previously incubated for 24 h with BMS-754807 1 and 10 μM, or untreated cells (control). A) Percentages of cells grouped according to the sample (untreated, treated with 1μM or treated with 10μM). B) Healthy cells of each sample C) Cells in early apoptosis of each sample D) Cells in late apoptosis of each sample. Data are mean ± SEM; Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs control condition.

4.1.2.5. MCC14/2 treated with BGJ-398

BGJ-398 is an RTK inhibitor whose primary target is the receptor for fibroblastic growth factor, or FGFR. This treatment has been shown to be effective in inhibiting cell proliferation, with IC50s of around 1 μ M in MCC14 /2 and MCC26, while for MCC13 and MKL1, IC50s were somewhat higher, reaching approximately 4 μ M. BGJ-398 treatment in MCC14 cells induce apoptosis in these cells. Results obtained (figure 13) show something unexpected: living cell population remains constant both in

the untreated cells and in the treated samples at two concentrations (1 and 10 μ M).; We can also see that cells in late apoptosis also remain constant. Cells in early apoptosis in samples treated with 1 μ M increase relative to untreated samples, but in samples at 10 μ M they decrease again. It is likely that throughout the processing of the samples and the stains and washes performed, a large number of living cells have been lost, and the death of healthy untreated cells has occurred. For this reason we cannot see differences between untreated cells and treated cells.

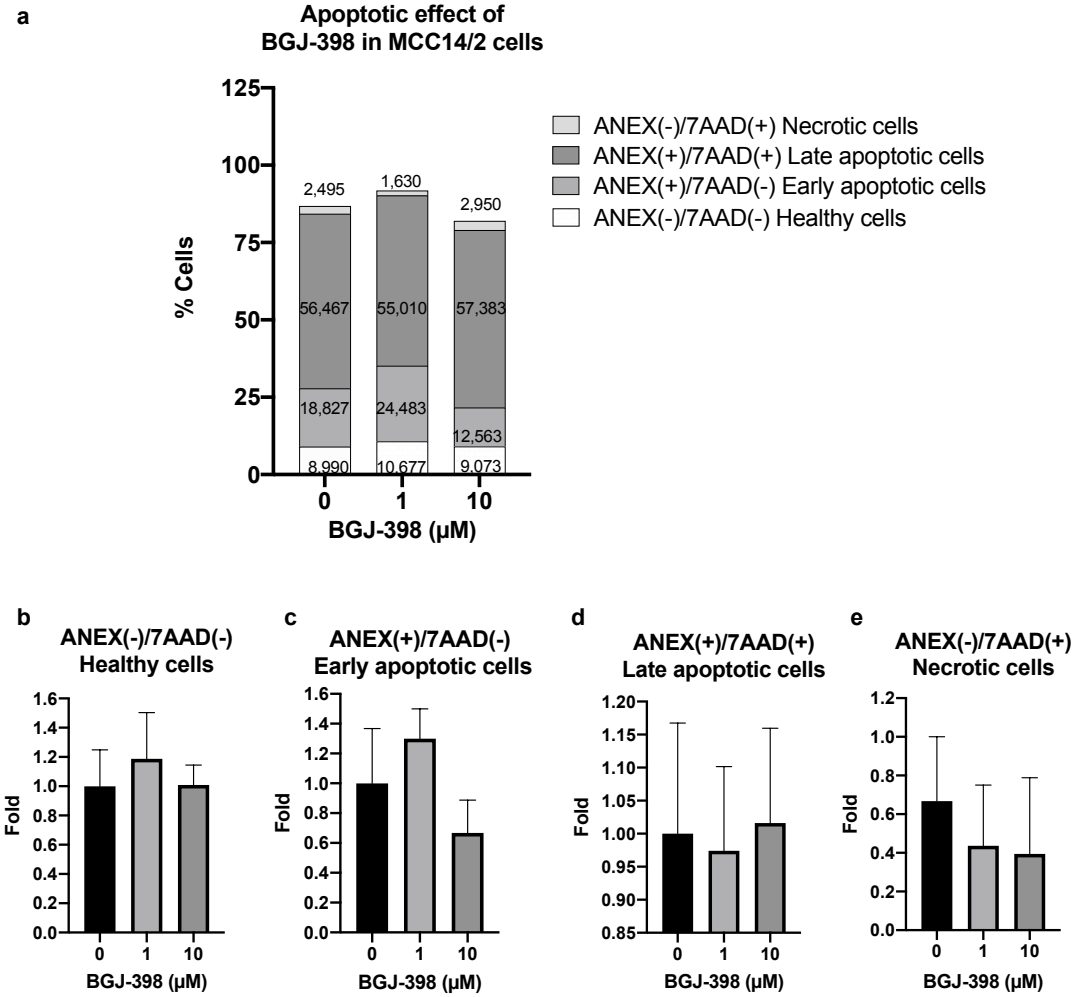


Figure 13. Pro-apoptotic effects of BGJ-398 in MCC14/2 cells. MCC14/2 cells previously incubated for 24 h with BGJ-398 1 and 10 µM, or untreated cells (control). A) Percentages of cells grouped according to the sample (untreated, treated with 1µM or treated with 10µM). B) Healthy cells of each sample C) Cells in early apoptosis of each sample D) Cells in late apoptosis of each sample. Data are mean ± SEM; Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs control condition.

4.1.2.6. MKL1 treated with BGJ-398

BGJ-398 treatment in MKL1 cells succeeds in inducing cell death. Figure 14 shows the results obtained where can verify that cells in early and late apoptosis increase in the treated samples with a final concentration of 10µM with respect to the untreated samples.

The results show how the population of healthy cells decreases significantly with the treatment. As well as the population of cells in early and late apoptosis increases, especially with the treatment of 10 μM. There are not many differences between the control cells and those treated with 1μM. This may be due to the IC50 of the treatment for MKL1 cells being 3,9 μM, therefore the treatment at 1μM is not enough to produce apoptosis of the cells.

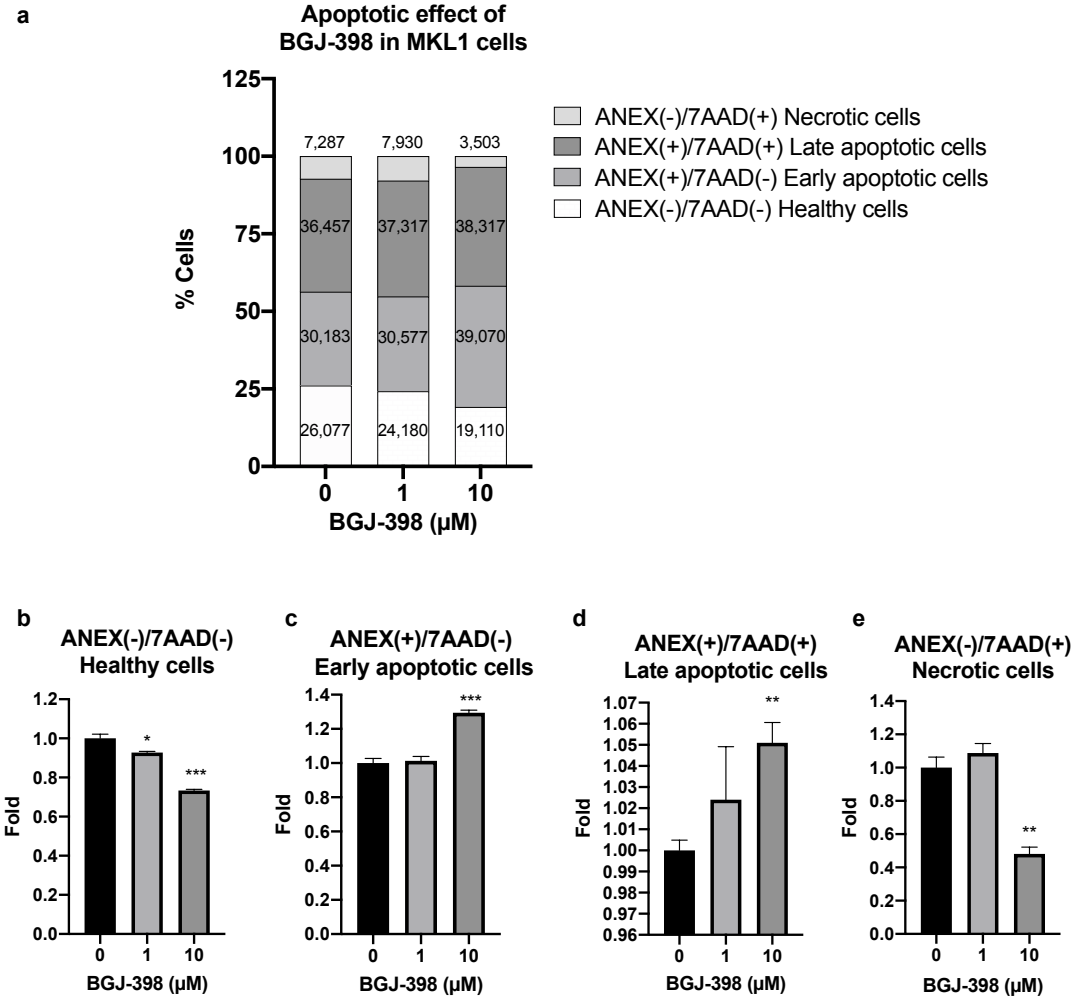


Figure 14. Pro-apoptotic effects of BGJ-398 in MKL1 cells. MKL1 cells previously incubated for 24 h with BGJ-398 1 and 10 μM, or untreated cells (control). A) Percentages of cells grouped according to the sample (untreated, treated with 1μM or treated with 10μM). B) Healthy cells of each sample C) Cells in early apoptosis of each sample D) Cells in late apoptosis of each sample. Data are mean ± SEM; Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs control condition.

In general, in all the tests it can be verified that the population of healthy cells, even in the samples without treatment, is a very small population. In the untreated samples we can see, in turn, how there are a large number of cells in early and late apoptosis. This may be due to all the processing, washing, and staining the samples undergo.

4.2. Transcriptional effects of RTKs pharmacological inhibition in MCC cells.

CREB is a transcriptional factor localized in nucleus. It is capable of binding to DNA and regulate transcription activity of its target genes. In basal conditions CREB is bound to DNA and basal activated in its non-phosphorylated form. When it is phosphorylated by upstream signaling pathways, its transcriptional activity is significant increased. We want to check whether RTK inhibitors capable of reducing cell proliferation and inducing apoptosis of MCC cell lines are also capable of inhibiting the binding of CREB and P-CREB with their consensus binding sites in DNA, and thus inhibit the expression of the genes it regulates.

To check the binding capacity of this transcription factor to its specific DNA binding sites, we take advantage of the commercial ELISA-based TransAM kit (see methods section for more information), studying both total and phosphorylated CREB.. For this, we used the MCC14 / 2 cell line and treated these cells with three different drugs(dacometinib, BMS-754807 and BGJ-398) at two concentrations of 1 and 10 μ M to see whether these treatments can reduce the binding capacity of CREB and P-CREB.
The figure 15 shows the results obtained for both the Elisa from CREB and P-CREB.

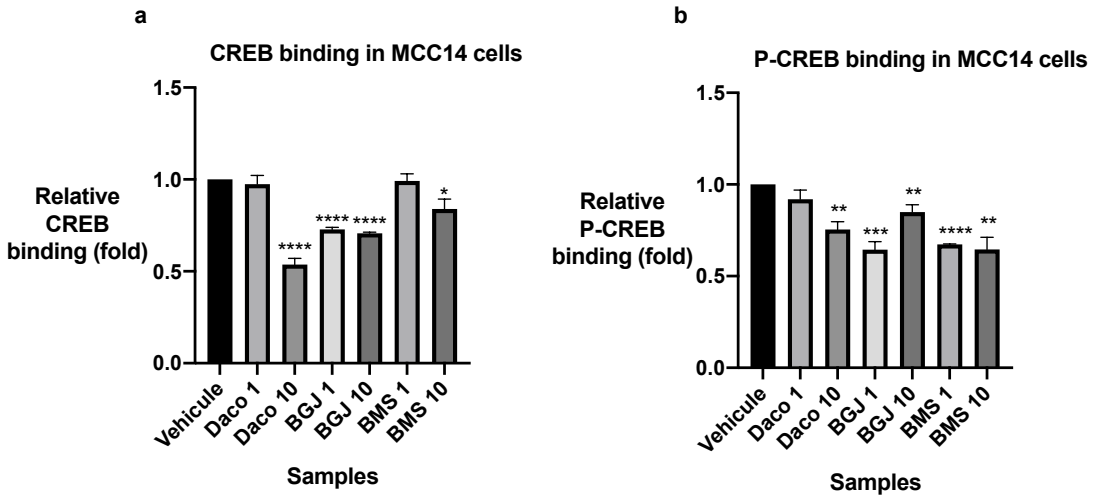


Figure 15. CREB-DNA binding quantification in MCC cells. MCC14/2 cells were incubated for 12 h with control vehicle or the following inhibitors: dacometinib, BGJ-398 and BMS-754807 (1 and 10 μM). Nuclear protein lysates were used to measure (A) CREB and (B) P-CREB DNA binding performing a TransAM ELISA-based (Active Motif, see methods). Data are mean ± SEM; Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs control condition.

Results can verify that in both ELISA-based assays (CREB and P-CREB), with all the treatments, it is possible to reduce the binding of CREB and P-CREB to the DNA consensus binding sites. In general the higher the concentration of treatment, the inhibition is greater. Furthermore, comparing both experiments, we can see that inhibitors are somewhat more effective when it comes to P-CREB than for total CREB.

5. Discussion

MCC is a neuroendocrine type of cancer affecting the skin. It is very aggressive and has a very high mortality, affecting up to 40% of its patients. Although the incidence of the disease is increasing, there is a lack of markers for molecular diagnosis and effective therapies. The main anti-MCC clinical activities are surgery and its combination with adjuvant radiotherapy or chemotherapy. Although the efficacy of immunotherapy for these patients has been demonstrated in recent years, through the use of anti-PD1 / anti-PDL1 antibodies, with a response efficacy of up to 50% of their patients (Tarabadkar et al., 2018) It is still unveiled which patients can benefit from these therapies. This means that half of the patients MCC do not have a real option for effective therapy, so it is necessary to continue investigating the mechanisms involved in the development and progression of MCC, to better understand the disease and to find new targets in order to develop effective therapy.

In previous investigations (González-Vela et al., 2017) they have been in charge of studying the molecular mechanisms of MCC of both MCPyV + tumors and MCPyV- tumors. They studied certain relevant pathways that shared both etiologies such as N-FAT, P-STAT, P-CREB, TP53 and P63. In addition, activated CREB was revealed as an independent factor for worsened prognosis regardless of the etiology of the tumor, sex, age and tumor staging.etc. Therefore, it seems relevant to further study the role of CREB in this disease alongside the mechanisms that might control its malignant activities in MCCs or that could be controlled by CREB, like for example CREB-mediated transcriptome. These have been shown to be involved in cell growth, cell cycle, tumor-mediated angiogenesis, tumor metabolism, etc (André Steven et al., 2016) and thus favor the development and progression of multiple types of human cancer and of course could do so in MCC.

Continuing with mechanisms involved in the development and progression of MCC, our laboratory group carried out a study analyzing the published exomes of tumors from MCC patients, and we saw that many of the mutations belonged to genes of RTK or of its signaling pathway, so that this pathway could be involved in the MCC carcinogenesis process. In addition, the RTKs pathway is one of the pathways that has been widely shown to activate CREB, so to find out if the activation of RTKs is capable of activating CREB, a series of experiments were carried out using a series of ligands that were capable of activating the RTKs and consequently their signaling pathway. Both CREB and ERK phosphorylation were analyzed (see tables 1 and 2) and showed that indeed the RTK pathway was capable of activating both ERK and CREB. Therefore, a conexion between the activation of specific members of the RTK family and CREB/ERK activation has been detected in MCC cells..

Moreover, In this work we provide evidence of the importance of the RTK pathway for the development and progression of MCC, which we show can be mediated by their ability to over-activate CREB. In this respect, we analyzed the ability of specific RTK inhibitors to inhibit proliferation and induce apoptosis in both MCPyV + and MCPyV- cell lines, as well as theirto inhibit CREB activitites downstream of these RTKs.

5.1 Biological effects of CREB and RTK inhibition in different lines of MCC

As previously explained, we carried out two analyzes on the biological effects of RTK inhibitors, on the one hand the antiproliferative effect and on the other the proapoptotic effect of both MCPyV- and MCPyV + MCC cell lines.

5.1.1 Antiproliferative effect of RTK and CREB inhibition in MCC cell lines

When analyzing the antiproliferative effects of the different RTK inhibitors in MCC lines, we calculated each inhibitor´s IC50s, for each line (see Table 5). In general, these drugs inhibited proliferation of MCC cells in a concentration-dependent manner thus supporting the idea that RTKs play important roles in maintaining the malignant proliferation of MCC cells..

In our hands, the most effective inhibitor was M666-15 (CREBi). Itinhibited proliferation in all MCC cell lines (both MCPyV- and MCPyV+) at low concentrations (nanomolar). In this sense, we believe that these data highlights the importance of CREB and its related signaling in the development and progression of MCC (Johannessen M. et al., 2004).

The second most effective inhibitors to reduce the proliferation of all cell lines are dacometinib (inhibits EGFR), BGJ 398 (inhibits FGFR) and BMS-754807 (inhibits IGF-1R/InsR). These treatments are capable of inhibiting the proliferation of cells from both MCC etiologies (MCPyV- and MCPyV +) at very small concentrations, in a concentration-dependent manner. This observation is in agreement with the number of mutated RTKs of the EGFR, FGFR and INSR-IGFR families (see introduction and previous data).

Another interesting result obtained is the effects of H89 (PKAi) on the different cell lines. As can be seen in table 5, H89 is not effective in inhibiting the proliferation of MCPyV- cell lines (MCC13, MCC14 and MCC26), but it is effective in inhibiting the proliferation of the line cell MCPyV + (MKL1). The effect of this drug is different according to the two etiologies of MCC. As previously explained, MCPyV negative tumors have a high mutational load, compared to virus positive tumors (Goh et al., 2016). Such a high mutational load can produce changes in the functioning of different signaling processes and pathways, for example causing hyperactivation of the PKA pathway, which is the inhibitory target of H89. It is probable that in the MCPyV- cell lines where this inhibitor does not exert such an important anti-MCC effect, there are alternative mechanisms that can target PKA pathway causing its deregulation and therefore might negatively affect the treatment The multiple genetic alterations observed in MCPyV- cells could help explain this observation. In support of this, it was was not the case of MKL1 (MCPyV+) cells hence suggesting that PKA inhibitors might exert better anti-MCC activities when used in a MCPYV+ context. This observation deserves further investigation.

In addition, it must be taken into account that PKA is perhaps the most known kinase to regulate CREB activity, whose presence activated in MCC tumors was related to a worse prognosis and a marker of death (Gonzalez-Vela et al ., 2017). So, deregulated PKA may also over-activate CREB in MCC-MCPyV-

tumors, therefore, although PKA inhibitors wasn´t effective at inhibiting the proliferation of MCPyV-negative cells, the CREB inhibitor, M666 proved to be effective in cells from both MCC etiologies, and therefore can be an interesting alternative to seek for future therapy of MCCs. Furthermore, the hyperactivation of PKA and of different kinases that are part of the signaling pathway of RTKs such as ERK, have already been directly associated with CREB hyperactivity in other melanoma cells (Lee et al., 2016), or in other studies, in those through the use of a tyrosinase inhibitor they were able to inhibit the cAMP / PKA / CREB pathway and decrease melanogenesis (Lee et al., 2013), therefore more effort needs to be invested in studying the PKA path and seeing if it is really over-activating CREB.

Finaly, the least effective inhibitors for MCC cell lines were sorafenib (inhibits Raf-1, B-Raf and VEGFR) and ruxolitinib (JAKi). Also other inhibitors that did not turn out to be very effective were selumetibib (MEKi) whose IC50 in MCC13 and MCC14 cells exceeded 20 μ M. Interestingly, Selumetinib (MEKi) and H89 (PKAi) wereboth effective at inhibiting proliferation of MKL1 (MCPyV+ cells). Although this is a preliminary data, it is possible that MCPyV+ MCC cells can be more sensitive to anti PKA/MEK inhibitors vs. MCPyV- MCC cells
On the other hand, ponatinib (PDGFRi, VEGFR2i and FGFR1i) does not seem to have a great effect in inhibiting proliferation in any cell line except MCC26. ,

In general, the results show that by inhibiting CREB, we were able to inhibit the proliferation of all cell lines, showing its importance for cell growth and division, as well as the key role in carcinogenesis and development of MCC. On the other hand, other key routes for the development and proliferation of MCC cells can be seen, such as the EGFR, FGFR, IGF-1R / InsR pathway, since the most effective inhibitors are those that inhibit these receptors (dacometinib, BMS -754807 and BMS-398). Furthermore the importance of such inhibitors has already been previously related to MCC. As the high number of EGFR mutations in samples from MCC patients, which also respond favorably to inhibitors of the aforementioned receptors (Veija et al., 2019).
On the other hand, there are other pathways whose inhibition does not cause large changes in the proliferation of MCC cells, such as the inhibition of intermediate kinases, which are responsible for carrying out signal transduction, such as MEK or JAK, as well as inhibition of other receptors with tyrosine kinase activity such as VEGFR2 or FGFR1. It can be verified that the most effective way to carry out the inhibition of MCC cells is through the direct inhibition of the tyrosine kinase receptors such as EGFR, FGFR or IGF-1R / InsR, and not through the intermediate points of the signaling route of RTKs, such as through the kinases that inhibitors such as ruxolitinib or sorafenib use as targets. Specifically, the most effective inhibitors regardless of the etiology of the tumor would be dacomitinib, BMS-754807 and BGJ 398.

As can be seen from the results, the RTKs pathway can be a way to investigate more deeply to know the mechanisms that promote the development and carcinogenesis of MCC in order to develop a specific and more effective therapy in the future. In addition, the use of RTK inhibitors to treat MCC has already been tested in previous studies, and its efficacy has been demonstrated. In the study by Krasagakis et al., 2011

they studied the co-expression of the KIT receptor and its stem cell ligand factor (SCF) in cells from an MCC biopsy. Although the role of this receptor and its ligand in the development and progression of MCC is still unknown, they saw that enrichment with SCF promoted cell growth and proliferation, as well as increased kinase signaling by ERK ½. In turn, the results showed that the use of an antibody that bound to the KIT ligand binding site, or the use of KIT kinase inhibitors such as imatinib and nilotinib inhibited the growth of MCC cells. And other inhibitors, such as U0126 that inhibits MEK ½, were able to inhibit the proliferation of MCC cells. So this study supports once again the importance of the RTKs pathway in MCC carcinogenesis, as well as the use of RTK inhibitors or their signaling cascade to inhibit the growth of merkel carcinoma in vitro, what that suggests new goals to develop a therapy for this cancer.

5.1.2 Pro-apoptotic effects of RTK inhibition in MCC cell lines

To analyze the proapoptotic effects of RTK inhibition, those treatments that had been shown to be more effective in inhibiting cell proliferation in the different lines of MCC were used. And those were chosen that were capable of inhibiting cell proliferation regardless of the etiology of the tumor, so dacomitinib, BMS-754807 and BGJ 398 were used.
These treatments were used in two representative MCC cell lines, on the one hand, MCC14 (MCPyV-) and MKL1 (MCPyV +).

Dacomitinib (EGFRi) was shown to have a great pro-apoptotic effect in both cell lines, since the population of healthy cells decreased with increasing drug concentration, as did the population of cells in early and late apoptosis. It can be seen how a higher concentration produces a greater proapoptotic effect since with the dacomitinib concentration of 1 μ M the differences with respect to the control are almost nil, but with the 10 μ M concentration, the proapoptic effect of said treatments can be clearly seen in both cell lines.

For the treatment of BMS-754807 (IGF-1R/InsR inhibitor) there are differences between the two cell lines. On the one hand we see that for MCC14 it has an effective pro-apoptotic effect, since for both concentrations we see how the population of healthy cells decreases and the population of cells in late apoptosis increases. On the other hand, for MKL1 this pro-apoptotic effect is not so evident. The results show that the population of healthy cells decreases with treatment but the population of cells in early and late apoptosis remains relatively constant with or without treatment. It should be noted that in all experiments the population of healthy cells is very small even in the untreated samples, due to all the processing of the samples, as well as the populations in late and early apoptosis of the untreated samples due to the same reason. Even so, it is not possible to see differences between the samples without treatment or as the concentration of treatment increases, so that said drug does not seem to have an evident proapoptotic effect on MKL1 cells.

In the case of BGJ 398 (inhibitor of FGFR) the opposite occurs, for the MKL1 cell line we can see a clear proapoptotic effect from this drug, since once again we see how the population of healthy cells decreases

with treatment, as well as the population of cells in early and late apoptosis, especially for the 10 μ M concentration. In contrast, for the MCC14 cell line both the healthy cell population and the population of cells in early or late apoptosis remain constant in the samples with and without treatment, no differences are seen as the concentration of treatment is increased (1 or 10 μ M). Therefore, treatment with BGJ 398 for the MCC14 cell line does not show a clear or evident proapoptotic effect as in the case of MKL1.

The results show that when inducing apoptosis of MCC cell lines regardless of their etiology (MCPyV + or MCPyV-), the most effective treatment is dacomitinib whose main target is the EGFR route. In contrast, for MCPyV- cell lines such as MCC14 / 2, another effective treatment would be BMS-754807 whose target is IGF-1R / InsR, while for MCPyV + cell lines such as MKL1, BGJ-398 whose main target is FGFR would be more effective.

Therefore, the results show that both to inhibit cell proliferation and to induce apoptosis of MCC cell lines regardless of tumor etiology, the most effective treatment is dacometinib, whose main target is EGFR receptors, which could be playing a very important role in the biological activity of MCC tumors.

As we can see, when inducing apoptosis of MCC cells, the use of RTK inhibitors seems effective, although other studies have used other strategies. As for example through the use of certain inhibitors, such as MAL3-101, which is an inhibitor of the heat shock protein 70 (Adam et al., 2014). This protein is used by MCPyV to integrate into the cell genome and the use of this inhibitor was able to induce apoptosis in 5 of the seven cell lines used in the study. Other studies used other pathways, such as through inhibition of the bcl-2 protein family (Verhaegen et al., 2014). They first analyzed the levels of said protein in eleven MCC cell lines and the results showed that all the cell lines had an overexpression of said protein family. The bcl-2 family are key proteins to ensure cell survival since they are responsible for maintaining the permeabilization of the mitochondria and inhibiting apoptosis (Danial et al., 2004). Inhibition was achieved through a small antagonist molecule, called ABT-263, and induced apoptosis in 10 of the eleven MCC lines, as well as halting cell growth of a representative MCC line.
Another way to induce apoptosis of MCC has been through the inhibition of microRNAs, such as MicroRNA-375 or miR-375, which is deregulated in many types of cancers and is implicated in tumorgenesis and metastasis (Kumar et al., 2018). Furthermore, said microRNA is highly expressed in MCPyV-positive MCC cells compared to healthy cells or MCC MCPyV- cells. Inhibition of miR-375 in MCPyV + cells reduced cell growth and apoptosis and in MCPyV- cells reduced cell migration, proliferation, and induction of apoptosis, although the effect was greater for MCPyV + positive cell lines.
Our study reveals another interesting way of inducing apoptosis in MCC cell lines, and not only valid for one of its etiologies but for both. As well as certain inhibitors that are more effective for one of the etiologies. Furthermore, these inhibitors are not only capable of inducing apoptosis of MCC cell lines, but also inhibit their proliferation, as previously seen. In addition, later on it will be possible to see another advantage of the use of these inhibitors that the other strategies used in the commented studies do not possess, and that is that RTK inhibitors such as dacometinib, BMS-754807 and BGJ-398 are capable of inhibit the binding of CREB and P-CREB with DNA in MCC14, and thereby reduce its activation,

something that makes it a very interesting target to study in the field of MCC, since the presence of active CREB has been related to a worse prognosis and a marker of death for MCC patients (Gonzalez-Vela et al., 2017).

5.2 Transcriptional Effects of RTKs Inhibition in MCC14

To analyze the transcriptional effects of RTKs inhibitors, two tests were carried out, to analyze the binding capacity of both CREB and P-CREB after the samples were treated with RTKs inhibitors. The chosen RTK inhibitors were once again dacomitinib (EGFRi), BMS-754807 (EGF-1R/InsR inhibitor) and BGJ 398 (FGFRi), since they are the two that have been shown to be more effective in inhibiting cell proliferation of MCC cell lines. The objective was to analyze whether the treatments were able to inhibit the binding of CREP and P-CREB with DNA in the MCC cell line, MCC14.

The results show that all treatments, both dacomitinib and BMS-754807 or BGJ 398, are capable of inhibiting the binding of both CREB and P-CREB with their DNA consensus sites. Furthermore, the treatments are slightly more effective in inhibiting the binding of P-CREB to DNA, rather than the binding of CREB.

Some of the treatments can be seen as being more effective, or their effect is more powerful using higher concentrations (10 μ M), as in the case of dacomitinib in both the CREB and P-CREB cases. For the rest of the treatments, the differences between the treatment with the lowest concentration and the treatment with the highest concentration are not very significant, and this may be due to the fact that with the least concentration we reach its maximum inhibition effect.
But the results show that in general all the treatments show a significant difference of less binding to receptor DNA than binding of the control sample to DNA.

Among all the inhibitors studied, the results have shown that the most effective are BMS-754807 BGJ-398 and dacometinib, the latter standing out as the most effective regardless of the etiology of the tumor. Therefore, although it is necessary to continue studying the molecular mechanisms of MCC, this study shows the importance of EGFR, FGFR and IGF / InsR receptors, as well as CREB as relevant malignant mechanisms in producing the development and progression of MCC, and therefore the inhibitors BMS-754807 BGJ-398 and dacometinib can be used for the development of a truly effective therapy, as they have been used on other occasions to treat metastatic MCC (Tarabadkar et al., 2018).

It is important to take into account the quality of life of patients, since many of them are elderly (Becker et al., 2017) with other pathologies, and treatments can be more dangerous in them. Some RTK inhibitors such as cabozantinib, which specifically inhibits many of the kinases involved in the RTKs pathway or receptors with tyrosine kinase activity such as c-MET or VEGFR, have been used in a study (Rabinowits et al., 2018) for treatment advanced MCC. It turned out that most of the patients were not tolerant to treatment and caused great side effects in them, such as ulcers, hyperthyroidism, anorexia, nausea,

fatigue, headache etc. Therefore, it is necessary to take into account the patient's well-being and what is the best option for each one. Although this drug has been tested in other studies (Tarabadkar et al., 2018) and did not show toxicity problems in patients. In the study by Tarabadkar et al., 2018, they tested the efficacy of two RTK inhibitors (pazopanib and cabozantinib) in patients with metastatic MCC. Such inhibitors specifically inhibited the receptor for growth endothelial factor, or VEGFR. Five of their patients responded beneficially to treatment, one of them responded completely at three months, four of them had the disease stabilized in a time range of five months to three and a half years, and not only that, one of them not only had the disease stabilized but also that the psoriatic arthritis that he suffered, was also improved with the treatment. Furthermore, none of them showed serious side effects or toxicity from VEGFR-TKIs. Thus, the use of RTK inhibitors may have a beneficial effect in patients with MCC in whom surgery is not an option, and immunotherapy has not been an effective treatment (approximately 50% of patients (Tarabadkar et al. , 2018)).

In conclusion, this study provides new evidences that the RTK pathway could have an important role in MCC tumor development in the context of CREB, especially the signaling pathways upstream controlled by EGFR, FGFR and IGF / InsR receptors. Further investigations can help to elucidate the molecular mechanisms involved in MCC carcinogenesis and develop a better specific therapy for this pathology.

6. Conclusion

6.1. RTK inhibition causes inhibition of proliferation and induction of apoptosis of MCC cell lines (MCPyV- and MCPyV +).

6.1.1. Dacometinib is the most effective RTK inhibitor for both inhibiting proliferation and inducing apoptosis in either of the two etiologies of MCC3.

6.1.2. BMS-754807 is an effective treatment to treat MCPyV- cells, both to inhibit proliferation and to induce apoptosis.

6.1.3. BGJ 398 is an effective treatment to treat MCPyV + cells both to inhibit proliferation and to induce apoptosis.

6.1.4. H89 is more effective in the MCPyV + cell line, showing that these cells are more sensitive to inhibition of the PKA pathway, suggesting that it probably finds a high mutational load in said pathway in MCPyV- cells.

6.2. Inhibition of RTK inhibits the binding of CREB and P-CREB to DNA and, therefore, its activation.

6.3. The RTK pathway is an important pathway for MCC carcinogenesis and tumorigenesis, specifically EGFR, FGFR and IGF / InsR receptors that have been shown to be relevant in biological and molecular mechanisms, contributing to their progression and development.

6.4. EGFR, FGFR and IGF / InsR receptors could be a target to develop an effective therapy for MCC.

6. Bibliography

Jürgen C. Becker, Andreas Stang, James A. DeCaprio, Lorenzo Cerroni, Celeste Lebbé, Michael Veness and Paul Nghiem. (2017) Merkel cell carcinoma. Nature review disease primer 3, 17077.

María del Carmen Gonzalez-Vela, Soraya Curiel-Olmo, Sophia Derdak, Sergi Beltran, Miguel Santibañez, Nerea Martínez, Alfredo Castillo-Trujillo, Martha Gut, Roxana Sánchez-Pacheco, Carmen Almaraz, Laura Cereceda, Beatriz Llombart, Antonio Agraz-Dobla , Jose Revert-Arce, Jose Antonio López Guerrero, Manuela Mollejo, Pablo Isidro Marrón, Pablo Ortiz-Romero, Lynnette Fernandez-Cuesta, Ignacio Varela, Ivo Gut, Lorenzo Cerroni, Miguel Ángel Piris and José Pedro Vaqué. (2017) Shared Oncogenic Pathways Implicated in Both Virus-Positive and UV-Induced Merkel Cell Carcinomas. Journal of Investigative Dermatology 137, 197-206.

Deepa B. Shankar, Jerry C. Cheng, Kentaro Kinjo, Noah Federman, Theodore B. Moore, Amandip Gill, Nagesh P. Rao, Elliot M. Landaw and Kathleen M. Sakamoto. (2005) The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia. Cancer cell 7, 351-362.

Ichiki T. 2006. Role of camp response element binding protein in cardiovascular remodeling: Good, bad, or both? Arterioscler Thromb Vasc Biol. 26(3):449-455.

Sun L, Zhao R, Zhang L, Zhang W, He G, Yang S, Song J, Du G. 2016. Prevention of vascular smooth muscle cell proliferation and injury-induced neointimal hyperplasia by creb-mediated p21 induction: An insight from a plant polyphenol. Biochem Pharmacol. 103:40-52.

Altarejos JY, Montminy M. 2011. Creb and the crtc co-activators: Sensors for hormonal and metabolic signals. Nat Rev Mol Cell Biol. 12(3):141-151.

Trinh AT, Kim SH, Chang HY, Mastrocola AS, Tibbetts RS. 2013. Cyclin-dependent kinase 1-dependent phosphorylation of camp response element-binding protein decreases chromatin occupancy. J Biol Chem. 288(33):23765-23775.

Richards JP, Bachinger HP, Goodman RH, Brennan RG. 1996. Analysis of the structural properties of camp-responsive element-binding protein (creb) and phosphorylated creb. J Biol Chem. 271(23):13716-13723.

Jiang M, Yan Y, Yang K, Liu Z, Qi J, Zhou H, Qian N, Zhou Q, Wang T, Xu X, Xiao X, Deng L. (2019) Small molecule nAS-E targeting cAMP response element binding protein (CREB) and CREB-binding protein interaction inhibits breast cancer bone metastasis. Journal Cell Molecular Medicine 23 (2), 1224-1234.

Johannessen M., Pedersen Delghandi, M., and Moens, U. (2004) What turns CREB on?. Cell Signal 10, 1211-1227.

Trenker R, Jura N. (2020) Receptor tyrosine kinase activation: From the ligand perspective. Current Opinion in Cell Biology 63, 174-185.

Yamaoka T, Kusumoto S, Ando K, Ohba M, Ohmori T. (2018) Receptor Tyrosine Kinase-Targeted Cancer Therapy. International Journal of Molecular Sciences 6; 19.

Tarik Regad. (2015) Targeting RTK Signaling Pathways in Cancer. Cancers 7, 1758-1784.

Paul William Harms, Pankaj Vats, Monique Elise Verhaegen, Dan R. Robinson, Yi-Mi Wu, Saravana Mohan Dhanasekaran, Nallasivam Palanisamy, Javed Siddiqui, Xuhong Cao, Fengyun Su, Rui Wang, Hong Xiao, Lakshmi P. Kunju , Rohit Mehra , Scott A. Tomlins , Douglas Randall Fullen , Christopher Keram Bichakjian , Timothy M. Johnson, Andrzej Antoni Dlugosz and Arul M. Chinnaiyan. (2015) The Distinctive Mutational Spectra of Polyomavirus-Negative Merkel Cell Carcinoma. Cancer Research 75 (18).

Stephen Wong, Kelly Waldeck,Ismael Vergara, Jan Schröder, Jason Madore, JamesS. Wilmott, Andrew J. Colebatch, Ricardo PaoliIseppi, Jason Li, Richard Lupat, Timothy Semple, Gisela Arnau, Andrew Fell owes, J.Helen Leonard, George Hruby, GrahamJ. Mann, John,F. Thompson, Carleen Cullinane, Meredith Johnston, Mark Shackleton, Shahneen Sandhu, David Bowtell, Ricky Johnstone, Stephen B. Fox, Grant A. McArthur, Anthony T. Papenfuss, Richard A. Scolyer, Anthony J. Gill, Rodney J. Hicks and Richard W. Tothill. (2015) UV-Associated Mutations Underlie the Etiology of MCV-Negative Merkel Cell Carcinomas. Cancer Research 75 (24).

André Steven and Barbara Seliger. (2016) Control of CREB expression in tumors: from molecular mechanisms and signal transduction pathways to therapeutic target. Oncotarget 7(23).

Dietrich J, Schindler M, Lampen A, Braeuning A and Hessel-Pras S. (2020) Comparison of long-term versus short-term effects of okadaic acid on the apoptotic status of human HepaRG cells. Chemico-Biological Interactions 1; 317.

Lee KS, Lee MG, Kwon YS and Nam KS. (2020) Arctigenin Enhances the Cytotoxic Effect of Doxorubicin in MDA-MB-231 Breast Cancer Cells. International Journal of Molecular Sciences 21; 8.

Erica S. Tarabadkar Hannah Thomas Astrid Blom Upendra Parvathaneni Thomas Olencki and Paul Nghiem Shailender Bhatia. (2018) Clinical Bene t from Tyrosine Kinase Inhibitors in Metastatic Merkel Cell Carcinoma: A Case Series of 5 Patients. American Journal of Case Reports 19: 505-511

Guilherme Rabinowits, Cecilia Lezcano, Paul J. Catalano, Patricia Mchuhg, Hailey Becker, Megan M.Reilly, Julian Huang, Ayushi Tyagi, Manisha Thakuria, Scott C. Bresler, Lynette M. Sholl, Geofrey I. Shapiro, Robert Haddad and James A. Decaprio. (2018) Cabozantinib in Patients with Advanced Merkel Cell Carcinoma. The oncologist 23:814–821.

Konstantin Krasagakis 1, Irene Fragiadaki, Maria Metaxari, Sabine Krüger-Krasagakis, George N Tzanakakis, Efstathios N Stathopoulos, Jürgen Eberle, Nektarios Tavernarakis and Androniki D Tosca. (2011) KIT Receptor Activation by Autocrine and Paracrine Stem Cell Factor Stimulates Growth of Merkel Cell Carcinoma in Vitro.Journal of Cellular physiology 226(4):1099-109.

Satendra Kumar, Hong Xie , Patrick Scicluna , Linkiat Lee , Viveca Björnhagen , Anders Höög , Catharina Larsson and Weng-Onn Lui. (2018) MiR-375 Regulation of LDHB Plays Distinct Roles in Polyomavirus-Positive and -Negative Merkel Cell Carcinoma. Cancers 10(11):443.

Monique E Verhaegen , Doris Mangelberger , Jack W Weick , Tracy D. Vozheiko , Paul W Harms, Kevin T Nash, Elsa Quintana, Paul Baciu , Timothy M Johnson , Christopher K Bichakjian and Andrzej A Dlugosz. Merkel cell carcinoma dependence on bcl-2 family members for survival. (2014) Journal of investigative dermatology 34(8):2241-2250.

Christian Adam, Anne Baeurle, Jeffrey L Brodsky, Peter Wipf , David Schrama , Jurgen Christian Becker and Roland Houben. (2014) The HSP70 Modulator MAL3-101 Inhibits Merkel Cell Carcinoma. Public library of science 9(4):e92041.

Nika N Danial and Stanley J Korsmeyer. (2004) Cell Death: Critical Control Points. Cell Press 116, 205–219.

Tuukka Veija, Mia Kero, Virve Koljonen and Tom Böhling. (2019) ALK and EGFR Expression by Immunohistochemistry Are Associated With Merkel Cell Polyomavirus Status in Merkel Cell Carcinoma. Histopathology 74(6):829-835.

Melissa Pulitzer. (2017) Merkel cell carcinoma. Surgical Pathology Clinics 10(2):399-408.

Dirk Schadendorf1, Céleste Lebbé2, Axel Zur Hausen3, Marie-Françoise Avril4, Subramanian Hariharan5, Murtuza Bharmal, Jürgen C Becker. Merkel Cell Carcinoma: Epidemiology, Prognosis, Therapy and Unmet Medical Needs. European Journal of Cancer 71:53-69.

Paul T Nghiem, Shailender Bhatia, Evan J Lipson, Ragini R Kudchadkar, Natalie J Miller, Lakshmanan Annamalai, Sneha Berry, Elliot K Chartash, Adil Daud, Steven P Fling, Philip A Friedlander, Harriet M Kluger, Holbrook E Kohrt, Lisa Lundgren, Kim Margolin, Alan Mitchell, Thomas Olencki, Drew M Pardoll, Sunil A Reddy, Erica M Shantha, William H Sharfman, Elad Sharon, Lynn R Shemanski, Michi M Shinohara, Joel C Sunshine, Janis M Taube, John A Thompson, Steven M Townson, Jennifer H Yearley, Suzanne L Topalian, Martin A Cheever. (2016) PD-1 Blockade With Pembrolizumab in Advanced Merkel-Cell Carcinoma. The New England Journal of Medicine 374(26):2542-52.

Miriam Becker, Melissa Dominguez, Lilo Greune , Laura Soria-Martinez, Moritz M Pfleiderer, Rachel Schowalter, Christopher B Buck, Bärbel S Blaum, M Alexander Schmidt, Mario Schelhaas. (2019) Infectious Entry of Merkel Cell Polyomavirus.Journal of Virology 93(6):e02004-18.

Gerald Goh, Trent Walradt, Vladimir Markarov, Astrid Blom, Nadeem Riaz, Ryan Doumani, Krista Stafstrom, Ata Moshiri, Lola Yelistratova, Jonathan Levinsohn, Timothy A Chan, Paul Nghiem, Richard P Lifton, Jaehyuk Choi. (2016) Mutational landscape of MCPyV-positive and MCPyV-negative Merkel cell carcinomas with implications for immunotherapy. Oncotarget 7, 3403–3415 (2016).

Marco Rastrelli, Beatrice Ferrazzi, Francesco Cavallin, Vanna Chiarion Sileni, Jacopo Pigozzo, Alessio Fabozzi, Saveria Tropea, Antonella Vecchiato, Alessandra Costa, Alessandro Parisi, Carlo Riccardo Rossi, Paolo Del Fiore, Mauro Alaibac. (2018) Prognostic Factors in Merkel Cell Carcinoma: A Retrospective Single-Center Study in 90 Patients. Cancers 10(10):350.

Alessia Villani, Gabriella Fabbrocini, Claudia Costa, Maria Carmela Annunziata, and Massimiliano Scalvenzi. (2019) Merkel Cell Carcinoma: Therapeutic Update and Emerging Therapies. Dermatology Therapy 9(2): 209–222.

Woan-Ruoh Lee, Shing-Chuan Shen, Pei-Ru Wu, Chia-Lun Chou, Yi-Hsien Shih, Chung-Min Yeh, Kun-Tu Yeh and Ming-Chung Jiang. (2016) CSE1L Links cAMP/PKA and Ras/ERK Pathways and Regulates the Expressions and Phosphorylations of ERK1/2, CREB, and MITF in Melanoma Cells. Molecular carcinogenesis 55(11):1542-1552.

Chang Seok Lee, Won-Hee Jang, Miyoung Park, Kyoungmi Jung, Heung Soo Baek, Yung Hyup Joo, Young-Ho Park and Kyung-Min Lim. (2013) A Novel Adamantyl Benzylbenzamide Derivative, AP736, Suppresses Melanogenesis Through the Inhibition of cAMP-PKA-CREB-activated Microphthalmia-Associated Transcription Factor and Tyrosinase Expression.Experimental dermatology 22(11):762-4.

A Nirenberg, H Steinman, J Dixon and A Dixon. (2019) Merkel Cell Carcinoma Update: The Case for Two Tumours. Journal of The European Academy of Dermatology and Venereology.