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Blockade of Epidermal Growth Factor Receptor, Cycloxygenase-2 (COX-2) and Mammalian Target of Rapamycin (m-TOR) in Animal Model of Lung Cancer

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ABSTRACT

To explore the effectiveness and possible toxicity of the use of epidermal growth factor receptor inhibitor (EGFR Inhibitor), Celecoxib (COX2 inhibitor) and Sirolimus (m-TOR inhibitor) as single agents and drug combinations for the treatment of lung cancer in an experimental model. Lung cancer was induced in Balb-C mice by intraperitoneal injection urethane. Mice were treated with water (control) , Erlotinib (E) (50 mg/kg), Celecoxib (X) (50 mg/kg), Sirolimus (R) (2 mg/kg) given alone and in the following doublet and triplet combinations in the same dosages for 7 days. The number of pulmonary nodules in the combined treatment was significantly inhibited compared with control (p=0.010); E (p=0.028), EX (p=0.010), ERX (p=0.040) showed a smaller number of statistically significant nodules. Regarding coat changes we observe statistically significant differences among groups (p<0.001) where ERX and ER had a higher occurrence of this change. There was a higher incidence of skin rashes in groups: E (p<0.001), ER (p<0.0001), and ERX (p<0.001). Regarding weight we identify weight loss in the ERX (p=0.025). The combination of EGFR inhibitor, COX-2 inhibitor and m-TOR inhibitor had anti-tumor activity in experimental lung cancer. The combination of Celecoxib treatment with Erlotinib is a suggestion for decrease of dermatological events in patients. The combination of EGFR inhibitor and Sirolimus does not decrease the number of lung nodules and potentiates adverse events.

Keywords: Experimental trials, Epidermal growth factor receptor, Lung cancer, Erlotinib Hydrochloride, Cicloooxgenase-2, m- TOR inhibitor.

INTRODUCTION

The introduction of new treatments target-driven for lung cancer in clinical practice has been recently used as therapies directed at vascular endothelial growth factor (VEGF) and Receptor Antagonists human epidermal growth factor (EGFR). [1-6] Moreover, targeted therapy has become important to study other ways such as: agents target receptor insulin growth factor-1 (IGF-IR), cyclooxygenase-2 (COX-2) inhibitors, mammalian target of rapamycin (m-TOR) inhibitors, histone deacetylase inhibitors, and inhibitors of anaplastic lymphoma kinase (ALK). [3, 5, 7-11]

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Celecoxib is a class of anti-inflammatory non-steroidal, and it was approved to assist therapy in patients with familial adenomatous polyposis high risk. [12-14] Several studies have indicated that COX2 is found in many tumors, and it acts in prostaglandin formation and stimulation mechanisms in angiogenesis, cell growth, adhesion, and differentiation. [15-19] This drug is capable of inhibiting cell cycle progression through inhibition of CDK-cyclin complexes and the ability to induce apoptosis. [20] The combination of an EGFR inhibitor and a COX-2 leads to a blockage of tumor progression in squamous cell carcinomas of head and neck with significantly improves the therapeutic response. It suggests that simultaneous inhibition of EGFR and COX-2 may be a more effective strategy to abrogate both signal transductions. [21]

The mammalian Target of Rapamycin (m-TOR) is a protein tyrosine kinase that regulates growth, cell proliferation, motility, protein synthesis and the cellular transcription. This protein is a central regulator of cell proliferation, angiogenesis, and cell metabolism. [22-25] In clinical studies, the inhibition of EGFR, COX2, and m-TOR has been studied as a treatment for cancers and had positive results in NSCLC. [22, 25-26]

Our aims were to evaluate the effect of the blockade of the epidermal growth factor receptor tyrosine kinase domain (Erlotinib Hydrochloride) in comparison to the use of inhibitors of cyclooxygenase-2 (Celecoxib) and m-TOR (Sirolimus), as well as the combination of these drugs against experimental lung cancer treatment.

MATERIAL AND METHODS

Experimental Design

After the approval from the Animal Research Ethics Committee, we used 93 adult male Balb-C mice in the experiment. The animals were kept in cages at a temperature of 23 ± 2ºC and relative humidity of 60% and subjected to 12 h light and 12 h of darkness per day, with water and food ad libitum. On the first day of experiment, 6 mg/kg urethane was injected intraperitoneally into each mouse, in four separate doses of 1.5 mg/kg with 48 h interval between them.

The animals were divided into eight groups:

C **–** 15 animals fed with mineral water (control group); ***E*** **–** 15 animals fed with Erlotinib Hydrochloride; ***X*** **–** seven animals fed with Celecoxib; ***R*** **–** seven animals fed with Sirolimus; ***EX*** **–** 15 animals fed with Celecoxib and Erlotinib Hydrochloride; ***ER*** **–** 15 animals fed with Sirolimusand Erlotinib Hydrochloride; ***RX*** **–** seven animals fed with Sirolimus and Celecoxib. ***ERX*** **–** nine animals fed with Erlotinib Hydrochloride, Sirolimus and Celecoxib;

The number of animals was defined according to the availability of our animal house and the difference in the number of animals per group did not bring any interference in the statistical analysis.

As in these mice, lung cancer does not develop spontaneously; there was no need to make a control group without urethane and without experimental treatment. Doses were calculated and programmed according to the individual weight of each animal on the first day of treatment and were treated for seven consecutive days. We used the following Erlotinib hydrochloride: 50 mg / kg [21] at a concentration of 10:1; Rapamune® (Sirolimus): 2mg/kg [22] with concentration 1:1; Celecoxib (Celebrex®): 50 mg/kg [21], at a concentration of 10:1, with use of mineral water to obtain the solutions.

All mice fasted for at least two hours daily, prior to receiving a total volume of 0.6 ml of drugs or mineral water as control, using the oral gavage technique. During the treatment period, in addition to daily weight measurement, the animals underwent physical assessment to identify possible adverse events such as coat change, skin rashes, and change in oral mucosa.

After 24 hours of the last dose of treatment, mice were weighed and sacrificed by inhalation of carbon dioxide (CO_2) and section of the abdominal aorta. Subsequently, the respiratory tract, heart, brain, kidney and spleen were removed and fixed by intratracheal instillation of 10% formalin solution. A complete autopsy was performed.

The histological analysis was performed counting the number of lung nodules and hyperplasias. All the other organs were also studied in search of any pathological changes, as done previously in similar studies from our group. [27-28]

Immunohistochemical assay

Consecutive 4.0µm-thick sections were cut from each trimmed paraffin block, and mounted in glass slides. In brief, following deparaffinization, sections were re-hydrated, treated with citrate buffer at 96°C for 30 minutes, and treated with 3% H_2O_2 in methanol (v/v) for 30 minutes to block endogenous peroxidases. The sections were then incubated for 1 hour at room temperature with specific antibodies: Ki-67 Antigen, Rabbit Monoclonal Antibody: Clone: MM1 (Novocastra, Newcastle, United Kingdom) 1:400 dilution; EGFR Rabbit Monoclonal Antibody, Clone ID: E114, 1:10 dilution, (Epitomics®).

For VEGFR analysis, we used Rabbit Anti-human FLT-1/VEGFR1 polyclonal antibody, (Spring Bioscience®), 1:50 dilution Rabbit Anti-Human FLK-1/KDR/VEGFR2 polyclonal antibody, (Spring Bioscience®)1:50 and Rabbit Anti-Human FLT-4 Polyclonal Antibody (Spring Bioscience®) 1:100 dilution Immunostaining was visualized with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) containing 0.005% H_2O_2 and counterstained with hematoxylin.

Immunohistochemistry analysis was performed counting the positive cell in a light microscope with a 400 magnification.

Statistical analysis

Statistical analysis was performed using non-parametric Kruskal-Wallis and Mann-Whitney for a nodule count, with a significance level less than or equal to 5%. To evaluate the incidence of adverse events, we used the Chi-square test and Fisher's exact

test with a significance level less than or equal to 5%. For numeric variable, we used weight Kruskal-Wallis test for comparison of all groups and Mann-Whitney test for comparison between groups, both with significance level less than or equal to 5%.

RESULTS

Histological evaluation of the number of lung nodules of the 93 BALB/c mice that received urethane and concluded the experimental treatment, 16 animals (17.2%) had pulmonary nodule. Table 1 shows the distribution of lesions per group. All the pulmonary nodules were from the papillary type (Table 1). No nodules were identified in groups EX, ERX, and X.

Group E with one nodule (p=0.028), EX (p=0.010) and ERX (0.040) showed a decrease in the number of nodules compared to group C (seven nodules). On the other hand, ER (three nodules) groups (p=0.345), R (three nodules) (p=0.907), RX (three nodules) (p=0.907) and X (p=0.067) showed no statistically significant difference (Mann Witney - $p \leq 0.05$).

Only one animal from ER group showed hyperplasia. Other findings in the histopathological analysis of lungs were a case of pulmonary vascular hypertrophy in one mouse from group X and one case of pulmonary emphysema in the group E. In the group ER, we identified five cases of pulmonary hemorrhage and in the group RX, only one animal had pulmonary hemorrhage. In the ER group, we identified four cases of pulmonary aspiration. In the group R, we observed a peri-bronchial inflammation in one in animal, and in the group RX hemorrhage was identified in lung of two mice.

We did not observe the presence of metastases or other significant morphologic changes in brain, heart, kidneys, spleen, and liver. Only in one specimen in the ER group, a mesenteric cyst was seen at histological evaluation, and it was considered to be unrelated to experimental drugs.

Assessment of adverse events related to antitumor therapies: Erlotinib Hydrochloride, Celecoxib, Sirolimus and associations

Physical assessment animal was performed, and findings were recorded in accordance with use of experimental drugs. The following adverse events were identified: change of coat (Table 2), skin rashes (Table 3) change in oral mucosa, and changes in body weight.

Regarding the change of the coat, we observed that no animal had such a change in groups C, EX, RX, while two animals in groups E (11.8%) and ERX (22.2%), ten animals in ER group (66.6%), and one animal in groups R (14.3) and X (14.3%) showed this manifestation (Table 2).

Groups C, EX, and RX expressed no such modification. Groups ERX (p<0.001) and ER (p<0.001) had a higher occurrence of this variable when compared with the group C. Even when compared with group C, groups E (p=0.486), X (p=0.268) and R (p=0.268) showed no statistical difference.

We noticed that no animal had skin rashes in groups C, X, and RX. Moreover, 66.6% (ten animals) of the group ER, 52.9% (nine animals) of the group E, 33.3% (three animals) of the group ERX and 14.3% (one animal) of the group R had this manifestation (Table 3).

Table 1: Number of animals and nodules per group

Group	Total number of animals per group	Total nodules per group	number of animals with nodules		*p*
			N	%	
C	16	7	6	37,5	
E	17	1	1	5,9	*p=0,028**
EX	15	0	0	0	*p=0,010**
ER	15	3	3	20	*p=0,345*
EXR	9	0	0	0	*p=0,040**
X	7	0	0	0	*p=0,067*
XR	7	3	3	42,9	*p=0,907*
R	7	3	3	42,9	*p=0,907*
Total	**93**	**17**	**16**	**17,2**	

Groups: *C* –animals fed with mineral water; *E* –animals fed with Erlotinib Hydrochloride; *EX* –animals fed with Celecoxib and Erlotinib Hydrochloride; *ER* –animals fed with Sirolimus and Erlotinib Hydrochloride; *ERX* – nine animals fed with Erlotinib Hydrochloride, Sirolimus and Celecoxib; *X* – seven animals fed with Celecoxib; *R* – seven animals fed with Sirolimus; and *RX* – seven animals fed with Sirolimus and Celecoxib. (* *Mann Whitney Test – p≤0.05).*

Table 2: Adverse events related to experimental drugs: change of coat

Group	Change of Coat						***p ≤ 0,05*** **Statistical difference**
	NO		YES		Total		
	N	%	N	%	N	%	
C	16	100,0	0	0,0	16	100,0	
E	15	88,2	2	11,8	17	100,0	0,489
EX	15	100,0	0	0,0	15	100,0	
ER	5	33,3	10	66,6	15	100,0	<0,001*
ERX	7	77,8	2	22,2	9	100,0	<0,001*
X	6	85,7	1	14,3	7	100,0	0,268
R	6	85,7	1	14,3	7	100,0	0,268
RX	7	100,0	0	0,0	7	100,0	
Total	76	82,8	16	17,2	93	100,0	

Groups: *C* –animals fed with mineral water; *E* –animals fed with Erlotinib Hydrochloride; *EX* –animals fed with Celecoxib and Erlotinib Hydrochloride; *ER* –animals fed with Sirolimus and Erlotinib Hydrochloride; *ERX* – nine animals fed with Erlotinib Hydrochloride, Sirolimus and Celecoxib; *X* – seven animals fed with Celecoxib; *R* – seven animals fed with Sirolimus; and *RX* – seven animals fed with Sirolimus and Celecoxib. * *Fischer's exact Test – p≤0.05*; N: number of animals.

Table 3: Adverse event related to medicinal products: skin rashes

Group	Skin Rashes						***p ≤ 0,05*** **Statistical difference**
	NO		YES		Total		
	N	%	N	%	N	%	
C	16	100,0	0	0,0	16	100,0	
E	8	47,1	9	52,9	17	100,0	<0,001*
EX	14	93,3	1	6,7	15	100,0	1,0
ER	5	33,3	10	66,6	15	100,0	<0,0001*
ERX	6	66,7	3	33,3	9	100,0	<0,001*
X	7	100,0	0	0,0	7	100,0	
R	6	85,7	1	14,3	7	100,0	0,346
RX	7	100,0	0	0,0	7	100,0	
Total	69	74,1	24	25,9	93	100,0	

Groups: *C* –animals fed with mineral water; *E* –animals fed with Erlotinib Hydrochloride; *EX* –animals fed with Celecoxib and Erlotinib Hydrochloride; *ER* –animals fed with Sirolimus and Erlotinib Hydrochloride; *ERX* – nine animals fed with Erlotinib Hydrochloride, Sirolimus and Celecoxib; *X* – seven animals fed with Celecoxib; *R* – seven animals fed with Sirolimus; and *RX* – seven animals fed with Sirolimus and Celecoxib. * *Fischer's exact Test – p≤0.05*; N: number of animals.

Evaluation of oral mucosa was performed at the time of performing the gavage technique and observed in groups E, ER and EX. The comparison among groups, showed no statistically significant difference (p=0.7326) (Fisher Exact Test - $p \leq 0.05$).

We identified weight gain in relation to the control group: group E (p<0.001), EX (p=0.001), ER (p<0.000), R (p=0.005), and group ERX showed weight loss (p=0.025) statistically significant.

Immuno-histochemicalanalysis

For immunohistochemistry evaluation from 17 nodules, only nine (52.94%) were subjected to this analysis due to technical problems.

DISCUSSION

Our data showed that the use of Erlotinib hydrochloride (group E) alone or in combination with Celecoxib (group EX) and Rapamune® (sirolimus) (Group ERX) decreased the number of lung nodules in an animal model with urethane compared with the control group (C). This did not occur when the association was made between Sirolimus and Erlotinib hydrochloride (group ER).

According to the literature, Erlotinib has been proven effective in clinical studies with patients who have a mutation in EGFR [11], as well as its performance inhibiting phosphorylation of EGFR. [29-30] It could be a therapeutic option for early and advanced stages of the disease. [11]

Therefore, our preclinical study showed that the EGFR inhibitor could decrease or delay the development of tumors except that addition of Sirolimus was favoring the development of pulmonary nodules.

Regarding Celecoxib, it is also known that it acts by inhibiting COX-2 and by preventing the formation of prostaglandins, which causes interference in the mechanisms of angiogenesis, cell adhesion, and proliferation. Thereby, it has antiproliferative and pro-apoptotic activity on these cells. [19, 31-33]

Otherwise, clinical trials showed no positive effect of the addition of the Celocoxib as a systemic antineoplastic therapy for metastatic disease. However, this does not mean that the inflammatory pathways play a nonsteroidal anti-inflammatory or antineoplastic activity, once clinical studies indicate that chronic use of aspirin may reduce the recurrence of new development of micrometastases or tumor. [34-35]

In contrast, Zhang [21] and colleagues (2005) showed that the combination of EGFR inhibitor with COX-2 slows the progression of the tumor. This study indicated that the EGFR inhibitor alone moderately inhibited tumor growth compared to control or Celecoxib alone. Thus, for these authors, a combination of the two drugs, Celecoxib and Erlotinib, can inhibit the angiogenic pathway with greater potency than either drug alone. [21]

However, in our study the combination of Erlotinib and Celecoxib was no more effective in reducing pulmonary nodules than Erlotinib alone. The use of Erlotinib hydrochloride (group E) or Celecoxib (Group X) alone ensured the reduction of these nodules in our animal model.

Our study showed that the m-TOR inhibitor when combined with the EGFR inhibitor and COX-2 might interfere with tumor development. Nevertheless, if only associated with Erlotinib, it showed no efficacy in tumor response when compared to the control group.

In the present study, the lack of statistical significance in the ER group compared to the control, suggests to us that this regimen has little influence on the evolution of lung tumors. Therefore, our result is similar to the other studies that showed the addition of an inhibitor of an m-TOR pathway to the treatment regimen with Erlotinib did not influence the development of lung tumors when compared with Erlotinib monotherapy. [7]

Despite conflicting data about the real effectiveness of an m-tor inhibitor, we cannot consider that Sirolimus would not be indicated for lung cancer. In our study, its association with Celecoxib and Erlotinib appeared to be beneficial and could be a promising therapeutic option. Thus, we suggest that further studies must be performed to define the optimal dose and plasma concentration of this molecule, which can inhibit m-TOR in animal models. It would also be necessary evaluation of the m-TOR inhibitor indicated more (Sirolimus, Everolimus, Tensirolimus).

Regarding adverse events, we cannot explain the lung hemorrhage found in groups that used Sirolimus. This could be secondary to the gavage, as the accumulation of macrophages also observed in the group ER could be to bronchial aspiration of material.

When we analyzed the effect of the urethane on blocking tumor development after the use of EGFR inhibitor, COX-2 and m-TOR alone and combined, we observed only a single tumor nodule in group E and in the group ERX no tumor was present. Such a finding was not statistically significant, but it was considered an important result and suggests that combination for future studies.

Adverse events related to experimental drugs

Although the anticancer drugs targeted to specific molecular tumors have been developed to be less harmful than cytotoxic agents, inhibitors of EGFR can generate various adverse events that may restrict or limit its use. [36-37]

Skin rashes were not present in groups C, X, and RX. On the other hand, they occurred in the groups ER, E, ERX, and R. These results are in agreement with the literature as the hydrochloride Erlotinib has the expected adverse event as rash [36, 38] and using Rapamune® (Sirolimus) can occasionally be related to this manifestation.

Compared to cytotoxic agents, myelosuppression occurs to a lesser charge. The use of EGFR inhibitor can cause advent events such as skin problems (rash, flaking, itching), gastrointestinal symptoms (diarrhea, nausea), and elevated liver enzymes. The skin rashes

are noticeable, and they can be an adverse event observed in two-thirds of patients receiving this therapy. [36, 38]

In this scenario, it became more evident the beneficial effects of Celecoxib associated with the treatment with Erlotinib, once the COX-2 inhibitor suppressed the skin manifestations related with Erlotinib hydrochloride. COX-2 inhibitor maintained its clinical effectiveness. This is the first time that result is shown in the literature. This demonstration is commonly found in patients on Erlotinib hydrochloride, and its slowdown by using an inhibitor of COX-2 could possibly be related to the anti-inflammatory effects from this drug. [16-18] The dermatological changes related to the use of Erlotinib can be minmized with the COX-2 inhibitor possibly due to its anti-inflammatory effects.

The expression "change of the coat," present in animals in group E and so exacerbated in the ER group was shown to be mitigated using Celecoxib in the treatment regimen (EX group animals showed no such manifestation).

On amendment of the oral mucosa is important to emphasize the technical difficulty encountered in his observation in male mice Balb/C, which can be very cooperative to its insignificance and little observation in this study. We expected an effect of this variable in the groups receiving Sirolimus.

An interesting finding was the decrease of change of the coat when Celecoxib was added to Erlotinib therapy. It has been shown that Celecoxib can decrease hand-foot syndrome caused by Capecitabine in clinical trials. Our finding suggested that Celecoxib could indeed decrease different types of skin reactions irrespective the source a common explanation for this skin protection by Celecoxib would possibly be related to the anti-inflammatory effects of this drug. [16-18]

In relation to the animals' weight gain, it was identified that all of them were in the growth phase. Thus, the weight gain would be an expected event not related to the drug administration. However, the group ERX presented weight loss. We have no literature to explain this weight loss and its relationship to the three drugs. We believe that the weight loss was due to the toxicity of the combination of three experimental drugs. Despite this activity, the weight loss brought attention to the toxicity of poly drug therapy.

The acquired EGFR in tumors contributed to the development of drugs and demonstrated promising response rates. However, are drugs that cause toxicity to the patient and may interfere with their quality of life. [8-10] The suggestion that Celecoxib can decrease skin toxicity by using Erlotinb without loss of efficacy is promising and merits further appraisal.

Animal model with urethane and immunohistochemical assessment

Our study presented technical difficulties in assessing immunohistochemistry (IHC) and prevented us to perform the correlation of protein expression EGFR, KI-67, VEGFR-1, VEGFR-2, and VEGFR-3 with responses to the treatments alone or in combination with Erlotinib, Celecoxib, and Rapamune®. On the other hand, the few tumors examined and immunohistochemical findings may propel other studies.

Our tumors were positive stain for EGFR, Ki-67, VEGFR1and VEGFR3, but not for VEGFR-2. As there were few cases available, more studies are needed to evaluate these findings.

We no identified preclinical research using urethane to assess markers VEGFR (VEGFR-1, VEGFR-2, VEGFR-3,). This study showed positive expression VEGFR-3 in the animal model. These results suggest that the urethane-induced model of lung cancer have changes in the lymphatic endothelium related to angiogenesis process.

In summary the hydrochloride Erlotinib has antitumor activity in animal models of lung cancer but when associated with Celecoxib, (cyclooxygenase-2 inhibitor) and m-TOR, has increased its effectiveness in relation to the tumor response; and the adverse effects observed mainly with EGFR inhibitor Erlotinib could be mitigated by expected one of the study medications (Celecoxib).

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